Techniques for Marine Chemical Ecology
Pawlik – Sep 2006 (adapted from student training guide written by Greg McFall)

GELLING AGENTS

(1) Sodium salt of Alginic acid - Used for Aquarium assays with fish predators to make pellets of food. Hardens on exposure to a calcium chloride solution. Advantages: does not need heat to harden. Disadvantages: can only be used with small amounts of extract or metabolite.

(2) Carageenan or Agar – Used for field assays with fish predators and short-term allelopathy assays. Hardens after heating. Different varieties available for varying gel hardness. Advantages: excellent miscibility of wet-extracted crude extracts into gel. Disadvantages: must heat to harden, will only last a few days exposure to seawater.

(3) Phytagel - Used for long-term assays of fouling and anti-overgrowth. Hardens after heating. Advantages: only gel that will survive 4+ weeks in field assays. Disadvantages: some problems with salt interactions with gel that prevent hardening – best to use extracts prepared from freeze-dried tissues.

SQUID MANTLE FOR ASSAY FOOD

Powdered, freeze-dried squid mantle is the preferred food added to the gel matrix for field and aquarium feeding assays. The food value of squid mantle approximates that of sponge tissue on a volumetric basis (see Chanas & Pawlik, 1995).

1) Buy frozen cleaned squid. You will only use the mantle (body covering, calamari) for making this food – the tentacles and internal organs can be discarded.
2) Chop the mantle into 2-cm squares.
3) Wash gently in fresh water and drain.
4) Add squid to a commercial blender and puree until the texture is smooth.
5) Pour (scrape) squid into a suitable container for freeze-drying (e.g., food storage containers, etc.) The thickness of the puree should not exceed 2 cm.
6) Place the container with squid puree into the freezer and let stand until completely frozen (ca. 4 hrs.)
7) Place container with squid into freeze-drier and lyophilize.
8) The squid powder is completely freeze-dried when it *is* brittle and *not* cool to the touch (ca. 48 hrs.)
9) When squid is completely lyophilized, place pieces in a dry blender set on puree and make a powder. Sift the resulting powderthrough a flour-sifter. *The pieces of squid that won't go through the sifter, will not go through the syringe when you try to make pellets. Place them back in the blender and try again.* Try to do Step (9) as rapidly as possible because the squid powder is very hygroscopic.
10) Place the sifted powder into Nalgene bottles, "cover" with N_2 gas to remove oxygen, seal lid tightly, and store in freezer. Powder will keep for many months at -20C.

ASSAY FOOD RECIPES

Tank Assay Recipe

Regular Assay Mixture:

In a 150 ml beaker mix the following ingredients:

5.0 g	freeze-dried squid mantle
3.0 g	Na salt of alginic acid (powder)
100 ml	distilled water

Mix all ingredients with a spatula and let it sit for about ten minutes; this will allow the squid matrix to set-up. After the set-up period, mix the matrix thoroughly again and you are ready to do tank assays.

***Note**: The first two ingredients can be added to a 50 ml plastic centrifuge tube and stored under N2 in the freezer for extended periods of time.*

Maintenance Diet - If you intend to keep assay fish for extended periods of time without conducting assays, you may wish to put them on a diet that is more nutritionally substantial than freeze-dried and reconstituted squid mantle. We use this formula (after capture) to get the fish feeding, and also at the morning and evening feeding (e.g. whenever you are *not* conducting an assay.)

In a 150 ml beaker mix the following ingredients:

5.0 g	freeze-dried squid mantle
3.5 g	alginic acid (powder)
5.0 g	powdered commercial tropical fish food mix
110 ml	distilled water

***Note**: The first three ingredients can be added to a 50 ml plastic centrifuge tube and stored under N2 in the freezer for extended periods of time.*

Calcium Chloride Solution - for hardening Sodium salt of alginic acid

A “noodle” of the two alginic acid-based foods can be made by extruding it from a 10 ml syringe into a 0.25 M solution of $CaCl_2$. To make a 0.25 M solution of $CaCl_2$, mix 27.75 g of anhydrous $CaCl_2$ (F. W. = 110.99 g/mol) with 1.0 liter of distilled water.

