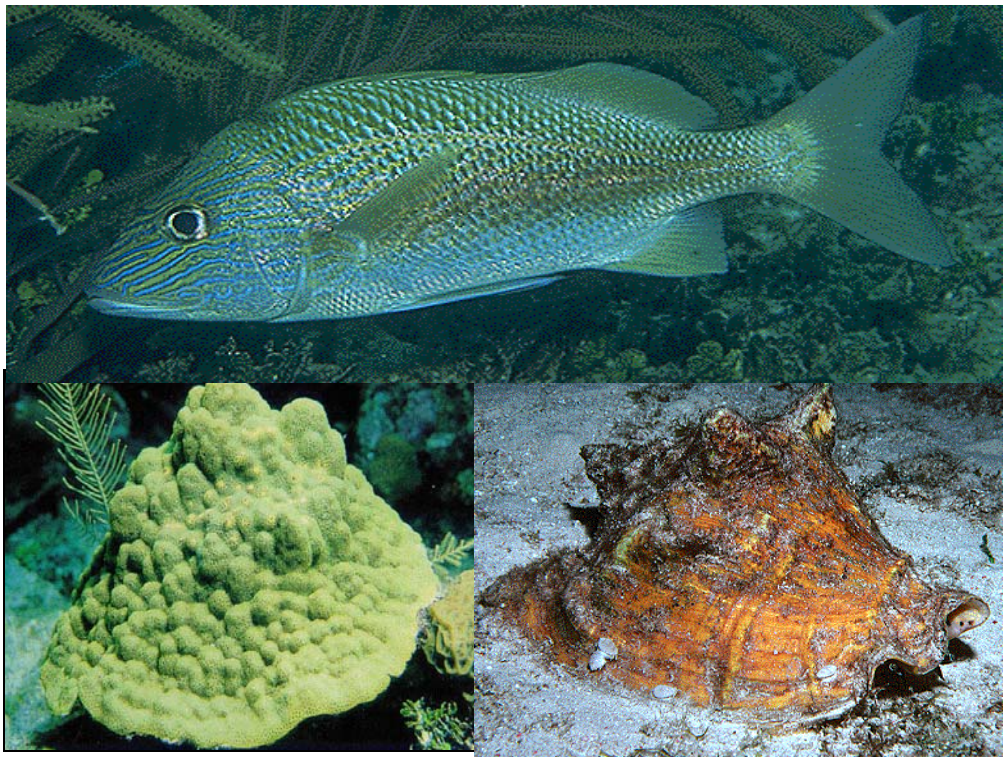
Sampling, Biomarker and Contaminant Chemical Target Analysis Protocol

Assessing the Effectiveness of Agricultural Better Management Practices in the Mesoamerican Reef



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Haereticus Environmental Laboratory

WWF

ICRAN International Coral Reef Action Network

UNEP

USAID FROM THE AMERICAN PEOPLE

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PREFACE

While excess nutrients and over-fishing are widely recognized as a major contributors to global coral reef decline, the role of chemical pollutants, including those derived from agricultural activities, as potential agents of reef ecosystem decline has rarely been studied or recognized as a significant threat.

However, recent studies within the largely offshore reef habitats of the Mesoamerican Reef of bioaccumulation of agrochemicals in a variety coral reef species have found that such contamination is present on offshore reefs. Our strategy, therefore, involves linking monitoring of bioaccumulation in marine organisms with the agrochemicals used by key industries in the agribusiness sector and our collaborative efforts to reduce those with the highest risks to marine life. The development of this monitoring protocol is in an effort to establish baseline conditions for subsequent monitoring of the success of these management interventions (better management practices or BMP's) aimed at reducing the contamination. It is the first in a series of protocols, and will be followed by protocols for the incorporation of targeted biomarkers and eco-toxicology assays. Together these will help to establish ecological risk framework appropriate to this marine ecosystem.

We encourage collaborations with researchers from conservation organizations, government and industry from the region and from the global community, who have similar aims and monitoring needs.

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Sampling, Biomarker, and Contaminant Chemical Target Analysis Protocols for Assessing the Effectiveness of Agricultural Better Management Practices in the Mesoamerican Reef

This document contains contaminant chemistry analysis protocols for organochlorine pesticides and polychlorinated biphenyls, including the collection and analysis of biological and sediment samples. It was prepared for WWF and the ICRAN MAR Alliance by Craig Downs, Executive Director of Haereticus Environmental Laboratory in collaboration with Melanie McField, WWF Centrral America.

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Monitoring Protocol for Assessing the Effectiveness of Agricultural Better Management Practices in the Mesoamerican Reef

This document contains sample collection, preparation and analysis protocols that can be used on the target organisms of grunts, conch, and coral. It was prepared for WWF and the ICRAN MAR Alliance by Craig Downs, Executive Director of Haereticus Environmental Laboratory in collaboration with Melanie McField, WWF Central America.

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Chapter 1
Sampling Protocol for Contaminant Chemical & Biomarker Analysis

1.1.1 Materials

Materials that are needed to collect *Porites asteroides*(coral), *Strombus gigas* (queen conch, mollusc), and *Haemulon plumieri* (white grunt, fish) for organic contaminant chemistry analysis. Units are suggested for a single sampling time-point for 4 sites

	Item	Source	Catalog #	Units
1.	Oak Ridge Centrifuge tube, 50 mL	Nalgene Labware	3114-0050	8
2.	Oak Ridge Centrifuge tube, 30 mL	Nalgene Labware	3114-0030	60
3.	Oak Ridge Centrifuge tube, 10 mL	Nalgene Labware	3114-0010	60
4.	Al Stohlman Brand Drive Punch 13/64”	www.sbeartradingpost.com	35005-06	10
5.	Stainless steel dissection probe	Fisher Scientific	NC9727105	10
6.	Stainless steel scissors	www.discountofficesupplies.com		1
7.	Surgical scissors	www.drinstruments.com	6SS	12
8.	Stainless steel cutlery knife			1
9.	Heavy duty aluminum Foil, clean	VWR	29952-200	1
10.	Hammer	?	?	2
11.	Chisel, 5”(125) long x 5/16”(8.0)	www.steritool.com	10280	8
12.	Scalpel, stainless steel	VWR	BD-371030	15
13.	Scalpel blades, stainless steel	VWR	BD-371111	1 case
14.	Forceps	VWR	25716-002	12
15.	Forceps #2	VWR	25728-001	5
16.	Nine-piece Dissecting kit	VWR	25640-002	2
17.	Acetone, GC-grade, 99.5%	VWR	VW0370-7	1
18.	Nitric acid (50%)	VWR	VW3335-1	2
19.	Mortar, 65 mL, case of 18	VWR	50420-223	2
20.	Mortar, 145 mL, case of 12	VWR	50420-245	1
21.	Pestle, case of 18	VWR	50420-427	2
22.	Pestle, case of 12	VWR	50420-449	1
23.	Nitrile gloves, small, box	VWR	40101-344	2
24.	Nitrile gloves, medium, box	VWR	40101-346	2
25.	Nitrile gloves, large	VWR	40101-348	2
26.	Hydrosorb 1 12x12, 150 count	VWR	33344-044	1
27.	MVE Doble 28 Cryo shipper	MVE	MVE11527730	2
28.	MVE Doble 28 shipping container	MVE	?	2
29.	No.2 graphite pencil	local store	?	10
30.	Liqui-Nox Phosphate-Free Detergent	VWR	21837-005	2
31.	Double distilled water, 30L	?	?	
32.	Empty, black 35mm film canisters with lid			80
33.	Cable ties, 6-8 cm in length			80
34.	Cable ties, 30 cm in length			4
35.	Aluminum carabiner 6-10 cm in length			4
36.	Igloo Marine 54 cooler	www.igloo-store.com		2
37.	Collapsible fish trap 32 in. x 24 in.	www.nylonnet.com	FT-B	3

A listing of materials necessary for conducting assays will be found in each assay section.

Need access to Liquid nitrogen and dry ice.

1.1.2 Sample Nomenclature

Samples will be identified by three category designations: Site, Species, and order of collection.

All sites are designated by a capital letter. For example, the site in Barbareta, Honduras will be referred to in all collections as "A"; the site in Turneffe, Belize will be designated as "C". Species in the collection will be designated as "X", "Y", or "Z".

"X" = *Porites asteroids*
"Y" = *Strombus gigas*
"Z" = *Haemulon plumieri*

Individual samples collected from each site from any species will be designated as a number; the designation reflected by the numerical order it was collected. For example, the first coral sample collected in Barbareta will be designated as "1". The second coral sample collected in Barbareta will be designated as "2", etc… The first grunt sample collected in Barbareta will be designated as "1", etc…

Hence, all samples in shipping/archival containers, notebooks, and manifests will be identified by the designation:

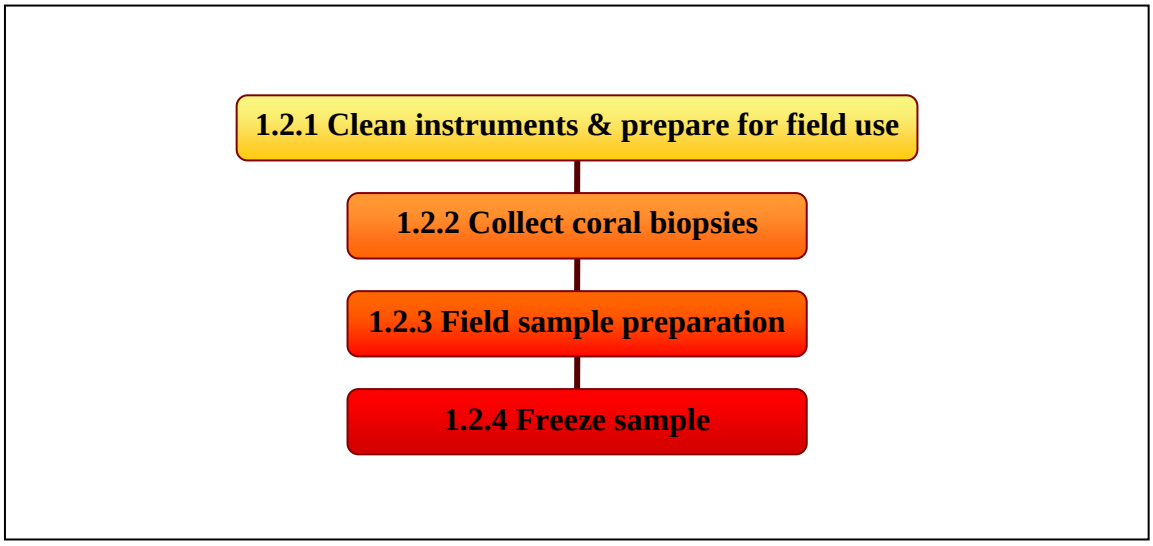
[Letter (site), Letter (species), Number (sample)]

For example, the first coral sample collected in Barbareta will be designated and labeled as [AX1]. The second coral sample collected in Barbareta will be designated and labeled as [AX2], etc… The first and second Queen Conch samples collected at Tuneffe will be designated and labeled [CY1] and [CY2], respectively.

1.1.3 Sampling Protocol Strategy

The sampling protocol for biological samples assumes that an individual that is collected will be assayed for both biomarkers and contaminant chemical target analytes. If biological samples are collected only for biomarker analysis, or if financial constraints make it difficult to obtain Teflon tubes, then an alternative step is provided. This alternative step wraps the samples in acetone-rinsed aluminum foil and places the sample in a polypropylene tube (in place of the Teflon tube). Using aluminum foil is not an ideal alternative, because it does not exclude the possibility of cross contamination between samples, and in some cases, between sites if the samples are all stored in the same freezer or liquid nitrogen cryo-shipper. Additional acetone-rinsed pieces of aluminum foil should be collected at the time of sampling and placed in a separate polypropylene tube. This foil will act as a control to determine if any of the residual target analytes may have been derived from the aluminum foil or the polypropylene tube.

1.2 Sample Collection & Preparation for Coral



Operations Chart for Coral Collection & Preparation for Contaminant Chemistry Analysis

Materials Needed:

- Al Stohlman Brand Drive Punch 13/64”
- Stainless steel dissection probe
- Heavy-duty Aluminum Foil
- 5 mm Teflon Layflat bags
- 35 mm film canisters with lids
- Both sizes of cable ties
- Polypropelene Falcon Tube, 50 mL
- Aluminum carabiner
- Clip-n-seal, large
- Liqui-nox detergent
- Pencil
- Acetone
- Double Distilled water
- Squirt bottle

1.2.1. Preparation for Cleaning

Arrange equipment and materials so they can be easily accessed once you start the cleaning process. Once you start cleaning, do not touch any other equipment or surfaces with the gloves you are wearing. If you must, remove the gloves and put on a new pair of clean gloves. Any stray contamination introduced during the cleaning process will affect the subsequent analysis.

1. Fill a new Teflon-coated squirt bottle with acetone. Identify the bottle with “acetone”.

2. Fill a new Telfon-coated squirt bottle with the distilled water. Identify the bottle with "water".
3. Fill a new squirt bottle with a 70% solution of acetone (ratio of 70 ml acetone to 30 ml distilled water). Identify the bottle with "70%".
4. Mix up a solution of 10% Liqui-nox detergent and place in a wash pan.
5. Place the roll of aluminum foil on a counter or table for cutting.
6. Cut a 30 cm x 30 cm section. In the top left-hand corner use a #2 pencil to inscribe an "A". The letter will let you know which side you have cleaned later.
7. Cut a piece of foil into 10 - 10 cm x 20 cm pieces. Use the pencil to inscribe an "A" in one corner of each piece.
8. Cut a piece of foil into 24 – 6 cm x 6 cm pieces. Use the pencil to mark the middle of each foil square with a sample designation number (e.g., [AX1], [AX2], [BX1], etc.) There should be 24 separate designation numbers.
9. Put on a clean pair of nitrile gloves.
10. Wash the side of the 30 cm x 30 cm piece of aluminum foil, with the "A", with acetone using the squirt bottle. Use acetone-cleaned forceps to manipulate the foil piece; handling of the aluminum piece with a gloved hand should be kept at a minimum.
11. Wash one side of each piece of 10 cm x 10 cm aluminum foil with acetone using the squirt bottle. Set the unwashed side face-down on a clean counter. Allow the foil to dry (15 minutes).
12. Rinse the forceps with acetone. Use the forceps to pick up each 6 cm x 6 cm piece of foil for cleaning. Wash both sides of the foil square with acetone using the acetone-squirt bottle. Allow to air dry on a 10 cm x 20 cm piece of acetone-rinsed aluminum foil.
13. Allow all pieces of foil to air dry, about 15 minutes.
14. Acetone-rinse four Oak Ridge 50 mL Teflon tubes/caps. Allow to air dry, then seal the tube.

1.2.2 Clean Instruments & Prepare for Field Use

1. Wearing the nitrile gloves, wash the Drive Punches and Dissection probes in the 10% Liqui-nox detergent.
2. Remove all residual detergent on the instruments by rinsing the instruments with distilled water using the squirt bottle.
3. Rinse instruments with acetone using the squirt bottle.
4. Place instruments on acetone-cleaned aluminum foil and allow to completely dry.
5. Place one Drive Punch and one Dissection Probe onto a single 10 cm x 20 cm aluminum foil piece (on the side of the foil that was washed with acetone).
6. Wrap the instruments with the acetone-cleaned aluminum foil.
7. Place one aluminum-covered instrument set into Teflon Layflat bag.
8. Seal the Layflat bag.
9. Punch a single hole in the lid of the 35 mm canister using the point of a sharp knife, about 1 mm in diameter and about 4 mm from the edge of the lid.

10. Punch a single hole in the side of the 35 mm canister, about 1 mm in diameter, and about 5 mm below the lip of the canister.
11. Punch a single hole at the bottom of the 35 mm canister, about 2 mm in diameter, and in the center.
12. Loop the lid and canister together with the 6-8 cm long cable tie. Make sure not to constrict the cable tie loop too tightly. The lid must be able to come off, and be put back on, the canister easily.
13. Quickly rinse the inside of the canister and the inside of the lid with the 70% acetone solution, followed very quickly with washing with double distilled water. Acetone can melt plastic.
14. Allow to air dry, then cap the containers.
15. Number each canister with a number, from 1 to 6, by etching the number to the outside of the canister with a sharp knife.
16. Assemble six canisters together by looping the 30 cm cable tie through the 6-8 cm cable tie loop (see Figure 1).
17. Gather the six sample-foil squares that pertain to a single sampling site into a Teflon Layflat bag that has been rinsed with acetone/air dried. Seal the bag.
18. Identify the four Oak Ridge 50 mL Teflon tubes/caps. Using a Dremmel with a fine point or a knife, etch the site and species designation onto the side of the tube. Each tube should have a different designation code.
19. Finally, place a carabineer onto the 30 cm cable-tie loop (see **Figure 1**).

Figure 1. Basic configuration design of 35 mm film canister loop for coral sampling

1.2.2. Collection of Coral Biopsies

Collection of samples is assumed to be conducted by SCUBA diving off a boat. There should be three people for the collection, the two collection divers and the 'boat person'.

1. Once collection divers have donned their dive gear, divers should put on nitrile gloves. The 'boat person' will latch the 35 mm film canister to the wing diver's buoyancy control device using the carabineer.

2. Remove a single set of drive punch/dissecting probe from its protective covering; the drive punch and the hammer are taken by the lead diver.