Field Assay Recipe

To make field assay food, mix the following ingredients into a 150 ml beaker:

3.0 g	freeze-dried squid mantle
1.5 g	Type I carageenan
60 ml	distilled water

***Note**: The first two ingredients can be added to a 50 ml plastic centrifuge tube and stored under N_2 in the freezer for extended periods of time.*

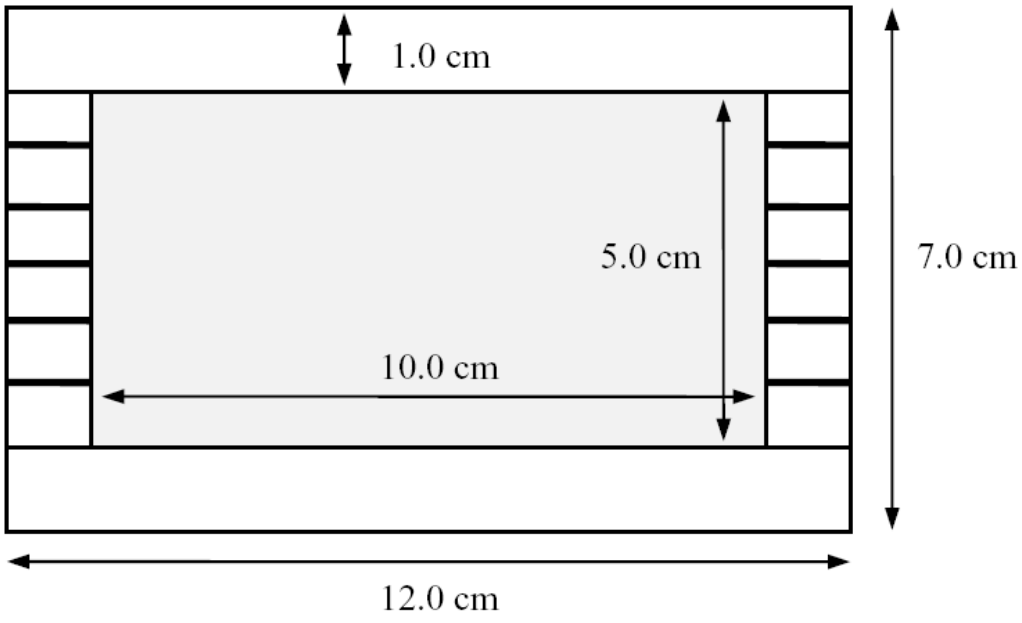
Field Assays of chemical defenses against generalist fish predators:

Field assays are conducted with a natural assemblage of reef fish to confirm results that were obtained from bioassay-directed fractionation using Aquarium assays. Field assays are usually conducted on 60 ml equivalent volumetric samples of the crude, semi-purified partitions, or pure metabolites by incorporating the extract, partition, or metabolites into a matrix of squid and carrageenan. Treatment and control food strips are tied ontto ropes that are secured to the substratum in an environment where generalist reef predators are common. Areas frequented by commercial dive boats are not good for these behavioral assays, because fish are fed by tourists and they will aggressively remove assay food from strings and spit-out unpalatable treatments. Predatory fishes are much more "picky" at sites where divers are uncommon, and assays in these areas will run well. Ropes with paired treatment and control strips should be deployed systematically for easy retrieval and should remain deployed until the fish have consumed at least 50% of either treatment or control (or both).

Required Equipment:
You will need the following items to run a field assay on *one* 60 ml equivalent:

20	assay ropes
20	35.0 cm lengths of cotton twine
4	food strip molds
2	150 ml beakers
2	microspatulas
2	field assay tubes*
1	food coloring set (R, G, B, and Y)
1	microwave oven
2	1 gallon zip-loc bags

Food-strip Molds - You will need to make four trays out of plexiglas in which to pour the molten food matrix .



The bottom of the mold can be made from any thickness (down to 1/16") plexiglas, but the sides of the mold should be constructed of 1/4" plexiglas. The simplest thing to do is use the same thickness of plexiglas for all the pieces. If you cut 1/4" plexiglas to the following dimensions, you will have all the parts necessary to make four molds:

4	12.0 cm x 7.0 cm
8	5.0 cm x 1.0 cm
8	12.0 cm x 1.0 cm

To construct the mold, use methylene chloride (DCM) as a glue to join the pieces of plexiglas together. It may be worth while to make a 5.0 cm x 10.0 cm blank out of wood to use as a gluing guide. *The exterior appearance of the mold is insignificant compared to the volume of the interior...it must be 60 cm^3 (= 60 ml).* Start with the long edges first, and then put in the end pieces. *Be sure to hold the material securely in place as the DCM dries, or you will have to start over!*

After the mold is glued and has dried, cut notches into the top surface of the end pieces as shown in Fig. 1. These notches will serve as a guide for cotton twine that is used to tie the food strips to the assay ropes. Use a hack-saw to cut notches at 1.0 cm intervals, starting 0.5 cm from the intersection of the end piece and the side piece. Cut the notches at least 1/8" inch (no deeper than 3/16") into the plexiglas end pieces; this will ensure that the twine is completely embedded in the food strips.

Assay Ropes - You will need a 10 meter (32.8 feet) length of 3/8" polypropylene line to make the ropes required for one assay. First, wrap a piece of electrical tape around the line at every 50 cm. In addition to marking the line for cutting, the tape will help prevent the line from fraying. Next, cut the line at the 50 cm marks right in the middle of the electrical tape. Cutting the line will be easier if you have a soldering gun with a line cutting tip; if not, use a sharp knife and cut the line on a solid surface. When you are finished cutting, you should have 20 ropes that are 50 cm each.

Making Squid Strips - In order to make the food strips, you will need to assemble the following items:

4	food strip molds
2	150 ml beakers
2	clean microspatulas
1	hot pad or thick glove
1	microwave oven
1	60 ml equivalent extract
2	field assay tubes
20	35.0 cm lengths of cotton twine
2	60 ml aliquots of *d*H_20
1	food coloring set (R, G, B, and Y)

Use the following procedure to make control food strips. Repeat this procedure with addition of crude extract, partition, or pure metabolite at step (5) to make treatment food strips.

1) Place ten lengths of cotton twine into the notches of two food molds and secure them on the opposite side. The strings should be tightly strung across the mold and have equal lengths (~10 cm) hanging from either end of the mold.

2) At this point you should decide how to mark the strings such that you will be able to tell the difference between the control and the treatment food strips. We usually mark the control strings close to the mold with a permanent marker; a single dot will suffice.

3) Pour the contents of a field assay tube (= 3.0 g squid powder, and 1.5 g Type I carageenan) into a clean, dry, 150 ml beaker. With a clean microspatula, dry mix the contents to ensure thorough blending of the two powders.

4) Add 60 ml of distilled water to the beaker containing the field assay powder and mix until it is homogeneous. ***You should be aware at this point that your mixture will be split into the two molds (= 30 ml each) in Step 7 below.***

**Note: Because we want the fish to make their choice based on the palatability of the extract and <u>not</u> because of the color, you need to consider controlling for the color of your extract. You can control the color in two ways: a) Use food coloring to make the control strips approximate the color of your extract; or, b) add food coloring to both the treatment and control to make them darker than the extract was originally (i.e. mask the original extract color). If you need to control for color, now is a good time to add food coloring to your mixture.*

5) Place the beaker into the microwave and heat on "High" for approximately 45 seconds; the mixture should begin to boil rapidly. ***You must be ready to mix and pour the contents of the beaker into the two molds as soon as the beaker comes out of the microwave; the mixture cools and sets-up very rapidly.*** Half-way through the heating process, remove the mixture and add the carrier solvent (usually ~10 ml MeOH) and stir vigorously – this often boils off rapidly, so be careful that it does not splash!

6) Using a *hot pad or thick glove*, remove the hot mixture from the microwave and begin to stir immediately.

7) Pour the mixture rapidly into the two molds, making sure that the cotton twine is completely submerged and that the corners of the mold are filled. You will have approximately 20 seconds before the mixture begins to "skin over."

8) Let the molds cool for approximately 5 minutes before you attempt to cut them into strips.

9) After the food mixture is cool, make a cut (with a scalpel or razor blade) *perpendicular* to the strings such that the food will be bisected into 2, 5.0 cm x 5.0 cm, blocks. Make sure that you cut completely through the strings.

10) Next, cut the food *parallel* to, and *between* the strings, to produce ten 1.0 cm x 5.0 cm food strips; a small ruler makes the task much easier. Make sure that you also run the scalpel down the inside, long edge of the mold, on both sides to loosen the strips from the mold.

11) ***Do not pull on the strings to remove the strips from the mold!*** The suction of the mold on the food strips is greater than the adhesion of strings to the strips; the strings will pull right out. Instead, insert a microspatula into the area of the first cut and gently pry the strips from the mold. Be careful not to break the strips when you pull the strings out of their notches.

12) Food strips are now ready to be tied to assay ropes. For 20 assay ropes, tie 10 with the treatment near to top end of the rope, with the control 10-15 cm from the end, and tie the remaining 10 with the order reversed.