3. The wing diver will bring with them the dissecting probe.

4. Once at the target site, the lead diver will place the drive punch on the apex of the coral colony. The apex of the coral is the area of the coral that is perpendicular to the surface of the water, and should receive the greatest amount of incident light (**Figure 2**). Do not collect the biopsy from the coral edge, as you may collect non-coral tissue, thereby introducing significant artifact into the study (**Figure 3**).

5. With several taps with the hammer, the drive punch should be driven into the colony about 1 cm. The diver must go deep enough into the coral colony so that the biopsy contains all visible tissue (**Figure 4**). For most boulder corals, the tissue can extend into the skeleton from 1mm to as much as 10 mm. If boring sponge is present, try not to collect the sponge with your biopsy – resample from the same coral colony, or sample from a different coral colony. The coral biopsy will remain in the drive punch.

6. The lead diver hands the drive punch that contains the biopsy to the wing diver. The wing diver, using the dissecting probe, pushes out the coral biopsy. Using a 5 cm x 5 cm square of acetone-cleaned aluminum foil, wrap the coral biopsy in the foil. Place foil-wrapped coral biopsy into the open 35 mm film canister. Once the biopsy is in the canister, seal the canister with the lid.

7. Wing diver will then return the drive punch to the lead diver.

8. Ideally, every sample should have a new drive punch/probe, though in most cases, this many not be financially possible. If this constraint is present, repeat sampling procedure with the next coral colony using the same coring device for all colonies at a given site.

Figure 2. Place drive punch at the apex or very near the absolute apex of the coral colony. The biopsy lesion should be completely surrounded by healthy coral tissue. Unlike this picture, diver should wear nitrile gloves.

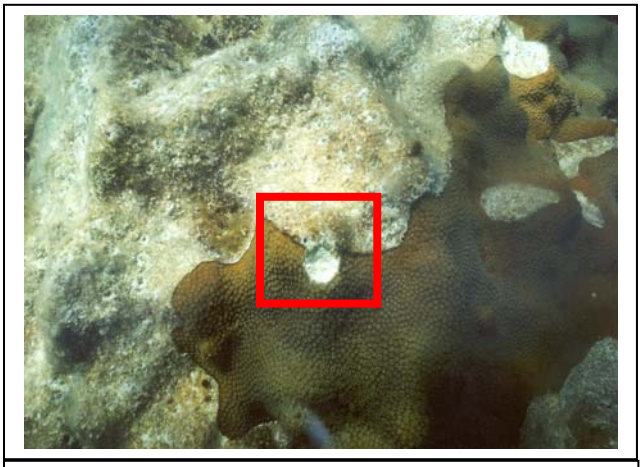


Figure 3. The biopsy SHOULD NOT be taken from tissue that is near the edge of the coral colony

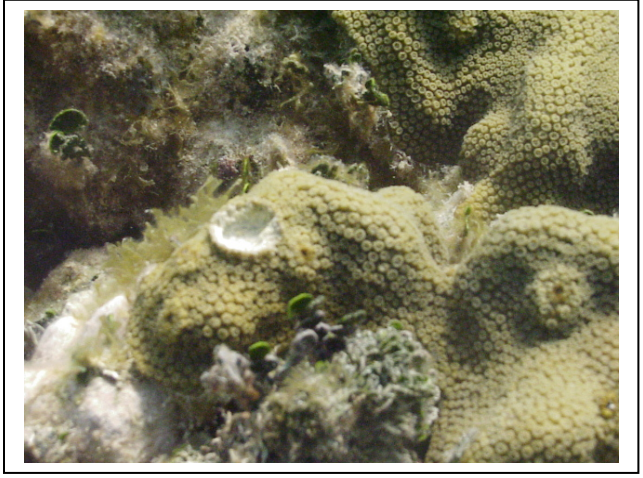


Figure 4. The divot created by the biopsy should tell you whether or not your biopsy went deep enough into the coral skeleton. You should see that the bowl of the divet extends below the tissue layer by about 2-3 mm.

1.2.3. Field Sample Preparation

1. During the diver interval, the boat person should make ready the 6 cm x 6 cm foil squares, a clean surface area to work on (a sheet of acetone-washed aluminum foil laid across the top of the igloo marine cooler). Boat person should be wearing clean nitrile gloves.

2. At the surface, the wing diver hands the film canister loop to the boat person.

3. Opening a single film canister, the boat person removes the coral biopsy from the canister using acetone-rinsed forceps. Remove the foil encasing the coral biopsy. Place biopsy on one of the 6 cm x 6 cm foil squares, place sample on top of the penciled number. Fold the foil square over the biopsy and crease with sharp edges and a rolled/pinched seal.

4. If coral is being used for contaminant chemistry analysis, place foiled biopsy into the 50 mL Oak Ridge Teflon centrifuge tube; the tube that is designated for this sampling site. If coral is being used only for biomarker analysis, place foiled biopsy into a 50 mL polypropylene Falcon Tube that has a 1 mm whole drilled into its side. This whole prevents the tube from exploding when it is removed from the cryo-shipper.

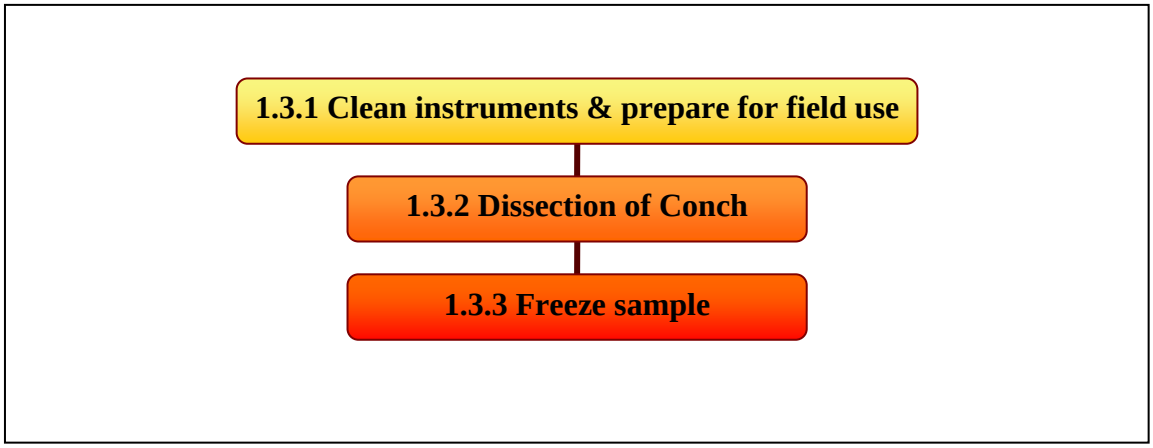
5. All six samples from a single site should fit into the single centrifuge tube. If worried about possible cross-contamination of target analytes if samples are placed in a single tube, then place samples in separate tubes.

6. Place the cap back onto the centrifuge tube, but only give the lid ¾ of a full rotation. An air-tight seal on the centrifuge tube could result in an exploding tube when the tube is removed from the cryo-vapor shipper.

1.2.4. Freeze Sample

1. Place the tube in the cryo-vapor shipper or in the Igloo Marine Cooler that contains dry ice.

1.3. Sample Preparation & Collection for Queen Conch



Materials

- Stainless steel hammer with a sparred end or stainless steel chisel
- Scalpel
- forceps
- Heavy-duty Aluminum Foil
- Oak Ridge Centrifuge tube, 30 mL
- Polypropylene Falcon Tube, 50 mL
- 5 mm Teflon Layflat bags
- Clip-n-seal, large
- Liqui-nox detergent
- Acetone
- Double Distilled water

If you have a stainless steel hammer with sparred end, you do not need the stainless steal chisel, and vice versa.

1.3.1 Clean instruments & prepare for field use

1. Wear nitrile gloves during the entire procedure.

2. Wash one side of a 30 cm x 30 cm piece of aluminum foil with acetone using the squirt bottle.

3. Allow sheet to air dry on the counter (15 minutes).

4. Wash the hammer/chisel, forceps and scalpel in 10% Liqui-nox detergent (dilute with double distilled water).

5. Remove all residual detergent on the instruments by rinsing the instruments with double distilled water using the squirt bottle.

6. Rinse instruments with acetone using the squirt bottle.

7. Place instruments on acetone-cleaned aluminum foil, allow to completely dry.

8. Cut aluminum foil into 30 cm x 20 cm pieces.

9. Wash one side of each piece of aluminum foil with acetone using the squirt bottle. Set the unwashed side face-down on a clean counter. Allow the foil to dry (15 minutes).

10. Place one hammer/chisel and one scalpel onto asingle 30x20 cm aluminum foil piece (on the side of the foil that was washed with acetone).

11. Wrap the instruments with the acetone-cleaned aluminum foil.

12. Place aluminum-covered instrument set into Teflon Layflat bag.

13. Seal the Layflat bag.

14. Acetone rinse 32 Oak Ridge 30 mL Teflon tubes/caps. Allow to air dry, then seal the tube.

15. Using a dremmel with a fine point or a knife, etch the site, species, and sample number designation onto the side of the tube. Each tube should have a different designation code.

1.3.2 Dissection of Conch

1. Once the conch has been collected, it can be placed in the cooler on dry ice or ice, or it can be dissected in the field.

2. Whether dissection occurs in the field or back in the lab, acetone clean one side of a 30 cm x 30cm piece of aluminum foil, and place the uncleaned side down on a clean benchtop or Teflon cutting board.

3. Wear nitrile gloves.

4. Position the conch shell on a clean surface and puncture or “crack” the shell with the stainless steel hammer or chisel at the location indicated in **Figure 5**.

5. With the scalpel, cut the retractor muscle off of the shell (**Figure 6**).

6. Pull the conch out of the shell.

7. Cut a 7 cm long x 1 cm in diameter swath of mantle tissue using the acetone-cleaned scalpel and forceps (**Figure 7**).

8. Place the mantel tissue into the labeled Oak Ridge Telfon 30 mL centrifuge tube that is appropriately labeled. Alternatively, wrap the tissue with an acetone-rinsed piece of aluminum foil; then place the tissue in a 50 mL polypropylene Falcon tube.

9. Be consistent with the area from which you dissect the tissue from one sample to another.

1.3.3 Freeze sample

1. Place the tube in the cryo-vapor shipper or in the Igloo Marine Cooler that contains dry ice.

Figure 5. Puncture or "crack" the shell at the location indicated in the figure. Use an acetone-cleaned stainless steel hammer with a spar or a stainless steel chisel.

Figure 6. Using the acetone-cleaned scalpel, cut the retractor muscle from the shell so that the conch can be removed easily from the shell.

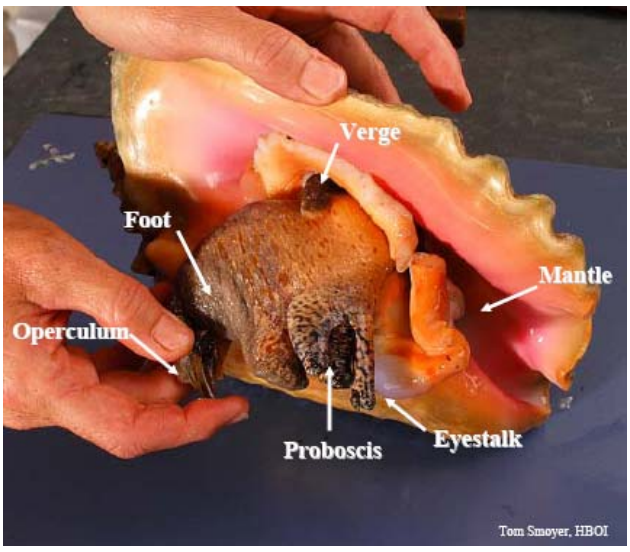
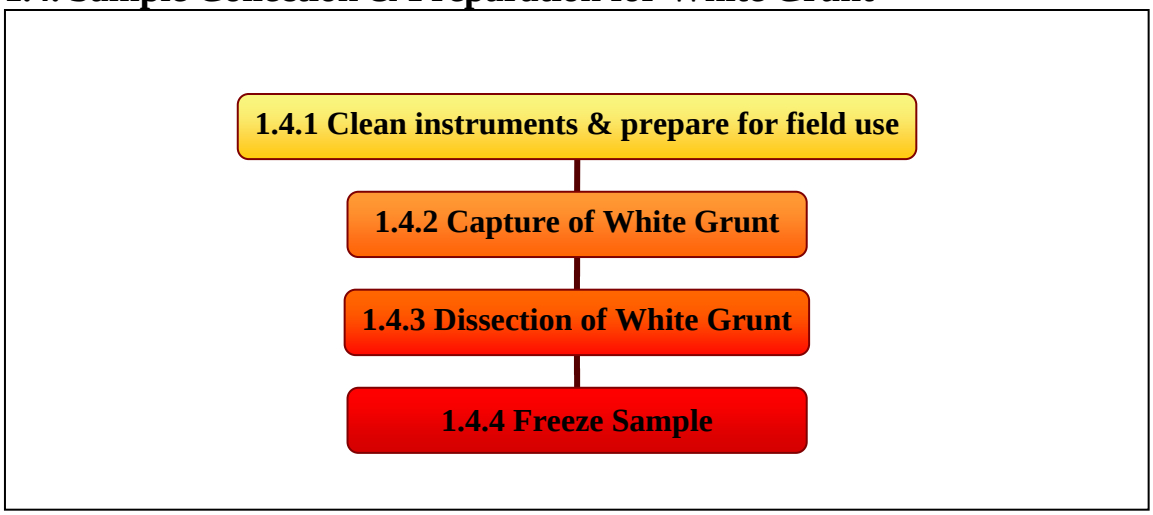


Figure 7. Cut a 7 cm long and about a 1-2 cm wide swath of mantle tissue using the acetone-cleaned scalpel and forceps.

1.4. Sample Collection & Preparation for White Grunt



Materials

- Fish cages
- Scalpel
- Forceps
- Surgical scissors
- Heavy-duty Aluminum Foil
- Oak Ridge Centrifuge tube, 10 mL
- Polypropylene Falcon Tube, 50 mL
- Teflon 12x12 cutting boards
- 5 mm Teflon Layflat bags
- Clip-n-seal, large
- Liqui-nox detergent
- Acetone
- Double Distilled water

1.4.1 Clean instruments & prepare for field use

1. Wear nitrile gloves during the entire procedure.
2. Wash one side of a 30 cm x 30 cm piece of aluminum foil with acetone using the squirt bottle.
3. Allow sheet to airdry on the counter (15 minutes).
4. Wash the surgical scissors, forceps, and scalpel in 10% Liqui-nox detergent (dilute with double distilled water).
5. Remove all residual detergent on the instruments by rinsing the instruments with double distilled water using the squirt bottle.
6. Rinse instruments with acetone using the squirt bottle.
7. Place instruments on acetone-cleaned aluminum foil, allow to dry completely.
8. Cut aluminum foil into 30 cm x 20 cm pieces.

9. Wash one side of each piece of aluminum foil with acetone using the squirt bottle. Set the unwashed side face-down on a clean counter. Allow the foil to dry (15 minutes).
10. Place one surgical scissors, forceps, and scalpel onto a single 20x20 cm aluminum foil piece (on the side of the foil that was washed with acetone).
11. Wrap the instruments with the acetone-cleaned aluminum foil.
12. Place aluminum-covered instrument set into Teflon Layflat bag.
13. Seal the Layflat bag.
14. Acetone rinse 32 Oak Ridge 10 mL Teflon tubes/caps. Allow to air dry, then seal the tube.
15. If only collecting samples for biomarker analysis, acetone rine 32 50 mL Falcon tubes/caps. Allow to air dry, then seal the tube.
16. Using a dremmel with a fine point or a knife, etch the site, species, and sample number designation onto the side of the tube. Each tube should have a different designation code.

1.4.2 Capture of White Grunt

1. Purchase bait from store.
2. Using an acetone-cleaned instrument, place approximately 5-10 grams of bait into a Oak Ridge Telfon 10 mL tube, and etch onto the side of the tube, "Fish bait." Freeze bait sample in -20C freezer or in the cryo-dry shipper.
3. When at the site location, place bait in trap. The fish-trap product suggested in this document was included because they were used in grunt studies in the Florida Keys. Other types of traps can be used, as long as they do not induce excessive stress on the fish. Excessive handling stress can cause artifactual results for some of the biomarker.
4. Have a diver set the trap at a location where a lot of white grunt are evident.
5. Allow trap to set for at least 30 minutes.
6. Seal entrance to trap, bring trap to surface.
7. Collect only White Grunts that are 25 cm and longer.
8. Wrap grunt in 36 x 36 cm aluminum foil square that has been acetoned-washed and labeled. Place Grunt directly over penciled label. Wrap Grunt in the aluminum foil square, making sure of creased edges and rolled-pinched seal.
9. Freeze same in Igloo Marine cooler with dry ice in it.
10. The fish will most likely exhibit intensive physical activity when you try to wrap the fish in foil, making it difficult and frustrating to carry out the procedure. There are a number of ways to reduce this effort. It is important to keep in mind that whatever method of reducing the physical exertion of the fish should be legally acceptable and institutionally approved. Placing the fish in an acetone cleaned tank with site water and a fish anesthetic may be the easiest and most socially acceptable method.

1.4.3 Dissection of White Grunt

1. Place acetone-cleaned 12 cm x 24 cm Teflon cutting board on a level and clean surface.
2. Partially thaw fish at room temperature (e.g., room temperature = 25°C-29°C).
3. Wear nitrile gloves.
4. Remove fish from aluminum foil covering and place on 12 cm x 24 cm Teflon cutting board.
5. Head of fish should be facing to your right, the tail facing to your left.
6. Make an incision with the scalpel that run from the dorsal edge to the bottom of the belly; the incision should be made in front of the pectoral fin (**Figure 8**).
7. Make a second incision that begins where the first incision ended at the fish's belly, and run along the belly to the vent (**Figure 8**; **Figure 9**). If it is easier for you, use the surgical scissors to make the cut.
8. Make a third incision from the vent across the side of the fish, ending the incision at the site of the beginning of the first incision (**Figure 8; Figure 10**).

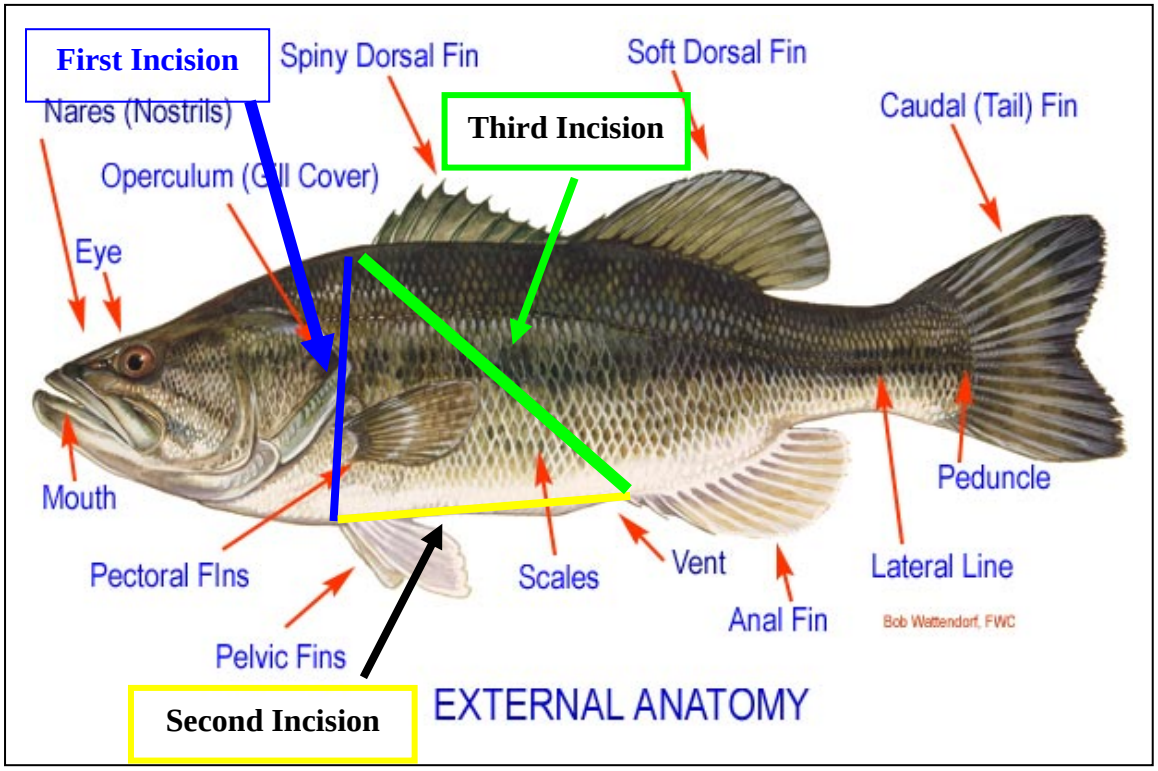


Figure 8. Make an incision along the blue line. The cut should only be deep enough to penetrate the muscle and break the gastrovascular fascia. DO NOT CUT into the organs. The yellow line is the second incision; again, penetrate the muscle and fascia, but do not cut into the organs. For the second incision, you may use your surgical scissors. The green line is the third incision; again, penetrate the muscle and fascia, but do not cut into the organs.

Figure 9. Second incision along the belly

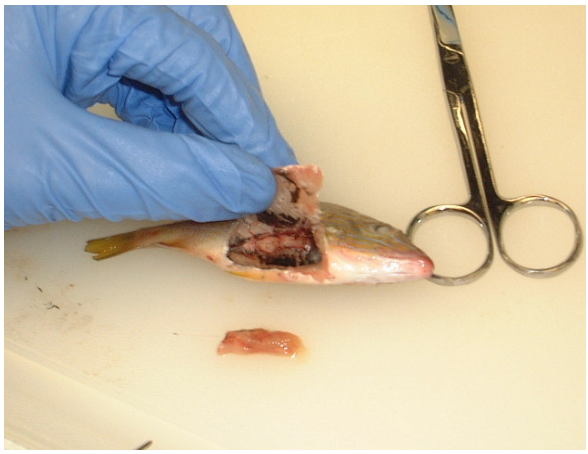


Figure 10. Third incision along the side

9. Identify the liver. It should be dark-red in color, and near the gills and immediately below the stomach (**Figure 11**).
10. Remove the liver with forceps and scalpel.
11. Place liver in acetone-cleaner Oak Ridge Telfon 10 mL centrifuge tube. Cap the tube with the lid, making sure that the lid is NOT on tight. Alternatively, wrap the tissue in acetone-rinsed aluminum foil. Place the wrapped tissue in a 50 mL polypropylene Falcon tube.

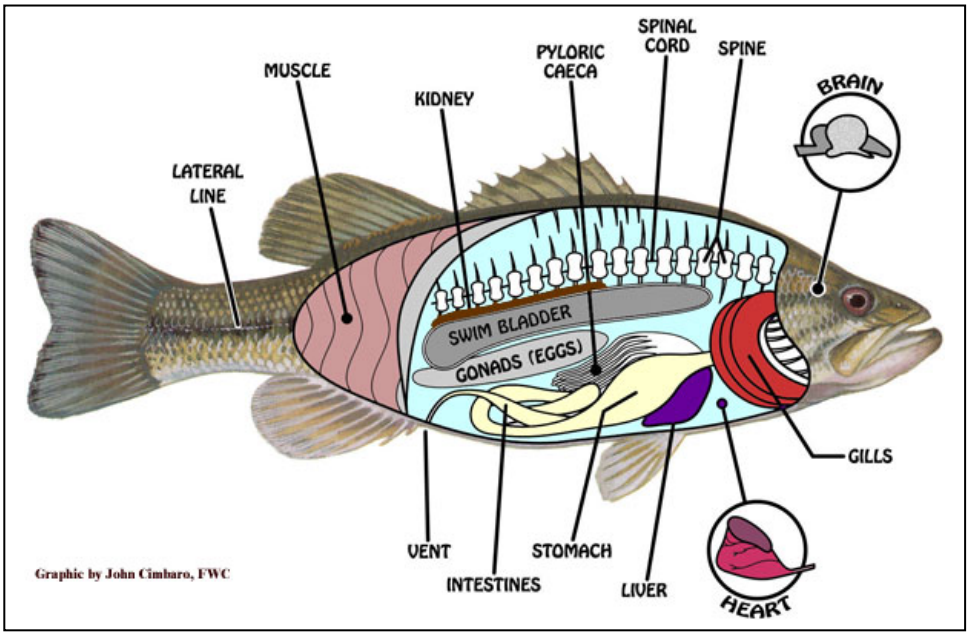


Figure 11. Liver is dark-red in color, and between the stomach and the gills

1.4.4. Freeze Sample

1. Place sample in cryo-dry shipper or in a -20C freezer. Samples can only remain at -20C for 28 days.

1.5. Sample Collection of Sediment for Organic Contaminant Analysis

Materials

- Sediment Jars, 500 mL

Collection Procedures

The particle composition and size of sediment may have an affect on the inferences that can be made from the contaminant chemistry data resulting from a sample site. The types of sediment at the sampling sites may vary from site to site. Oftentimes, inexperienced or uneducated sampling teams will sample sediments containing large sized stones (e.g., 1 centimeter or larger). Particulate size within a sediment sample should be, on average, smaller than two millimeters. Stones larger than 1 cm should be excluded from the sample. Exclusion of large stones requires that the sediment be resampled, and to not manually remove the stones from the sediment jar.

It is also suggested that the divers make note in the field book a description of the texture, color and scent of the matrix of each sediment sample.

1. At each sampling site, place six jars into mesh bag. Jars should have lid on them. If you do not dive deeper than 15 meters, the pressure on the jar should not break the jar. Do loosen the lid on the jar so that it can be opened easily underwater.
2. Diver should wear nitrile gloves.
3. At depth, open jar above sediment, and scoop the sediment into the jar using the lip of the jar. Only collect the top 1-2 centimeters of sediment.
4. Try to ensure that the jar is completely filled with sediment, and contains little residual seawater.
5. Cap jar with lid.
6. Once on deck, label the jar with a permanent marker or with laboratory tape, and chill the sample. Place jar in an Igloo cooler with normal ice – not DRY ICE.
7. If possible, open the jar and decant any excess seawater.
8. Once back in the lab, place the jar in a -20°C freezer.

If desired, grain size distribution for each sediment sample may be determined so that contaminants analysis results can be normalized against this grain size distribution. Grain size should be determined either by sieve fractionation or by gravimetric pipetting. The major fractions of particle distribution (based on the Wentworth scale) include the following classes:

- cobble (-6 phi and above)
- gravel (-2 phi to -5 phi)
- sand (+4 phi to -1 phi)
- combination of both silt and clay (+5 phi and below)

Most coastal marine systems have a diversity of sediment types, and hence, normalization against a single grain size distribution may be unrealistic and fallacious. This is not to say that this information might not be beneficial during the final analysis.

1.6. Option 1 – Short-term management of samples

Option 1 assumes that none of the samples will be archived or sent to a secondary analysis lab.

Samples from all three species will be in their capped, centrifuge tubes frozen in the cryo-vapor shipper.

1. Create a manifest of all contents in the cryo-vapor shipper.

2. Obtain the necessary export CITES permits.

3. Obtain the necessary import CITES permits.

4. DO NOT USE **DHL** as a courier service. Use air freight with an airline, FEDEX, or an air-freight service.

5. The day before shipping, make sure the cryo-vapor shipper is charged.

6. Notify the receiver five days in advance, then again at 48 hours in advance, and before the day the cryo-shipper is being sent to the receiver.

7. It is best that the receiver meets personally with the U.S Fish and Wildlife and the U.S. Customs inspection agents during the inspection of the cryo-shipper to expedite the processing and ensure that the cryo-shipper is delivered to the receiver in a timely fashion.

1.7. Option 2 - Long-term management of samples

Option 2 is a long-term management plan for samples collected as part of the Meso-American reef system. Option 2 is based on U.S. EPA's split-sample analysis inter-laboratory program. Samples are collected and prepared (as described in Section 1) and shipped to a central processing and storage facility. Samples are ground frozen to a fine powder, and aliquoted into three separate tubes. The purpose of each tube is as follows:

- Tube 1 is sent to the primary analysis facility.
- Tube 2 is archived for future reference.
- Tube 3 can be sent to the secondary analysis facility if there is a dispute, or for analysis of cellular end-points and contaminant target analytes that are not included in the primary biomarker and chemistry analysis.

Each tube will be given a new identification code that will ensure that all of the receiving facilities will be 'blind' to the true identification of the sample.

This Central Processing and Storage Facility is required to have the following items

- -80°C freezer or liquid nitrogen freezer storage
- Back-up power supply for cryo/freezer storage

- Secure entry
- System tracking of samples/identification
- Sample preparation equipment and supplies

Procedures for Option 2

Clean Instruments and prepare for use

1. Ship samples to Central Processing and Storage Facility as in Option 1.

2. Samples processed by ID input into data management system and stored either in a -80°C freezer or in a liquid nitrogen cryo-storage container.

3. Wear nitrile gloves for all instrument cleaning steps.

4. Mortar and pestles are washed in Liqui-nox detergent, rinsed in distilled water.

5. Optional: Mortar and pestles are set in a solution of 50% nitric acid for 2 hours, and then liberally rinsed in distilled water.

6. Mortar and pestles are rinsed with acetone.

7. Mortar and pestles are air-dried for five minutes, and then wrapped in acetone-cleaned aluminum foil for storage.

8. Stainless steel spatulas are washed with Liqui-nox detergent, rinsed with distilled water, then washed with acetone and air-dried. Wrap spatulas in aluminum foil for storage.

Sample preparation

1. Wear nitrile gloves.

2. Unwrap mortars, pestles, forceps, and spatulas from their aluminum foil covering and place on a clean lab bench.

3. In the three 1.8 mL cryo-vials, appropriately label the side of each tube with the Sample ID, and the storage tube Number (1-3). Labeling should be done with a black permanent marker or using printed cryo-tag labeling tape.

4. Place the three 1.8 mL cryo-vials into the tube racks, and place the racks in the ceramic ‘cool’ box with dry ice in the box. This is so that once you put the ground, frozen sample into each of the tubes, the samples do not thaw.

5. Place the 'cool' box with tubes onto a clean lab bench. It is assumed that the cool box and the tube racks have been cleaned with Liqui-nox detergent and rinsed in distilled water.

6. Fill cryo-dewar with liquid nitrogen.

7. Remove the samples from the cryo-storage container and place in a second 'cool' box that is filled with dry ice.

8. Set the second 'cool' box with the sample in it onto the lab bench.

9. Pour liquid nitrogen into a mortar that has the pestle in it. Fill the mortar to the rim, and allow the mortar to boil off (**Figure 12**).

10. Again, pour liquid nitrogen into mortar that has the pestle in it. Fill the mortar to the rim, and allow the mortar to boil off.

11. Rest the pestle on the lip of the mortar, and for the third time, pour liquid nitrogen into mortar (**Figure 13**).

12. As the liquid nitrogen is boiling in the mortar, remove the sample from its 3114 Oak Ridge Teflon Centrifuge tube with forceps, and place the entire sample into the mortar that has liquid nitrogen in it.

13. With the pestle, fracture the sample into smaller pieces before the liquid nitrogen completely boils off.

14. With 1-4 mL of liquid nitrogen remaining in the mortar, quickly grind the sample into the mortar applying significant pressure. Continue to do this until about 5-10 seconds after the liquid nitrogen completely boils off from the mortar.

15. Carefully add liquid nitrogen into the mortar, filling it to 1/3 to ¼ full.

16. Carefully grind the samples into a powder that is of a granulated consistency between ground sugar and finely milled flower (**Figure 14**).

17. With an acetone-clean, liquid-nitrogen cooled spatula, place contents of the mortar equally into the three cryo-vials that are in the 'cool' box that has dry ice in it (**Figure 15**).

18. Cap the samples, but do not make the seal air-tight, as nitrogen gas that is escaping from the sample can build up pressure within the tube and burst the vial.

19. Place sample in -80°C freezer or cryo-storage facility.

Figure 12. Pour liquid nitrogen into the mortar/pestle configuration and fill to the brim of the mortar

Figure 13. For the third filling of quid nitrogen into the mortar, remove the pestle and rest the pestle on the lip of the mortar.

Figure 14. Grind samples into a fine powder, making sure that the sample never thaws. Be careful when pouring liquid nitrogen into the mortar with granulized sample, as the liquid will sometime splash the sample out of the mortar and onto the bench.

Figure 15. While still frozen and in near liquid form, pour frozen, pulverized sample into a 1.8 mL cyo-vial that has been pre-chilled with liquid nitrogen. Using a liquid nitrogen or dry ice pre-chilled spatula will facilitate transfer of sample from the mortar to the vial.

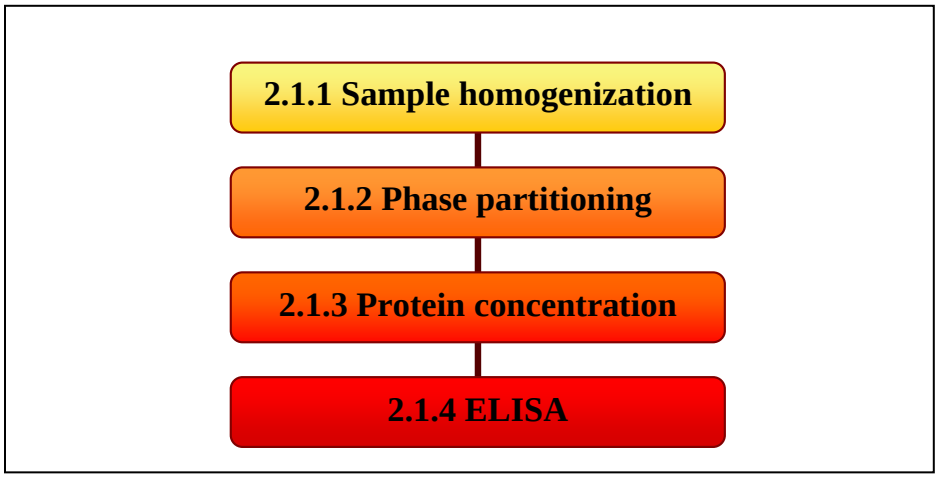
Some of the pulverized sample may escape from the mortar or cryo-vial and land on the lab bench, lab coat, etc. DO NOT place this piece of sample back into the mortar/cryo-vial with the remainder of the sample. This piece must be discarded.

Laboratory Safety

It is assumed (and strongly encouraged) that all technicians conducting laboratory preparation and assay protocols described in this document have taken a laboratory safety training course sanctioned by the laboratory's federal/state government and institution, and have read all Materials Safety Data Sheets for all of the chemicals used in the protocols. Special safety training will also be required for handling of liquid nitrogen and dry ice (solid carbon dioxide). The laboratory room where liquid nitrogen and dry ice are being handled should be well vented and precautionary measures should be taken to safeguard against asphyxiation. A number of safety guidelines, training documents, and information concerning specific laboratory activities can be found or requested at the U.S. Department of Labor Occupational Safety and Health Administration (www.osha.gov). It is the responsibility of the principle investigator and executives at the laboratory's institution to comply with all safety regulations indicated by institutional policy and federal/state laws.

Chapter 2:
Sample preparation and assays for biomarker analyses

2.1 Sample preparation and protocol criteria for ELISA



This document is not meant to introduce and instruct the novice laboratory technician. It is assumed that the technician has a minimal amount of education and training in biochemistry and molecular biology and has the ability to solve issues (trouble-shoot) concerning sample preparation and assay protocols. The purpose of Chapter 3 is to provide a basis for standardization of biomarker end-points used in the MAR study area as well as standardization of assay protocols. This document also assumes a level of competence in making diagnostic interpretations for the results of any of these biomarkers. Use of biomarkers should be conducted within a diagnostic context. It is beyond the scope of this document for the proper training and education in the theory of cellular diagnostics and biomarkers.

2.1.1 Homogenization of sample in Denaturing Buffer

1. Within two hours of commencing the homogenization of samples, prepare the Denaturing Buffer fresh, using the solid form of each ingredient, not a previous solubilized form of the ingredient.

2. All glassware, spatulas, and Teflon-coated magnetic stirrers should be cleaned with Liquid Nox detergent and thoroughly rinsed with distilled water.

3. In a 50 mL Pyrex bottle, make up a 100 mM stock solution of Disodium ethylenediaminetetraacetate (EDTA). First add 25 mL of distilled water to the vessel, then a teflon-coated magnetic stir bar. Then add 1.86 grams of disodium EDTA. Slowly titrate with 4 M sodium hydroxide (NaOH) and monitor the pH of the solution with a pH meter. The final pH of the solution when all of the EDTA is dissolved will be about 8.0. Solubilizing by titration for this amount of EDTA should take about 10 minutes. This solution of EDTA should be good for about two weeks if kept at 4°C.

4. In a 15 mL polypropylene Falcon tube, add 0.138 grams of salicylic acid. Then add 10 mL of distilled water to the tube, and mix until dissolved. This is a 10 mM solution of salicylic acid.

5. To a 100 mL Pyrex flask or Pyrex bottle, add 25 mL of distilled water.

6. To the vessel with water in it, add a small Teflon-coated magnetic stir bar. Place vessel on magnetic stirrer.

7. To the vessel, add 0.48 grams of tris base (also known as Trizma base).

8. To the vessel, add 8 mL of 100 mM EDTA solution to the vessel.

9. To the vessel, add 0.185 grams of fresh dithiothreitol (DTT).

10. To the vessel, add 800 microliters of 10 mM salicylic acid solution.

11. Once the tris, salicylic acid, and DTT have been dissolved, titrate the solution to pH 7.8 using 3 N hydrochloric acid (HCl).

12. Add 1.6 grams of sodium dodecyl sulfate from a bottle purchased within the last three months and has not been opened for more than five days.

13. To the vessel, add 800 microliters of dimethyl sulfoxide.

14. To the vessel, add 0.0015 grams of D-sorbitol.

15. If working with coral samples, add 2.4 grams of polyvinylpolypyrrolidone (PVPP) to the vessel.

16. Allow the solution to mix on the magnetic stirrer for about 15 minutes at a temperature above 23°C.

17. Label your locking 2.0 mL microcentrifuge with the appropriate sample identification.

18. Purchase a protease inhibitor cocktail solution from any reputable commercial source. The protease cocktail should contain the following ingredient and have a minimal stock concentration: 0.4 mM Bestatin, 0.1 mM E-64, 2 mM phenylmethylsulfonyl fluoride, 2 mM benzamidine, 5 mM a-amino-caproic acid, and 1 μg/100 μL pepstatin A.

19. Add 10-20 microliters of the protease inhibitor cocktail solution to the inside of the open lid that caps the microcentrifuge tube. This location is on the side of the lid that closes within the microcentrifuge tube.

20. For each species, the volume or weight of the frozen ground sample powder that will be homogenized in the microcentrifuge tube must be experimentally determined. The final total soluble protein concentration of the sample should not be more than 2 mg/ml and should not be less than 0.125 mg/mL.

21. Add the appropriate volume/weight of frozen powdered sample to the locking microcentrifuge tube using a pre-chilled spatula (**Figure 16**).

22. Add 1,700 microliters of fresh denaturing buffer in which the PVPP has been maintained in a equally distributed, suspended state in the buffer.

23. Close the lid on the microcentrifuge tube and lock the lid.

24. Vortex the tube with a mechanical vortex for about 20 seconds (**Figure 17**).

25. Incubate the tube in a temperature-controlled metal block or water bath that is set at 93°C for three minutes.

26. Vortex the sample tube for about 20 seconds.

27. Incubate the tube at 93°C for three minutes.

28. Set the tube out on the bench and allow cooling to room temperature (e.g., 25-28°C) for about 10 minutes.

Figure 16. Remove frozen, pulverized sample from cryo-vial with a pre-chilled spatula. Place sample on spatula into a locking microcentrifuge tube. Photograph courtesy of Phillip Dustan.

Figure 17. Before 93°C incubation and after the third minute of incubating at 93°C, vortex the sample for about 20 seconds, insuring thorough mixing of the sample with the Denaturing Buffer. Photograph courtesy of Phillip Dustan.

2.1.2 Phase partitioning of sample by centrifugation

1. Place the microcentrifuge tube into a microcentrifuge and centrifuge the tube at about 12,000 g or higher for 10 minutes.
2. Three phases will be evident after centrifugation: the bottom phase is the insoluble phase consisting predominantly of coralline skeleton, extra-cellular matrix, other insoluble tissue material and PVPP (**Figure 18**). The middle phase should be transparent, brownish (coral, conch) or reddish (fish) in color, and free of a whitish film that characterizes the top phase. The top phase is a very viscous matrix composed predominantly of cross-linked polysaccharides and fats.
3. Aspirate 200-300 µL of the middle phase; be careful not to collect any supernatant that may be contaminated with the whitish matrix that is the top phase.
4. Deposit the middle-phase supernatant into a new tube that is labeled with the same identification.

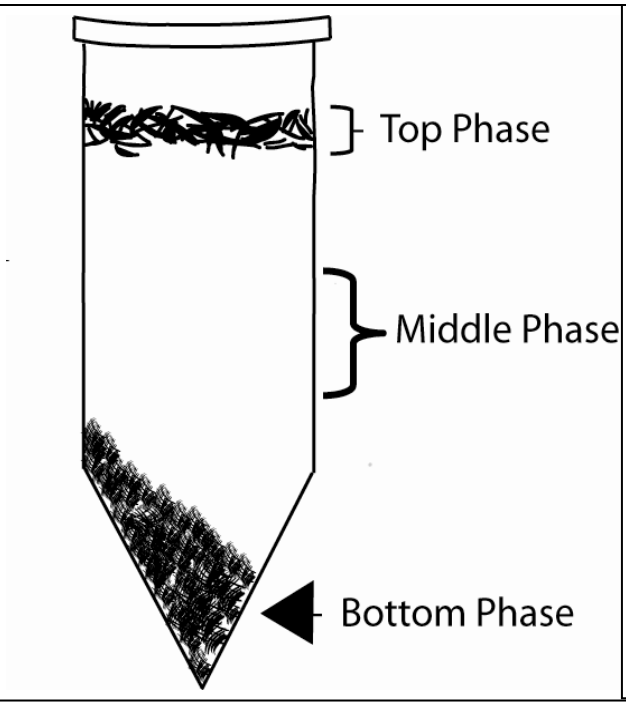


Figure 18. Three phases are formed after centrifugation of the sample. The bottom phase is insoluble debris, primarily calcium carbonate skeleton and insoluble PVPP. The middle phase should be relatively transparent and should be absent of residual mucus. It should be non-viscous. The top phase should be somewhat viscous and sticky, whitish in color. The demarcation between the middle and top phase is not always apparent, and care should be taken that when aspirating the middle phase, there should be no contamination from the top-phase matrix. From Downs, C.A. (2005) Cellular Diagnostics and its application to aquatic and marine toxicology. In: Techniques in Aquatic Toxicology, vol. 2: G. Ostrander (ed). CRC Press, Inc. Boca Raton, Florida. Pp 181-208.

2.1.3 Total soluble protein concentration determination of a sample

Lowry-based, bicinchoninic acid-based, and coomassie blue-based spectrophotometic protein concentration assays cannot be used to determine the protein concentration in the samples because the concentrations for many of the components in the Denaturing Buffer will cause significant artifact for these assays. An alternative assay platform was developed by Dr. Sibdas Ghosh in the late 1980s to overcome interfering substances from plants (e.g., chlorophyll, xanthophylls) that used cuvette-based spectrophotometric protocols. This assay was later modified by Ghosh and Downs in the mid-1990s to account for high concentrations of dithiothreitol and sodium dodecyl sulfate in the Denaturing Buffer which significantly interferes with both copper and silver dyes from binding to proteins.

1. Two solutions should be made: Stain Solution and a Destain Solution. The recipe for both solutions are:

   Stain Solution:
   - 200 mL of distilled water
   - 50 mL of glacial acetic acid
   - 250 mL of methanol
   - 4 g of Coomassie Blue RR 250

   Destain Solution:
   - 800 mL distilled water
   - 200 mL of glacial acetic acid
   - 1000 mL of methanol

   Make each solution in a pyrex bottle with a plastic lid. The staining solution has a shelf life of about 20 days. The destain solution has a shelf life of about four months. Be careful with both solutions and conduct this assay in a well-ventilated area or under a chemical hood. Methanol is a cumulative poison. Inhalation of the vapors of both solutions should be avoided.

2. Place clean lab mat down on in the area on the lab bench where you will be conducting the protein concentration assay.

3. Wearing latex or nitrile gloves, take a piece of Whatman #5 filter paper using forceps and the filter paper on the clean lab mat. Whatman filter paper disks that are 9 cm to 11 cm are the best to use.

4. With a #2 pencil, mark the filter paper indicating the location of the standards, placement of the samples, and the identification of the project and the date, similar to that in **Figure 19**.

5. Using a 1-20 uL pippettor, place 1 uL of sample and standard in an ordered fashion as indicated by the sample locations marked with pencil. Standards and samples should be spotted on the Whatman filter paper in triplicate (**Figure 19**).

The pipette tip should be changed for a new tip between spotting each sample and each standard. You can use the same tip for spotting the triplicate sample spots.

6. Allow the filter paper to air dry for about 20 minutes. You can accelerate the drying process by using a hair dryer to dry the filter paper. Use forceps to hold the filter paper in place while using the hair dryer. Do not use your fingers.

7. Once dried, place the filter paper in an incubation boat that contains enough Stain Solution to completely cover the filter paper.

8. Place the incubation boat on a rocker platform or hand agitate for 10 minutes (**Figure 20**).

9. After the 10 minute incubation, pour the Stain Solution back into its bottle.

10. Wash the filter paper with about 20 mL of Destain Solution. Decant the Destain Solution into the sink or into the proper waste receptacle.

11. Incubate the filter paper in about 30 mL of Destain Solution for about five minutes, and then decant the Destain Solution. Repeat this procedure until the non-spotted filter paper is white (**Figure 21**).

12. Gently wash the filter paper in distilled water, then allow to incubate in water for two minutes on the rocking platform or by hand agitation. This will remove the overwhelming smell of the acetic acid and allow the filter paper to be placed in the laboratory notebook.

13. Once the filter paper is dried, it can be scanned into a digital image by a scanner. Optical densities for each replicate can be determined using a densitometry program such as NIH Image developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/.

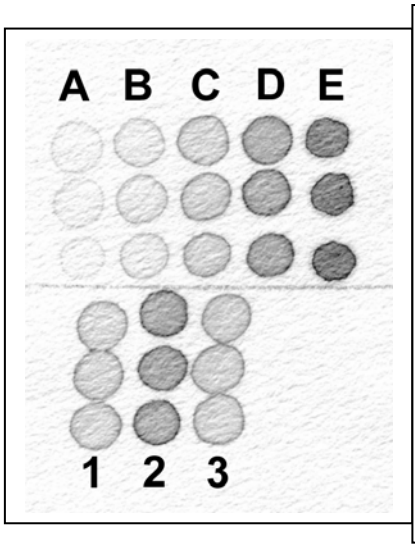


Figure 19. Protein Concentration Assay. Results of protein concentration assay using a modified method of Ghosh et al. (1988). Concentration calibrants are plated on the Whatman No. 5 filter paper as in Columns A - E. Column A = 0.125 µg/µL. Column B = 0.250 µg/µL. Column C = 0.5 µg/µL. Column D = 1.0 µg/µL. Column E = 2.0 µg/µL. Samples done in triplicate indicated in columns 1-3. All calibrants and samples are plated (hence assayed) on the filter paper in triplicate. Assay paper can be scanned by a scanner to create a digital image. Optical densities for each replicate can be determined using a densitometry program such as NIH Image developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/. From Downs, C.A. (2005) Cellular Diagnostics and its application to aquatic and marine toxicology. In: Techniques in Aquatic Toxicology, vol. 2: G. Ostrander (ed). CRC Press, Inc. Boca Raton, Florida. Pp 181-208.

Figure 20. Incubate the filter paper in an incubation boat. The boat can be anything: e.g., petri dish or weigh boat, and be made from plastic or glass. Stain solution should cover the filter paper either in its entirety or so that each rocking slant covers at least 50% of the filter paper in the Stain Solution.

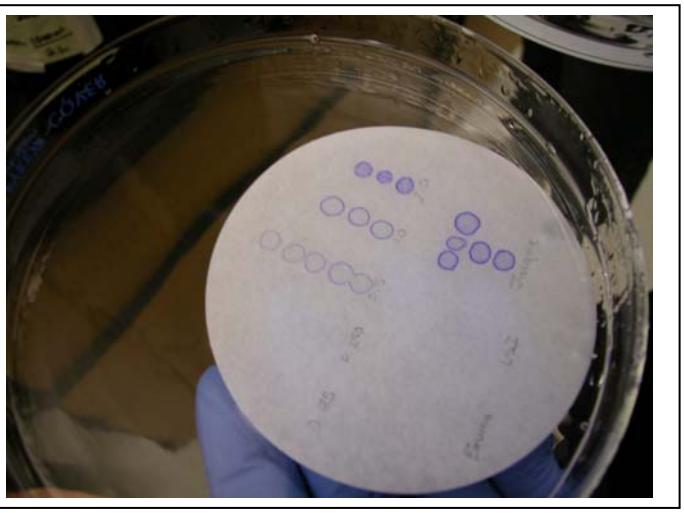


Figure 21. There should be a distinct contrast between the blue of the sample and standard spots and the rest of the filter paper. The area of the filter paper that has not been spotted should be white. Be careful! It is possible to over destain the filter paper.

Once the protein concentration assay is done, Denatured Buffer-extracted samples can be frozen. The best temperature to preserve the samples is -80°C or colder. The samples can only be thawed once, so if you plan to conduct your ELISAs for more than a one-day time period, you need to aliquot the samples into new tubes so that a single tube can be removed from cold storage only once according to your work schedule.

2.1.4. Enzyme Linked Immuno-sorbent Assay (ELISA)

There are a number of different ELISA methods that can be used. This document does not advocate a specific method, but only methods that have a measure of validation and quality control. Before an ELISA is run on a sample or a set of samples, the antibodies and solution reagents need to be optimized and validated. This should include, at the very least, testing of the samples and the reagents using an SDS-polyacrylamide gel electrophoresis (PAGE) and western-blotting assessment system. A detailed description of SDS-PAGE and western blotting for validation of the ELISA system can be found in Downs, C.A. (2005) Cellular Diagnostics and its application to aquatic and marine

toxicology. In: Techniques in Aquatic Toxicology, vol. 2: G. Ostrander (ed). CRC Press, Inc. Boca Raton, Florida. Pp 181-208. Besides validation of the antibody and the ELISA reagents, SDS-PAGE/western blotting also helps in determining the integrity of the sample. Oftentimes, artifacts can befall a set of samples as a result of insufficient protease inhibition during the homogenization of the sample, freeze/thaw exclusion of SDS from proteins which results in protein aggregation, and adduction of target proteins with secondary compounds resulting in masking of epitopes and/or polymerization of heterogeneous protein species.

One of the least expensive methods to conduct an ELISA is a Dot-Blot/Direct ELISA method. A protocol for this method can be found Downs, C.A. (2005) Cellular Diagnostics and its application to aquatic and marine toxicology. In: Techniques in Aquatic Toxicology, vol. 2: G. Ostrander (ed). CRC Press, Inc. Boca Raton, Florida. Pp 181-208. This method requires a primary and secondary antibody. The secondary antibody can be conjugated to any number of different reporter enzymes that catalyze a colorimetric or chemiluminescent signal. Alternatively, the secondary antibody can also be conjugated with a fluorescent probe that can be used in a fluorescence detection system. The least expensive is a secondary antibody conjugated to either alkaline phosphatase or horseradish peroxidase that uses nitroblue tetrazolium and an electron donor compound. Samples and the standard calibrant curve should be replicated in triplicate (**Figure 22**). A five-point calibration curve should at least be used, especially when examining biomarkers such as heat-shock proteins and cellular damage products. These end-points can often have concentration differences of several fold to an order of magnitude.

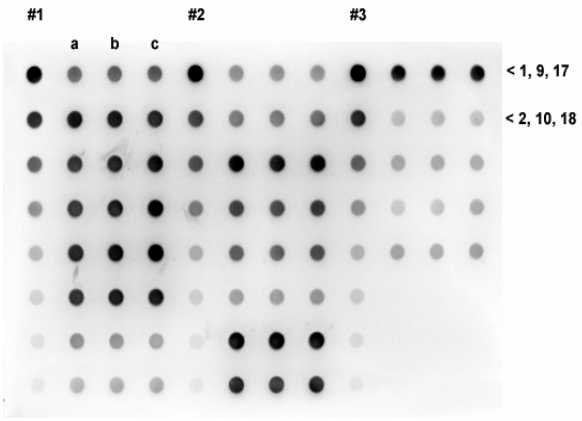


Figure 22. ELISA result using a 96-well dot blotter**.** # = calibrant standard curve. The calibrant with the highest concentration is plated in row 1, the calibrant with the lowest concentration is plated in row 8. Optimally, replicates of the standard curve should be evenly distributed across the dot blotter. In row 1, triplicate assays of sample 1, 9, 17. In row 2, triplicate assays of sample 2, 10, 19. In row 3, etc. In column ***a***, replicate one of sample 1, 2, 3, etc... are plated. In column ***b***, replicate two of samples 1, 2, 3, etc… are plated. In column ***c***, replicate three of samples 1, 2, 3, etc… are plated. Plate represents data for the chloroplast small heat-shock protein in samples of the hard coral, *Montastrea annularis*.

A second type of ELISA method uses a polystyrene, 96-well microtiter plate. This method often requires a microplate washer instrument and a microplate reader instrument. Samples and calibrant standards can be configured the same way as the dot-blot ELISA system (**Figure 23**). A good methodology resource for this ELISA method can be found in Crowther, J.R. (2001) The ELISA Guidebook: Methods in Molecular Biology. Humana Press. Totowa, New Jersey.

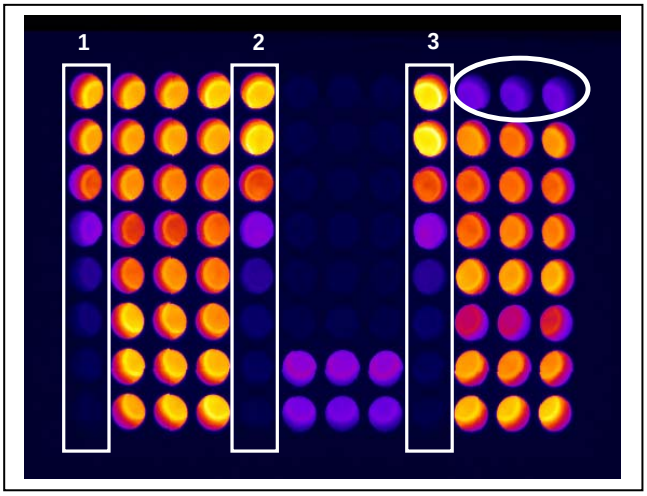


Figure 23. ELISA result using a 96-well microtiter plate**.** 1,2,3 = calibrant standard curve. The calibrant with the highest concentration is plated in row 1, the calibrant with the lowest concentration is plated in row 8. Optimally, replicates of the standard curve should be evenly distributed across the microtiter plate. The three wells that are circled are the triplicate of sample #17. The detection system used here is based on a chemiluminescent reporter system. This is the most sensitive detection system you can use; The lower calibrant-curve detection limits are often in the range of 1 to 400 picomoles for a target.

It should be recognized that ELISA is based on the kinetic characteristics of its components (e.g., binding of sample to solid phase, primary antibody binding to target protein, secondary antibody binding to primary antibody, washes, etc.). Because of the nature of this assay platform, conducting an ELISA should not be conducted like a "cook book" exercise. Each step requires optimization to ensure consistency in assay repetition, confidence in raw data generation, and consistency for inter-laboratory assessment and validation. This optimization can only be done experimentally.

2.1.5 ELISA end-points (biomarkers) for grunt, conch, and coral samples

The biomarkers in this section have been selected because changes in their concentration indicate a significant change in the physiological condition of an organism or a specific homeostatic response of that organism to environmental conditions. It must be cautioned that the life history of a specific organism/population must be considered when making a diagnostic interpretation using any of these biomarkers. For example, sampling during the reproductive season or mating season of an organism may produce a biomarker result that is determined, in part, not only by environmental conditions, but also because of life history conditions. The age of the organism may also influence biomarker results. A wise sampling assessment and monitoring design can overcome these hurdles: for example, collecting samples that are of the same age, or sampling a population outside of its spawning season.

Another important issue that must be addressed is establishing a normal range for a biomarker. A "normal range" can be defined as the concentration or activity of a biomarker that can be found in a population that is in a physiologically healthy condition, or a homeostatic condition that is not responding to a stressor. Determining and defining the "normal range" can be accomplished by incorporating a reference population within the assessment or monitoring design. Criteria for a reference population may be based on aspects of physiological performance and environmental conditions. For example, a potentially good candidate reference population should possess a level of reproductive fitness that can sustain the population. Disease incidence should be relatively low.

Environmental conditions should be consistent with a habitat that has historically sustained populations.

The ELISA biomarkers presented in this document include:

- Ubiquitin
- Multiple Drug/Xenobiotic Resistance Protein
- Cytochrome P450 2-class Proteins
- Invertebrate Small Heat-shock Proteins (for corals and conch)
- Fish Small Heat-shock Protein
- Metallothionein
- Cytochrome P450 6-class Proteins (invertebrates only)

A display in the levels of these biomarkers outside of their established normal range indicates a significant change in the physiological condition or homeostatic response of the organism/population. Any one of these biomarkers examined by itself, or with consideration of the any of the other ELISA-based biomarkers described in this section, is not meant to establish an etiology of pathology. These biomarkers are meant to be used as harbingers of physiological and population change if the organism/population remain in a persistent condition.

Ubiquitin – This end-point can be assessed in all three target species most likely using the same antibody source. This is because of the extreme conservation of this polypeptide and its ubiquitous presence and function in all eukaryotes. Ubiquitin is a 76-residue protein found in most phyla of life and used in a process for marking proteins for rapid degradation (Hershko and Ciechanover, 1998. The ubiquitin system. *Annu Rev Biochem* 67: 425-479.). Ubiquinated proteins are degraded by proteolytic enzymes known as proteosomes (Hershko and Ciechanover, 1998. The ubiquitin system. *Annu Rev Biochem* 67: 425-479.). Proteins, during stress, are targeted for degradation usually because these proteins have undergone an irreversible denaturation (Iwai, K. 1999. Roles of the ubiquitin system in stress response. *Tanpakushitsu Kakusan Kuso* 44: 759-765). Increases in ubiquitin levels are an indication of increased levels of protein degradation, and hence, increased protein turnover (Goff et al., 1988. Protein breakdown and the heat-shock response. In *Ubiquitin* (ed. M. Rechsteiner). New York, NY: Plenum Press: 207-238.). Consequently, to compensate for decreased functional protein levels due to stress, the cell will increase production of these same proteins (Iwai, 1999). Thus, measurement of levels of ubiquitin is an index of the structural integrity of the protein component of the superstructure of the cell (Mimnaugh et al., 1999. The measurement of ubiquitin and ubiquinated proteins. *Electrophoresis* 20: 418-428). Increased ubiquitin levels above a normalized range indicates: (1) a protein denaturing stress is occurring; (2) increased expenditure of energy is required to compensate for this stressed-induced protein turnover; and (3) in comparison to baseline data of this parameter for a particular species, may act as an indicator of individual fitness (Hawkins, 1991. Protein turnover: a functional appraisal. *Funct Ecol* 5: 222-233).

For this assay, it is important to use an antibody that can recognize the ubiquitin monomer and polyubiquinated chains. Without this type of recognition, detecting changes in ubiquitin equilibrium may not be possible. Antibody to ubiquitin and purified ubiquitin can be purchased from over half-dozen commercial sources (e.g., Sigma-Aldrich).

Multiple Drug/Xenobiotic Resistance Protein (ABC family of proteins) – This end-point will be measured in all three target species most likely using the same antibody source. This is because of the extreme conservation of this protein's primary structure, function and its ubiquitous presence and function in all eukaryotes. P-glycoprotein plays a role in removing xenobiotics and toxins from the cell by acting to transfer intracellular xenobitics and toxins across the cytoplasmic membrane. Sustained exposure to certain xenobiotics causes an increase in the cellular level of P-glycoproteins. Animals deficient in P-glycoproteins are viable and do not exhibit any obvious abnormalities. The pharmokinetic activity and relative toxicity of several compounds are, however, altered in these P-glycoprotein-deficient animals. For example, P-glycoprotein in mammals plays a significant role in preventing certain xenobiotics from crossing the blood-brain barrier: a decrease in P-glycoproteins would result in an increase in the amount of xenobiotics reaching the brain. P-glycoproteins are members of a superfamily of proteins called the adenosine triphosphate binding cassette that act as channels and transporters of solutes across membranes. Induction of certain P-glycoproteins indicates a response to xenobiotic exposure. See Ueda et al., (1999). Comparative aspects of the function and mechanism of SUR1 and MDR1 proteins. Biochem. Biophys. Acta 1461: 305-313; Borst et al., (1999). The multidrug resistance protein family. Biochem. Biophys. Acta 1461: 347-357; Bard (2000) Multixenobiotic resistance as a cellular defense mechanism in aquatic organisms: a review. Aqua. Toxicol. 48: 357-389. A simplified interpretation for this biomarker is that if levels of this protein increase, it may mean that the organism is responding to an exposure of a xenobiotic or a toxin. As always, there are a number of caveats: some xenobiotics do not elicit an increase in P-glycoprotein levels, such as mercury.

Cytochrome P450 2 homologue (CYP P450-2) – An antibody that cross-reacts with the mammalian homologue of cytochrome P450 2-class, but not CYP 1-class or CYP 3-class can be used to measure the accumulation of an antigenically similar CYPs in the three target species. Cytochrome P450 2-class enzymes are known to specifically oxidize ethanol to acetaldehyde via a monooxygenase mechanism, as well as other xenobiotics such as imidazole-based derivatives (Lieber, 1997. Cytochrome P-4502E1: its physiological and pathological role. *Physiol Rev* 77: 517-544). Cytochrome P450 2-class has both physiologically relevant oxidative and reductive reactions and is known to associate and catalyze as many as 60 xenobiotic-based substrates (Lieber, 1997). For example, it causes the demethylation of N, N-dimethylnitrosamine and the hydroxylation of p-nitrophenol and chlorzoxazone (Koop, 1992. Oxidative and reductive

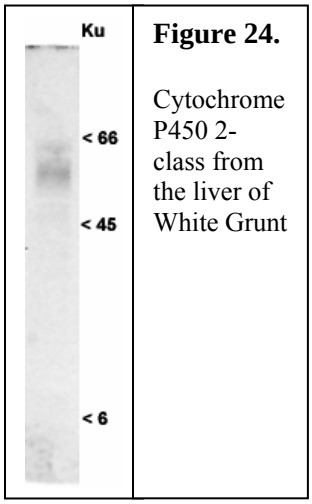


Figure 24. Cytochrome P450 2-class from the liver of White Grunt

metabolism by cytochrome P450 2E1. *FASEB J* 6: 724-730). Reduction reactions include reduction of a number of different lipid types. This enzyme homologue is conserved in all three target species, though the function may be less conserved. This enzyme is known to be induced in all three species by a variety of crop protection products. This is not to say that these enzyme homologues function by detoxifying these crop protection products, though in some species they may play a central role in Phase I of Xenobiotic Transformation (e.g., Hanioka, N., Jinno, H., Tanaka-Kagawa, T., Nishimura, T., Ando, M., and Ogawa, K. 1998. In vitro biotransformation of atrazine by rat liver microsomal cytochrome P450 enzymes. *Chem Biol Interact* 116:181–198).

Changes in the expression level from the nominal range for this biomarker indicate a changed physiological response, especially a changed endocrine and metabolic condition.

Antibody to cytochrome P450 2-class is readily available from a number of commercial sources. Alternatively, this enzyme can oftentimes be assessed by enzymatic activity. Several commercial sources provide protocols and kinetic assay kits to analyze enzymatic activity. In-house production for an antibody against CYP P450 2-class homologues is also straightforward since this enzyme has three evolutionarily conserved domains that define this class of enzyme.

Invertebrate sHsp (total small heat-shock protein isoforms) – This biomarker would only be used for the invertebrate target species, not for the grunt. αB-crystallin, Hsp22, Hsp23, Hsp26, and Hsp28 share domains of common homology to one another, but have different cellular functions (de Jong et al., 1993). In bivalves, as many as 5-6 major sHsp isoforms exist. Small Hsps from all phyla share a common motif near the carboxyl-terminal end of the protein, known as the "heat-shock domain" or α-crystallin domain (de Jong et al., 1993). Other areas of these proteins are not homologous and are specific to the sub-family of sHsps. In most cases, the small heat-shock proteins are not present during optimal growing conditions and are only elicited by stress (de Jong et al., 1993).αB-crystallin is a small heat-shock protein found only in the cytosol of animals, where it protects cytoskeletal elements during stress (Derham and Harding, 1999). Evidence indicates that Hsp22 localizes to neural-type cells and follicular cells in *Drosophila melanogaster*. Furthermore, this protein localizes to the mitochondria in arthropods and has been suggested to have a functional role similar to that of plant and mammalian mitochondrial sHsps (Downs & Heckathorn, 1998; Downs et al., 1999a). Thus, the presence and concentration of different small heat-shock proteins reflects the physiological status of several metabolic and structural pathways in the cell. In this study, it can be used as an indicator of a severe stress response. One caveat for this biomarker is that it has been shown to be expressed during spawning and at key

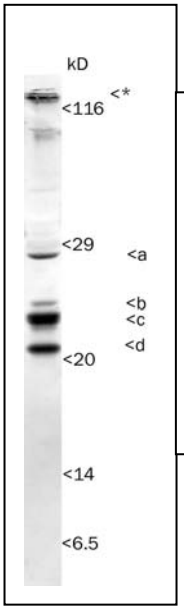


Figure 25. Invertebrate small heat-shock proteins

30 μg of total soluble protein from ribbed mussel
* = multimeric structure
a = Hsp28
b = Hsp25W or Hsp26
c = Hsp23
d = Hsp22

stages of early development – extremely stressful events in-and-of themselves. Care should be taken not to sample target species during these developmental stages.

Fish small heat shock protein – This biomarker could only be used for a fish species. Like the invertebrate small heat-shock proteins, this protein is usually induced only as a result of severe cellular stress. Less is known about the function of this protein as compared to other heat-shock proteins. Antibody for this protein will need to be manufactured "in-house" by the laboratory that is running this assay. The sequence to this gene is known from a number of species of fish and the epitope can be made against an evolutionarily conserved sequence in the carboxyl-terminal end of the protein known as the "crystalline" domain.

This protein is usually absent during normal physiological conditions and is usually induced under extremely stressed conditions. Detection or an increased accumulation of this protein may signify that the population/individual is under metabolic duress.

White CN, Hightower LE, Schultz RJ. 1994. Variation in heat-shock proteins among species of desert fishes (Poeciliidae, Poeciliopsis). Mol Biol Evol. 11(1):106-19.

Metallothionein – Metallothionein is often used as a biomarker of heavy metal exposure, though it is argued that this may be a misuse of this biomarker (*e.g.*, Aspholm and Hylland, 1998; Soazig and Mark, 2003; Galloway et al., 2004). In the context of cellular diagnosis, changes in metallothionein levels can be interpreted as an indicator of broad changes in mitochondrial functional equilibrium. This position is based on recent work demonstrating that metallothionein type 1 localizes to the inter-membrane space of mitochondria and can regulate oxidative phosphorylation (Simpkins et al., 1994; Ye et al., 2001). Studies showing the interaction of zinc, metallothionein, and mitochondrial function further support the role of metallothionein as a regulator of cellular energy production and redox state (Maret, 2000; Coyle et al., 2003; Maret, 2003). This interpretation is further justified by toxicology studies with cadmium and metallothionein that demonstrate the interaction between cadmium and zinc metallothionein and mitochondrial dysfunction (*e.g.*, Simpkins et al., 1998a; Klassen et al., 1999; Tang and Shaikh, 2001). Metallothionein can be induced by a number of different stressors, for example chronic oxidative stress; metallothionein gene expression in fish can be induced by exposure to chlorinated aromatic hydrocarbons (Gerpe et al., 1998).

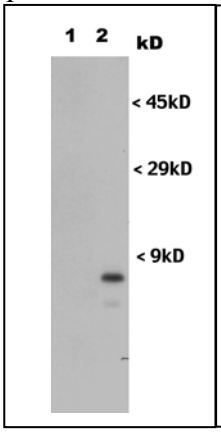


Figure 26. Metallothionein
Lane 1: 30 ug of total soluble protein from control *Mytilus edulitus.*
Lane 2: 30 ug of total soluble proteim from *Mytilus edulitus* exposed to 50 uM CdCl2 for eight hours. Two-three bands appear, depending on the type of metal exposure. Detects novel copper-inducible isoform.

Metallothionein gene sequences are known from a number of invertebrate species (including gastropods and cnidarians) and from more than 15 species of fish. Gene sequence can be aligned for the target phylum and be used to create an antigen that can be immunized into a host for antibody production (Figure 26).

A simplified interpretation for this biomarker is that levels of this protein outside the nominal range for this organism indicate a homeostatic response to maintain mitochondrial oxidative phosphorylation equilibrium. This marker should not be used as a biomarker of exposure to heavy metals.

Cytochrome P450 3 homologue (CYP P450-3) – An antibody that cross-reacts with the homologue of cytochrome P450 3, but not CYP 1A, CYP 2 or CYP 6 classes can be used to measure the accumulation of an antigenically similar CYP in all three target species. Cytochrome P450 3 homologues are known to play a role in steroidal biogenesis and porphryn metabolism. Induction of CYP P450-3 is oftentimes associated with cellular xenobiotic response. This may be true, but alterations in the accumulation and activity of this enzyme class can also result from changes in cellular demand for porphyrins and for steroid metabolism. The value of CYP P450-3 may not always be associated with it being a "biomarker of exposure", but as a biomarker of effect.

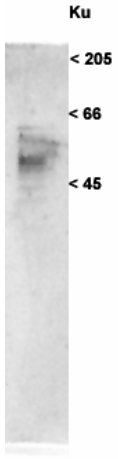


Figure 27. CYP P450-3 from liver of White Grunt

An antibody against CYP P450-3 homologues can be obtained from a number of commercial sources, but should be validated for each of the target species. Several polyclonal antibodies from at least three different commercial sources were tested by the author and found to work in all three of the target species.

A simplified interpretation for this biomarker is that if levels are beyond the nominal range, this indicates a change in the endocrine and metabolic condition of the organism. Oftentimes, an increase in the level of this protein is associated with an organic-based xenobiotic exposure.

Cytochrome P450 6 homologue (CYP P450-6) – This enzyme is specific to invertebrates and is recognized as a major contributor to insecticide resistance (Scott JG, Wen Z. 2001. Cytochrome P450 of insects: the tip of the iceberg. Pest Mang. Sci. 57:958-967). This class of CYP P450s is known to oxidize and be up-regulated by pesticides such as aldrin, dieldrin, diazinon, chlorpyrifos, deltamethrin, and a wide range of pyrethrin-like compounds. This enzyme has been used as both a biomarker of exposure and as a biomarker of effect. Assaying for this biomarker can only be done with coral and conch, not with White Grunt since vertebrates are not known to have this enzyme class.

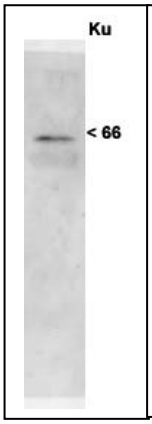


Figure 28. CYP P450 6 class.

From *Montastrea Annularis*

Courtesy: U.S. NOAA

A simplified interpretation for this marker is that if levels of this protein are higher than the nominal range, this organism is potentially responding to a specific class of pesticides. Decrease of this protein below the nominal range may indicate a compromised metabolic condition.

2.2. Non-ELISA-based Biomarker Assays

The following biomarkers are non-ELISA-based assays and will require additional equipment, different sample homogenization and preparation procedures, and different methods for detection. Like the ELISA-based biomarkers, these biomarkers display a dynamic quantitative range, and that the levels of these biomarkers outside of their established normal range indicate a significant change in the physiological condition or homeostatic response of the organism/population. Any one of these biomarkers examined by itself, or with consideration of the any of the other ELISA-based and non-ELISA-based biomarkers described in this section, is not meant to establish an etiology of pathology. These biomarkers are meant to be used as harbingers of physiological and population change if the organisms remain in a persistent condition.

2.2.1. Porphyria Species Assay

Materials

- Fluorescent 96-well microplate reader or a spectrofluorospectrophotometer
- Multichannel pipettor, 20-200 microliters
- Single channel pipettor set
- 3 N hydrochloric acid
- Black-walled 96-well microtiter plates
- Uroporhyrin standard (Porphyrin Products, Inc., Logan, Utah, U.S.A.)
- Ethyl acetate (99% purity)
- Sodium dodecyl sulfate
- Trizma base
- Sodium dodecyl sulfate

Porphyrins are prosthetic structures usually associated with proteins that contain a metal for functionality (**Figure 29**). A porphryin with an iron in it is heme. A porphyrin with a phytol chain and a manganese atom is chlorophyll. The porphyrin synthesis pathway begins in the cytosol and concludes within the mitochondria (**Figure 30**). At least six different enzymes are directly involved in the porphyrin synthesis pathway. Each one of these enzyme expresses differential sensitivity to the inhibitory power of environmental contaminants, such as heavy-metals, organochlorine compounds, organophosphorous compounds, and polycyclic aromatic hydrocarbons. A porphyrin synthesis enzyme inhibited by a toxicant results in the accumulation of precursor porphryn species. Accumulation of these precursors can lead to pathological effects - porphyria. Measurement of porphyria species is a classical clinical diagnostic test with straightforward diagnostic interpretation. Measurement of porphyrin-precursor (porphyria) species can be conducted in almost any coral reef species with little modification to the method. This method measures total porphyrin precursors and is unable to distinguish which porphyrin species is accumulating.

The method can be validly conducted using two protocols. The first method capitalized on the sample preparation conducted for ELISA. The second method can be employed on a sample independent of the ELISA-sample-preparation protocol. Both

methods should be conducted under dimmed light or a red light. Direct sunlight needs to be excluded from the laboratory.

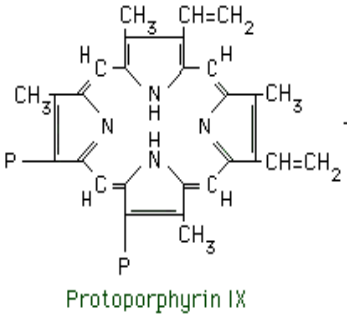


Figure 29. A precursor porphyrin species

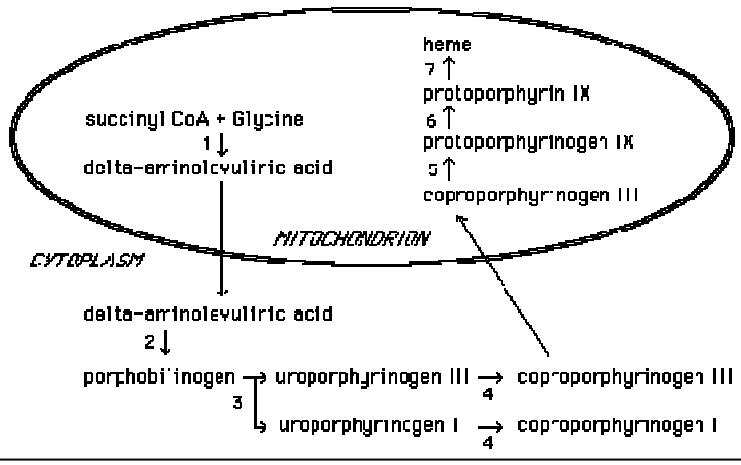


Figure 30. Schematic of the porphyrin/heme synthesis pathway. Courtesy of **medlib.med.utah.edu/ NetBiochem/hi3.htm**

Concentration of Total Porphyria Species using ELISA sample preparation

1. One-hundred and fifty micrograms of total soluble protein from sample supernatants prepared in Section 3.1.1. are diluted in a solution containing 1% SDS, Tris-HCl (pH 7.8), 5 mM EDTA into a volume of 310 microliters.

2. Dispense one-hundred microliter aliquots of each sample in triplicate to a clear-bottom with black walls 96-well microtiter plate.

3. Dispense 50 uL of 3 N hydrochloric acid into each sample well.

4. Place plate in a container that exclude light and allow incubation for fifteen minutes.

5. Mix the contents in each sample well by gently rocking the plate.

6. Uroporphyrin standards can be obtained from Porphyrin Products (Logan, Utah, U.S.A.), and diluted using an 8-point calibrant standard curve from 0 pmole of protoporphyrin to 1000 pmole of uroporphyrin. Dispense standard on the micro-titer plate in triplicate using a format similar to **Figure 23**.

7. Detect fluorescence signal using a fluorescent/luminescent microplate reader with the excitation filter set for 405 nm and the emission filter set for 610 nm. Band length for both excitation and emission filter should be less than ±20 nm.

Concentration of Total Porphyria Species using ethyl acetate extraction

1. Take about 40 microliters of frozen sample powder from the sample cryovial using a pre-chilled spatula and place into a locking microcentrifuge tube.

2. Add 700 microliters of 99% pure ethyl acetate.

3. Vortex sample for about two minutes until semi-homogenized.

4. Add 700 microliters of 3 N hydrochloric acid to the sample tube.

5. Vortex sample for about two minutes.

6. Incubate in dark for five minutes

7. Vortex sample for one minute.

8. Centrifuge sample at 12,000 *g* or higher for five minutes.

9. Aspirate the acidified phase from the tube and place in a new tube. This volume should be about 400 microliters. You will need to determine experimentally for your protocol which is the acidified phase. The acidified phase should be absent of any mucilage. Do not confuse the acidified phase with the ethyl acetate phase.

8. From the new tube, dispense one-hundred microliter aliquots of each sample in triplicate to a clear-bottom with black walls 96-well microtiter plate.

9. Uroporphyrin standards can be obtained from Porphyrin Products (Logan, Utah, U.S.A.), and diluted using an 8-point calibrant standard curve from 0 pmole of protoporphyrin to 1000 pmole of uroporphyrin. Dispense standard on the micro-titer plate in triplicate using a format similar to **Figure 23**.

10. Detect fluorescence signal using a fluorescent/luminescent microplate reader with the excitation filter set for 405 nm and the emission filter set for 610 nm. Band length for both excitation and emission filter should be less than ±20 nm.

2.2.2 Ethoxyresorufin-*O*-deethylase (EROD) activity assay

Materials

- Trizma Base
- EDTA
- Hydrochloric acid
- Ethoxyresorufin
- nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)
- acetonitrile

- Fluorescent microplate reader or spectrofluorophotometer

This biomarker can represent a number of different assessment end-points. In the literature, this biomarker is predominantly used as an end-point that reflects a receptor-mediated induction of cytochrome P450 mono-oxygenase activity. This putative receptor is usually identified as the aryl hydrocarbon receptor. This biomarker is also used as a "response indicator" to contaminant uptake; the contaminant most likely a hydrocarbon-based compound or mixture of compounds. Another value of the EROD assay is associated with its decline-in-activity behavior. A number of environmental contaminants and conditions can either inhibit enzyme activity directly or depress gene expression, potentially reducing protein expression of this enzyme. One caveat with this assay is that induction of EROD activity should not be confused with it being a reflection of toxicity. Activity may increase without any pathological symptoms. The catalysis of ethoxyresorufin by a cytochrome P450 monooxygenase action results in the production of resorufin – a compound that can be detected through spectrofluorimetric means (**Figure 31**).

This biomarker should only be conducted in fish (White grunt). Though the assay has been tested in some invertebrate species, the EROD assay would need to undergo extensive validation in coral and conch.

To derive the greatest sensitivity for the EROD assay, most protocols recommend purification of the microsomal fraction via differential sub-ultracentrifugation or ultra-centrifugation methods. Unfortunately, the microsomal integrity has been compromised as a result of the pulverization of the fish liver sample using the liquid nitrogen grinding technique. EROD activity is often normalized against total soluble protein of the microsomal fraction. Since this cannot be accomplished as a result of the initial sample preparation, EROD activity will be normalized against total soluble protein of liver tissue.

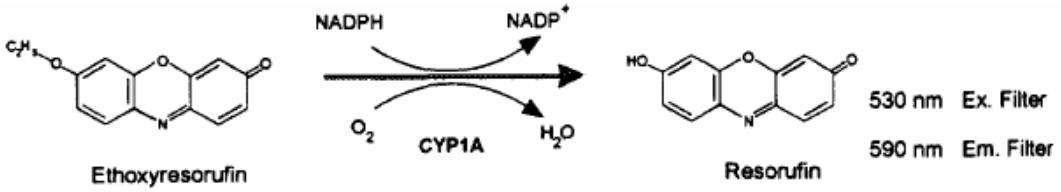


Figure 31. Mechanism of catalysis of ethoxyresorufin to resorufin by Ethoxyresorufin-*O*-deethylase activity.

Ethoxyresorufin-*O*-deethylase (EROD) activity assay protocol

Before conducting this protocol on the target species, the lab must conduct an optimization step before conducting this assay on field samples. Since the EROD assay is an enzymatic assay based on enzymatic kinetics, the Michaelis constant (K_m) and maximum velocity (V_{max}) must be determined for each species, since one cannot assume that the K_m of EROD is the same for every species. This can be determined by keeping the total soluble protein of the sample constant while varying the substrate (ethoxyresorufin and NADPH) concentration. Final concentration of both substrates

should be at least twice the V_{max} concentration to account for varying concentrations of the field sample starting material. Kinetic behavior determination and kinetic modeling for EROD can be determined following principles set forth in Segal, I.H. 1975. Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems. John Wiley & Sons, Inc: New York, New York.

1. Add about 30 microliters of pulverized fish liver sample into a microcentrifuge tube.

2. Add 500 microliters of EROD buffer with is a solution of 50 mM Trizma base and 20 mM EDTA that has been titrated to a pH of 7.8 with 1 M hydrochloric acid.

3. Vortex for 1 minute.

4. Add pre-determined volume of a fixed concentration of ethoxyresorufin solution to the sample.

5. Vortex for 20 seconds.

6. Add pre-determined volume of a fixed concentration of NADPH solution to the sample.

7. Vortex for 20 seconds.

8. Incubate for sample to the experimentally determined time that equals the catalysis of 25% of the available substrates.

9. Stop the reaction with 200 microliters of acetonitrile.

10. Centrifuge sample at 6,000 g for five minutes.

11. Aspirate one-hundred microliter aliquots of supernatant from each sample in triplicate to a clear-bottom with black walls 96-well microtiter plate.

12. Detect fluorescence signal using a fluorescent/luminescent microplate reader with the excitation filter set for 530 nm and the emission filter set for 590 nm. Band length for both excitation and emission filter should be less than ±10 nm.

2.2.3. DNA Abasic quantitative lesion assay

Materials:

- Multi-channel pipettor 20-200 microliter volume
- Single-channel piepptors, 20-200 microliter & 100-1000 microliter
- Double distilled water
- N’-aminooxymethylcarbonylhydrazino-D-biotin

- Streptavidin conjugated to horseradish peroxidase
- Luminol/hydrogen peroxide-based chemiluminescent solution or,
- Horseradish peroxidase nitroblue tetrazolium dye
- DNA AP quantitative standards
- 96-well microplates
- Spectrophotometric or luminescent/fluorescent 96-well microplate photometers

This system can also be purchased as a commercial kit for colormetric-based detection system from:

Dojindo Molecular Technologies, Inc.
211 Perry Parkway, Suite 5
Gaithersburg, MD 20877
Phone: 301-987-2667 Fax: 301-987-2687
E-mail: info@dojindo.com
Web site: www.dojindo.com

Dojindo Laboratories
Kumamoto Techno Research Park
2025-5 Tabaru, Mashiki-machi, Kamimashiki-gun
Kumamoto 861-2202, JAPAN
Phone: +81-96-286-1515 Fax: +81-96-286-1525
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Oxidative damage to DNA is a result of the interaction of DNA with reactive oxygen species (ROS), in particular, the hydroxy radical which is converted from superoxide and hydrogen peroxide by the Fenton reaction. Hydroxy radicals produce a multiplicity of modifications in DNA. Oxidative attack by hydroxyl radical on the deoxyribose moiety will lead to the release of free bases from DNA, generating strand breaks with various sugar modifications and simple abasic sites (AP sites). In fact, AP sites are one of the major types of damage generated by ROS. It has been estimated that endogeneous ROS can result in about 2x105 base lesions per cell per day. Aldehyde Reactive Probe (ARP) reagent (N'-aminooxymethylcarbonylhydrazino-D-biotin, Fig. 33) reacts specifically with an aldehyde group which is the open ring form of the AP sites. This reaction makes it possible to detect DNA modifications that result in the formation of an aldehyde group. After treating DNA containing AP sites with ARP reagent, AP sites are tagged with biotin residues. By using an excess amount of ARP, all AP sites can be converted to biotintagged AP sites. Therefore, AP sites can be quantified using avidin-biotin assay followed by a colorimetric detection of peroxidase or alkaline phosphatase conjugated to the avidin (Fig. 34). **DNA Damage Quantification Kit** contains all the necessary solutions, enabling the determination of 1 to 40 AP sites per 1x105 bp.

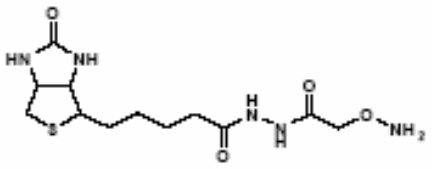


Figure 32. Aldehyde Reactive Probe

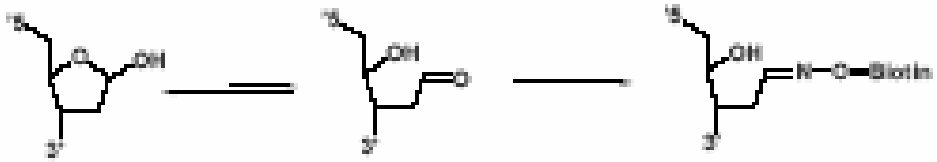


Figure 33. Reaction of ARP with AP site.

Purification and quantitation of genomic DNA

Several different methods and products are available for the isolation of genomic DNA from samples; such as the membrane binding method, the guanidine/detergent lysis method, and the polyelectrolyte precipitation method, to name just a few. Among these methods, the guanidine/detergent lysis method is simple, and it gives highly purified genomic DNA for the ARP-based abasic sites detection. During the purification process, avoid heating of the DNA solution.

Contamination of DNA by protein and other UV absorbing compounds (e.g., polyphenols) should be determined using the 260 nm absorbance/280 nm absorbance spectrophotometric assay. This ratio should be higher than 1.7.

The Absorbance 260nm method for determining DNA concentration is inaccurate and, thus, inappropriate to be used with the DNA AP site assay. A fluorescent-based method for determining double-stranded DNA concentration is much more accurate and precise. Double-stranded DNA quantification assay kits can be obtained from a number of commercial sources.

ARP reaction with sample genomic DNA

This method is based on the Dojindo AP assay kit system that uses a colormetric-based detection platform. If you use a chemiluminescent-based detection platform, the amount of DNA need per sample is three µg/mL for triplication (step 1 of section 3.3.2).

1) Mix 10 µl of purified genomic DNA solution (100µg/mL) and 10 µl of ARP Solution in a 0.5 ml tube, and incubate at 37°C for 1 hour.

2) Wash the inside of the Filtration Tube cup with 100 µl of TE twice.

3) Add 380 µl of TE to the reaction solution, and transfer the solution to the Filtration Tube.

4) Centrifuge the Filtration Tube at 2500 g for 15 min, and discard the filtrate solution.

5) Add 400 µl of TE to the Filtration Tube and resuspend the DNA on the filter with a pipette.

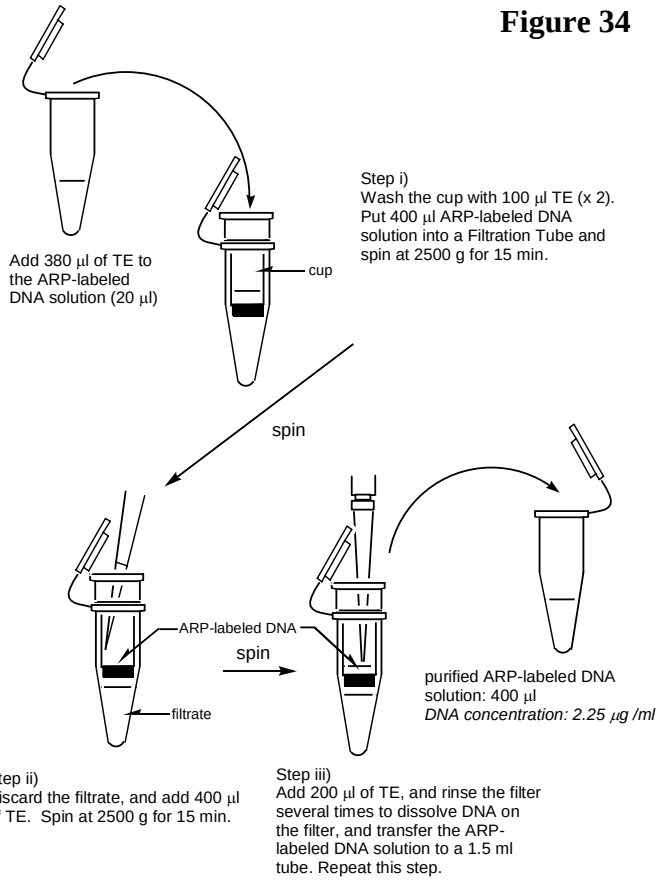
6) Centrifuge the Filtration Tube at 2500 g for 15 min.

7) Add 200 µl of TE to the Filtration Tube to resuspend the DNA on the filter with a pipette.

8) Transfer the DNA solution to the 1.5 ml tube, and add 200 µl of TE again to the Filtration Tube to transfer the ARP-labeled DNA on the filter completely to the 1.5 ml tube.

9) Store the ARP-labeled genomic DNA solution at 0°C to 5°C.

Figure 34



Day 1:

1) Dilute 90 µl of the ARP-labeled genomic DNA with 310 µl of TE.
2) Add 60 µl of Standard ARP-DNA Solution per well. Use three wells per 1 standard solution (use the same format as in **Figure 23**).

3) Add 60 µl of the diluted ARP-labeled genomic DNA solution per well. Use at least three wells per 1sample.

4) Add 100 µl of the DNA Binding Solution to each well, then allow the plate to remain at roomtemperature overnight.

5) Discard the DNA Binding Solution in the wells, and wash the well with 250 µl Washing Buffer 5 times.

6) Add 150 µl of diluted HRP-avidin solution to each well, and incubate the plate at 37°C for 1 hour.

7) Discard the solution in the well, and wash the well with 250 µl Washing Buffer 5 times. After discarding the solution, invert the plate and tap it on a paper towel several times to remove the solution completely.

8) Add 100 µl of Substrate Solution to each well, and incubate at 37°C for 1 hour.

9) Measure the O.D. at 650 nm within 1 hour after the incubation is finished, and prepare a calibration curve using the data obtained with standard ARP-DNA solutions.

10) Determine the number of abasic sites in the genomic DNA using the calibration curve.

2.2.4 Acetylcholinesterase activity assay

This assay can only be used on fish and conch. It will not work in corals. This protocol is a slight modification of the methods:

- Ellman, G. L., Courtney, K. D., Anders V., Jr., and Featherstone, R. M. (1961). A new and rapid coloremetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.
- Hamm, J. T., Wilson, B. W., and Hinton, D. E. (1998). Organophosphate induced acetylcholinesterase inhibition and embryonic retinal cell necrosis in vivo in the teleost (Oryzias latipes). Neurotoxicology 19, 853–869.

It should be noted that the Ellman method is very susceptible to artifact as a result of numerous factors. There are better, more accurate chemiluminescent-based and fluorescent-based assays that are commercially available.

Materials

- Dounce microcentrifuge tube homogenizer, 2 mL (Teflon pestle)
- Acetylthiocholine iodide
- 5,5'-dithiobis-2-dinitrobenzoic acid (DTNB)
- Tetra(monoisopropyl)pyrophosphor-tetramide (iso-OMPA)
- Trizma base
- Sodium chloride
- Dithiothreitol
- Disodium ethylenediaminetetraacetate (EDTA)

Fish sample preparation

1. Make up a Homogenization solution consisting of 50 mM Trizma base, 13 mM sodium chloride, 10 mM EDTA and 1 mM dithiothreitol. The solution should be titrated with 2 N hydrochloric acid for a final pH of 8.0.

2. Immediately prior to adding the pulverized fish sample, add 20 microliters of solution containing 0.4 mM Bestatin, 2 mM phenylmethylsulfonyl fluoride, and 1 µg/100 µL pepstatin A.

3. With a pre-chilled spatula, place about 100 microliters of frozen pulverized fish liver (from Section 2.2.2) into a microcentrifuge tube.

4. Add 1,200 microliters of homogenization buffer to the microcentrifuge tube.

5. Homogenize the sample using the Teflon homogenizer. Make sure that at the end of the homogenization, each sample has the same consistency. Close the lid on the microcentrifuge tube.

6. Centrifuge the microcentrifuge tube at 7,000 g for five minutes.

7. Aspirate 400 microliters of supernatant with a pipettor and place supernatant in a new tube.

8. Conduct protein concentration of soluble protein on each sample using the modified Ghosh method described in section 3.1.3.

9. Sample should be flash frozen, and can be stored until you are ready to commence the acetylcholinesterase activity assay.

Conch sample preparation

1. Make up a Homogenization solution consisting of 50 mM Trizma base, 10 mM EDTA, 13 mM sodium chloride, and 1 mM dithiothreitol. The solution should be titrated with 2 N hydrochloric acid for a final pH of 8.0.

2. Immediately prior to adding the pulverized conch sample, add 20 microliters of solution containing 0.4 mM Bestatin, 2 mM phenylmethylsulfonyl fluoride, and 1 μg/100 μL pepstatin A.

3. With a pre-chilled spatula, place about 100 microliters of frozen pulverized conch (from Section 2.2.2) into a microcentrifuge tube.

4. Add 1,200 microliters of homogenization buffer to the microcentrifuge tube.

5. Homogenize the sample using the Teflon homogenizer. Make sure that at the end of the homogenization, each sample has the same consistency. Close the lid on the microcentrifuge tube.

6. Centrifuge the microcentrifuge tube at 7,000 g for five minutes.

7. Aspirate 400 microliters of supernatant with a pipettor and place supernatant in a new tube.

8. Conduct protein concentration of soluble protein on each sample using the modified Ghosh method described in section 3.1.3.

9. Sample should be flash frozen, and can be stored until you are ready to commence the acetylcholinesterase activity assay.

Acetylcholinesterase activity assay

1. Dilute samples to a concentration of 1.0 microgram/microliter with Homogenization solution.

2. To each well in the 96-well microplate, add 10 microliters of 0.8 mM iso-OMPA. This compound inhibits non-specific cholinesterases.

3. Add 100 microliters of sample supernatant to a well in the 96-well microplate. Each sample will be triplicated, so you will need to add 100 microliters of sample to three well.

4. Incubate the microplate at 25°C for 15 minutes.

5. Add 100 microliters of a 300 μM of DTNB to each well with sample. Incubate the microplate for five minutes.

6. Add 50 microliters of 90 mM acetylthiocholine iodide

7. Measure activity for five minutes in a microplate spectrophotometer at 415 nm.

8. Divide final O.D. by five to calculate concentration on a per minute basis.

Chapter 3
Contaminant Chemistry Protocol

Contaminant chemistry analysis for both tissue and sediment is assumed to be conducted using gas chromatography (GC) with an electron-capture detector. Extraction procedures for both tissue and sediment can be done using standardized liquid-solid extraction methods, such as the Soxhlet Extraction method (EPA Method 3540) or an Accelerated Solvent Extraction (ASE) method (EPA Method 3545). Other extraction methods can also be used, such as Microwave Accelerated Extraction Method (EPA Method 3546) and Super-Critical Fluid Extraction Method. The Soxhlet and ASE methods seem to predominate in labs within the Meso-American Reef system and it is suggested that participating laboratories use the Soxhlet or ASE methods.

Samples will be normalized against wet weight and against lipid weight so that the data can be compared to the data in other studies.

3.1. Normalization

3.1.1. Gravimetric determination of lipid content of tissue sample

As noted by the below quote from the EPA document (Guidance for Assessing Chemical Contaminant Data for Use In Fish Advisories. Volume 1: Fish Sampling and Analysis - Third Edition), standardization of an extraction solvent is paramount. Based on current accepted methods in the literature, the broad array of difference extraction methods, and the availability of obtaining pesticide-grade solvents in the Meso-American Region, North America, and South America, it is recommend that all laboratories that participate in this study use dichloromethane mix as the initial extraction solvent (EPA Method 8290 and 1613).

It is recommended that a gravimetic method be used for lipid analysis. This method is easy to perform and is commonly used by numerous laboratories, employing various solvent systems such as chloroform/methanol (Bligh and Dyer,1959), petroleum ether (California Department of Fish and Game, 1990; U.S. FDA, 1990), and dichloromethane (NOAA, 1993a; Schmidt et al., 1985). The results of lipid analyses may vary significantly (i.e., by factors of 2 or 3), however, depending on the solvent system used for lipid extraction (Randall et al., 1991; D. Swackhamer, University of Minesota, personal communication, 1993; D. Murphy, Maryland Department of the Environment, Water Quality Toxics Division, personal communication, 1993). Therefore, to ensure consistency of reported results among fish contaminant monitoring programs, it is recommended that dichloromethane be used as the extraction solvent in all lipid analyses.

In addition to the effect of solvent systems on lipid analysis, other factors can also increase the inter- and intralaboratory variation of results if not adequately controlled (Randall et al., 1991). For example, high temperatures have been found to result in decomposition of lipid material and, therefore, should be avoided during extraction.

Underestimation of total lipids can also result from denaturing of lipids by solvent contaminants, lipid decomposition from exposure to oxygen or light, and lipid degradation from changes in pH during cleanup. Overestimation of total lipids may occur if a solvent such as alcohol is used, which results in substantial coextraction of nonlipid material. It is essential that these potential sources of error be considered when conducting and evaluating results of lipid analyses.

NOAA (National Oceanic and Atmospheric Administration). 1993c. Sampling and Analytical Methods of the National Status and Trends Program, National Benthic Surveillance and Mussel Watch Projects 1984-1992. Volume IV. Comprehensive Descriptions of Trace Organic Analytical Methods. NOAA Technical Memorandum NOS ORCA 71. Coastal Monitoring and Bioeffects Assessment Division, Office of Ocean Resources Conservation and Assessment, National Ocean Service, Silver Spring, MD. July.

Randall, R.C., H. Lee II, R.J. Ozretich, J.L. Lake, and R.J. Pruell. 1991.Evaluation of selected lipid methods for normalizing pollutant bioaccumulation. Environ. Toxicol. Chem. 10:1431-1436.

Due to the difference in laboratory equipment and extraction methods, a single standardized protocol may be difficult to implement. Instead, an example using a single method (one that is conducted in the author's laboratory) is provided that demonstrates the principle of obtaining the parameter of wet weight and lipid weight.

- Measure out five grams of fish on a pre-tared aluminum weight dish or in a pre-tared 250 mL pre-cleaned glass beaker.
- Add five grams of ASE prep material (dionex p/n 062819) to the 250 mL pre-cleaned glass beaker with the fish tissue in it.
- Place mix in microwave for 2.5 minutes (depends on moisture content of sample).
- Transfer dried sample to ASE extraction cell using acetone-cleaned stainless steel spatula.
- Conduct ASE extraction (dichloromethane, pressure at 1500 psi, 105 degrees Celsius).
- After ASE processing, transfer extract to 40mL pre-cleaned glass graduated vial
- Dry extract to 10mL, measure precisely using.
- Transfer 0.5 mL (10%) to pre-cleaned, pre-tared aluminum weighing dish.
- Dry 0.5 mL to dryness and weigh dish for 10% weight of lipids from this five gram sample.

3.1.2. Dry weight determination of tissue sample

The purpose of this procedure is to be able to normalize the concentration of contaminants against a parameter that has consistency from one sample to another.

All instruments and glassware should be cleaned with a phosphate-free micro-detergent, and acetone rinsed. Samples will be thawed, and using a cleaned spatula, one to three

grams of thawed material will be placed in a clean 10-25 mL beaker that has been pre-tared, and pre-dried (in the drying oven at the same temperature the samples will be dried at). The 'wet-weight' of the beaker is weighed, the amount recorded.

Samples are then placed in a drying oven and incubated to dryness. Drying oven temperature should be between 100°C and 115°C. The temperature should be consistent for all incubation of samples. Samples in beakers are weighed again, the amount recorded.

Ask for certification in the accuracy of all balances and thermometers used in this procedure. Accuracy of balances and thermometer should be checked before and after processing of the samples. Also ask for guarantee that the lab cleans all instruments and glassware appropriately so as prevent the occurrence of cross-contamination of samples.

Equation for calculating the percent dry weight of a sediment sample.

$$\text{Dry Wt.\%} = \frac{(\text{Beaker Wt.} + \text{Dry Wt.}_{\text{sample}}) - (\text{Beaker Wt.})}{(\text{Beaker Wt.} + \text{Wet Wt.}_{\text{sample}}) - (\text{Beaker Wt.})} \times 100$$

3.1.3. Dry weight determination of sediments

The purpose of this procedure is to be able to normalize the concentration of contaminants against a parameter that has consistency from one sample to another.

All instruments and glassware should be cleaned with a phosphate-free micro-detergent, and acetone rinsed. Samples will be thawed, and about one to two grams of tissue be homogenized in a homogenizer. Once homogenized, the homogenate is transferred to a clean 10-25 mL beaker that has been pre-tared, and pre-dried (in the drying oven at the same temperature the samples will be dried at). The 'wet-weight' of the beaker is weighed, the amount recorded.

Samples are then placed in a drying oven and incubated to dryness. Incubation should be between 24-36 hours. Drying oven temperature should be between 100°C and 115°C. The temperature should be consistent for all incubation of samples. Once samples are dried, they should be allowed to cool to room temperature. This should take less than one hour. Samples in beakers are weighed again, the amount recorded.

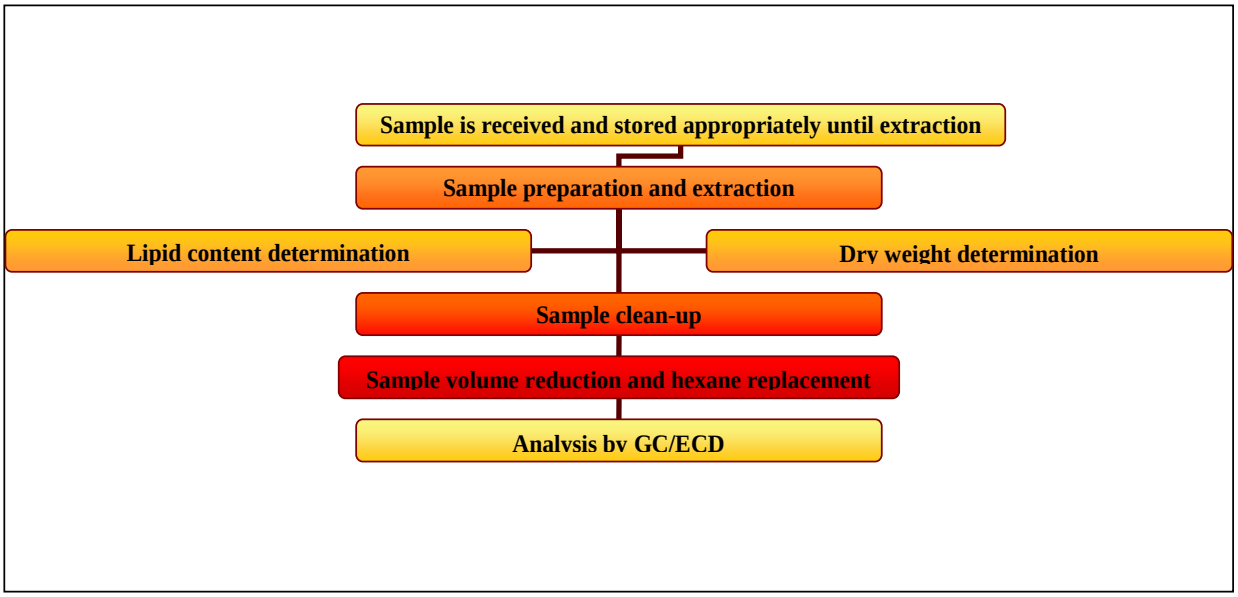
Ask for certification in the accuracy of all balances and thermometers used in this procedure. Accuracy of balances and thermometer should be checked before and after processing of the samples. Also ask for guarantee that the lab cleans all instruments and glassware appropriately so as prevent the occurrence of cross-contamination of samples.

Equation for calculating the percent dry weight of a sediment sample.

$$\text{Dry Wt.\%} = \frac{(\text{Beaker Wt.} + \text{Dry Wt.}_{\text{sample}}) - (\text{Beaker Wt.})}{(\text{Beaker Wt.} + \text{Wet Wt.}_{\text{sample}}) - (\text{Beaker Wt.})} \times 100$$

3.2. Example Protocol of Method

Because of the diversity of instruments and acceptable methods that can be used in the Analytical Laboratory for gas chromatography/electron capture detection, the document provides a single protocol for the extraction and detection of target analytes using a GC/ECD instrument. It can be used as a template for examining key attributes that any analytical laboratory's methodology should possess. The extraction method and the instruments used in this example *should not* be taken as that every analytical laboratory must use this method and this specific instrument. Coral will used as the sample example. The flow of the process is diagrammed below.



3.2.1 Sample Preparation and Extraction

1. Corals are ground in liquid nitrogen in a mortar and pestle as described in Section 2.2.2. of this document and it is assumed that this process is being done by a competent technician in a clean lab with clean instruments.

2. About one gram of frozen powder is transferred to a clean container using a frozen spatula for determination of dry-weight.

3. Frozen sample are then dried. This step can be done using a variety of methods, but most laboratories dry samples using Hydromatrix, a commercial chemical desscicant.

4. The dried sample is then loaded into a Dionex ASE (accelerated solvent extraction) cell and using dichloromethane as the extractant solvent. All samples, blanks, matrix spikes, and reference samples are prepared in the same fashion.

5. The ASE cells are closed and loaded onto the Dionex ASE extraction instrument.

6. Extracts are collected into extraction vials, and then evaporated to a volume of about 10 milliliters using a heating block or hot-water bath.

7. An aliquot of the sample is removed (usually 100 uL to 500 uL) to determine lipid content of the sample. The method for determining lipid concentration can be found in Section 3.1.1. of this document.

8. This extract is 'cleaned' of contaminating debris and lipid. Addition of samples with high lipid content onto the GC can create significant artifact. 'Clean-up' of samples can be done using a variety of methods, and even a combination of methods. For this sample example, clean-up is conducted using alumina/silica gel chromatography columns from a commercial source that comes pre-packed and pre-equilibrated in dichloromethane. Columns are washed with a 1:1 solution of hexane:dichloromethane. The elutant is collected, and its volume reduced via evaporation. The final sample is diluted to 0.5 milliliters with hexane.

9. The sample is then transferred to a one milliliter vial and stored at -80°C until analysis.

3.2.2. Sample Analysis

Samples are loaded unto an autosampler that is attached to the gas chromatograph. The autosampler will inject a designated volume of a sample into the gas chromatograph. Besides the target samples, the autosampler will also inject method blanks, calibrant standards, matrix standards, internal standard, and the surrogate spiked standard in a single run. A run is defined as a set of samples and standards that are loaded onto the instrument over a discrete amount of time (usually 36 hours).

3.3. Standards

There are four different standards that any analytical laboratory must have in their methods. These four standards are necessary to provide a reasonable level of confidence in the data generated for the samples.

3.3.1. Internal Standard

This standard is added to all samples and quality control samples and is used to provide a reference for the calculation of analyte concentrations. Most often, the internal standard is a 1 nanogram/microliter concentration of tetrachloro-m-xylene. This compound can be obtain from a number of commercial sources and is certified/designated for use as an internal standard in contaminant chemistry analysis.

3.3.2. Surrogate Spiking Standard

This standard is used to determine the efficiency of analyte recovery of the method used in the analytical laboratory. The surrogate spiked standard *cannot* be a target analyte (e.g., PCB 105), and can be compounds such as 4,4,'-dibromooctoflurobiphenyl, tetrachloro-m-xylene, and 2,2',4,5',6 pentachlorobiphenyl. This compound can be obtain from a number of commercial sources and is certified/designated for use as a standard in contaminant chemistry analysis.

3.3.3. Calibration Standards (aka working standards)

The purpose of Calibration Standards is to calibrate the instrument and to determine the range of linearity for the instrument. These standards are purified/known concentration of the target analytes. They are obtained from a commercial source and are certified/designated for use as calibrant standards. The calibrants are actually a series of dilutions of a mastermix of target analytes are loaded directly into the GC-ECD instrument. Calibrants for each target analyte may range from 1 to 500 picograms/microliter. For organochlorine analysis that is usually analyzed in most U.S. EPA survey programs, the calibrant mastermix should contain the following target analytes:

1,2,4,5-tetrachlorobenzene	2,4'DDD	dieldrin
1,2,3,4-tetrachlorobenzene	4,4'DDD	endrin
hexachlorobenzene	Heptachlor	mirex
α-HCH	Heptachlor epoxide	endosulfan II
β-HCH	Oxychlordane	endosulfan sulfate
γ-HCH	α-chlordane PCB66	PCB8
δ-HCH	PCB101	PCB18
pentachlorobenzene	PCB105	PCB52
chlorpyrifos	PCB118	PCB170
pentachloroanisole	PCB128	PCB180
2,4'-DDE	PCB138	PCB187
4,4'DDE	PCB153	PCB195
2,4,'DDT	γ-chlordane	PCB206
4,4,'DDT	trans-nonachlor	PCB209
	cis-nonachlor	PCB28
	aldrin	PCB44

3.3.4. Matrix Spike Standard

The purpose of the matrix spike standard is to determine the efficiency of the recovery of target analyte from an actual sample. To conform to U.S. EPA methods, a matrix spike duplicate should be included in the analytical laboratory's methodology. The matrix spike duplicate is used to determine the precision of matrix recovery. These matrix spike standards can be obtained from a commercial source and are certified/designated for use as standards. The concentration of analytes in the matrix spike solution should be about 10 times greater the concentration of the methods detection limit. The matrix spike standards are added to a sample. The matrix spike solution usually contains the same target analytes found in the working standard solution or a subset of those target analytes.

3.3.5. Blanks

Included in the Analysis Laboratory's methodology should be a number of Laboratory Method Blanks. This blank, actually a set of blanks, includes components used for analysis. For example, one blank should include the analysis of the dichloromethane and hexane from the original reservoir. Glassware used during the extraction should also have a blank. The Teflon sample containers, and ever the aluminum foil used to contain some samples should have blanks of their own. To counter possible criticism, the the Hydrosorb wipes should be used to on 'clean' Teflon cutting boards, dissecting instruments, and counter-tops to determine the extent of possible sample preparation contamination.

3.3.6. Standard Reference Material

Standard reference material is usually obtained from a government repository or some other certified source whose sample and sample matrix is similar to the samples. This material is processed through the entire extraction method, determined for lipid content and dry weight, and analyzed with the samples usually in the same batch of samples that are loaded onto the analytical instrument.

3.4. Quality Control/Quality Assurance Parameters

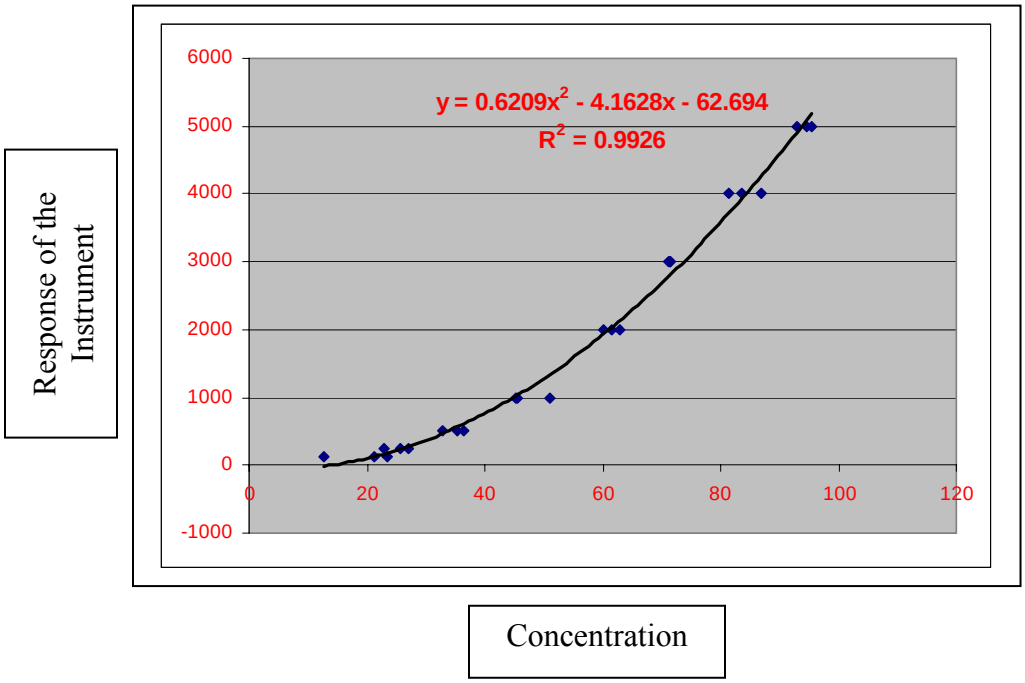
Whatever laboratory is responsible for conducting the contaminant analysis for your samples, it should have a Quality Assurance (QA) program in place that meets the demands of specific Quality Control (QC) criteria. Quality Assurance is a 'management strategy' that meets the demands of the study for a level of confidence in the validity of the data being generated.

As part of the Quality Assurance Program for the analysis laboratory, the laboratory should be able to provide you (on demand) documentation that addresses the following items (which is what expected as part of an US EPA QA/
QC program and is copied almost verbatim from Guidance for Assessing Chemical Contaminant Data for Use In Fish Advisories. Volume 1: Fish Sampling and Analysis - Third Edition appendix I):

1. Detailed descriptions of laboratory procedures for sample receipt, storage, and preparation.
2. Detailed descriptions of the analytical methods used for quantitation of target contaminants, dry weight of sample, and percent lipid determination.
3. Detailed descriptions of methods routinely used to assess data accuracy, precision, and completeness.
4. Detailed descriptions of preventive maintenance procedures for sampling and analysis equipment.
5. Detailed descriptions of calibration procedures for all measurement instruments, including specification of reference materials used for calibration standards and calibration schedules.
6. Detailed descriptions of procedures for internal QC performance and/or systems audits for sampling and analysis programs.
7. Detailed descriptions of procedures for external QA performance and/or systems audits for sampling and analysis programs, including participation in certified QA proficiency testing or interlaboratory comparison programs

Every analysis laboratory should be able to meet five separate QC criteria. They can present their QC in different ways, but you should be able to feel satisfied that they have met these criteria.

1. Demonstration of capability – The laboratory must demonstrate that all of the instruments are in sound working order, that the technicians are competent, and that the lab has a quality assurance plan in place.
2. Laboratory background contamination – the laboratory must demonstrate that benchtop, the sample preparation instruments, solvents, the analytical instruments are free of traces of target analytes. The lab should provide method blank data and nonzero blanks for both sample analysis and analyte percent recoveries.
3. Determining surrogate and targeted analyte recovery – the laboratory must demonstrate that the surrogate and targeted analytes can be recovered within an acceptable range by the methods preparation in that laboratory. It is beyond the scope of this Protocol, but an excellent review for theory and application of surrogate and targeted recovery can be found in Loconto, P.R. (2005) Trace Environmental Quantitative Analysis: Principles, Techniques, and Applications, 2nd edition. CRC Press. Boca Raton. Pp 82-91. What you need to understand from the analytical laboratory is the range of recovery of a target analyte using the laboratory's methods, and what you require to be an acceptable range of %recovery.
4. Experimental calibration and the range of linearity - The analytical laboratory will provide you with data that has quantitative units. There is a method for which they determined concentration of each target analyte. That method includes the addition of known concentrations of a target analyte that is analyzed by the analytical instrument. This data represent two characteristics: (1) the response of the instrument and (2) concentration of the analyte. This data can be represented as a relationship (quadratic relationship).



The correlation coefficient, ***r***, of 0.9990 is the goal to achieve between the instrument response and the amount of analyte. The coefficient of determination, r^2, is the measure of the variation of the instrument response (dependent variable) that can be accounted for by the concentration of the target analytes (independent variable). These two coefficients are necessary to determine the range of linearity and the goodness of fit in the calibration of an instrument for measuring the target analytes. The lab should establish what the linear dynamic range of their instrument is for a target analyte, whether the linear range be determined by a first-order least squares regressions or a second order least-squares regression (polynomial), you should understand that for your sample to have a validly determined concentration of a target analyte, the instrument's response for your sample's target analyte must fall within the linear range of the calibration curve. If your sample's target analyte falls outside the linear range of the calibration curve, it produces an increased level of uncertainty in measuring accurately the true concentration of the analyte in that sample.

5. Precision and Accuracy – The laboratory should provide you documentation of the precision of the method/instrument in measuring the target analyte in each sample, and the accuracy of the measurement. Precision can be evaluated by conducting replicate measurements on the same sample for the same target analyte. Most assays are conducted in triplicate so that a meaningful standard deviation can be determined for you sample, as well as determining the %coefficient of variation.

Accuracy can be determined by the use of a known, certified, reference standard. Your sample's response/calculated concentration can be compared to the reference standard, and a standard error determined. The accuracy should be reported as a "% standard error."

%Error=[Xi(unknown) – Xi(known)]/Xi(known)

3.5. Reports of contaminant chemistry analysis

Following U.S. EPA suggestions for data reporting of contaminant chemistry analysis, documentation of analytical data for each sample or batch of samples should include the following information:

1. Study identification (e.g., project number, title, phase).
2. Description of the procedure used, including documentation and justification of any deviations from the standard procedure.
3. Method for the detection and quantitation limits for each target analyte.
4. Method for the accuracy and precision for each target analyte.
5. Discussion of any analytical problems and corrective action taken.
6. Sample identification number.
7. Sample weight (wet weight).
8. Lipid content (as percent wet weight).
9. Final dilution volume/extract volume.
10. Date(s) of analysis.
11. Identification of analyst.
12. Identification of instrument used (manufacturer, model number).
13. Chromatograms for each sample analyzed by GC/ECD.
14. Raw data quantitation reports for each sample.
15. Description of all QC samples associated with each sample (e.g., reference materials, field blanks, rinsate blanks, method blanks, duplicate or replicate samples, spiked samples, laboratory control samples) and results of all QC analyses. QC reports should include quantitation of all target analytes in each blank, recovery assessments for all spiked samples, and replicate sample summaries. Laboratories should report all surrogate and matrix spike recovery data for each sample; the range of recoveries should be included in any reports using these data.
16. Analyte concentrations with reporting units identified (as ppm or ppb wet weight **and** ppm or ppb lipid weight; two significant figures unless otherwise justified). **Note:** Reported data should not be recovery-corrected or blank-corrected.
17. Data qualifications (including qualification codes and their definitions, if applicable, and a summary of data limitations).