# SPECIMEN PROCESSING

Below is a quick guide to methods of specimen preparation for marine invertebrates.

**Relaxation:** Objective is to get specimen anaesthetized, so that it is unable to respond when placed in a fixative. Relaxants can knock out both muscles and nerves, and make fixation more humane as well. Relaxation is important in many groups because identification is hampered or made impossible if animals are preserved in a contracted form; in other groups (e.g. crustaceans, some echinoderms) autotomy may occur if animals are dropped straight into fixative. When relaxing a specimen make sure it 1) expands if it started out contracted (ascidians, anthozoans) (this may not always happen) and 2) that animal is fully anaesthetized, so that it does not respond to even strong stimulation. Do not stimulate strongly until you are sure it is fairly unresponsive, as otherwise a strong initial poke will send the animal into contraction from which it may not come back out again. Relaxants are often group specific; some useful chemicals are listed below (there are many others). You may need to experiment with various methods before you hit one that works well for a particular taxon. Even closely related groups may relax better with different relaxants.

Magnesium chloride ($MgCl_2$): prepared in distilled water at 7.5 weight % (of the hexahydrate form that is usually sold) makes an isoosmotic solution with seawater. Note that $MgCl_2$ crystals are highly hydrophilic and you may need to mix a generously greater amount if they have absorbed water and are moist/wet. Exact percentage is not that important. $MgCl_2$ works by virtue of Mg replacing Ca in muscles, thus making muscles unable to contract. This is why you need to mix it in low-Ca water, ideally distilled water unless you know that your tap water has low Ca content (not the case where karstic aquifers are tapped). $MgCl_2$ works well with most marine invertebrates, but can take an hour or more for larger animals. For animals that contract, it can be added gradually to seawater, typically for a final 50-50 concentration.

Menthol: add to dish with animals by either sprinkling crushed crystals on top or adding drops of concentrated menthol solution prepared in ethanol. Menthol works especially well for cnidarians and ascidians. Even better, add menthol in addition to $MgCl_2$; this method was initially proposed for holothuroids, and is the quickest and most effective technique for relaxing cukes.

Clove oil: a few drops in a bowl will knock out many crustaceans (especially decapods & stomatopods). A clove oil bath quickly slows down and knocks out shrimp and makes their photography easier as well. Clove oil can glom onto animals, especially finely setose appendages, so it is best used in a pretty dilute emulsion in seawater, and animals that are photographed should be rinsed in clean water before they are put in the photo tank. Clove oil is also used in 25% mixture in ethanol, in which case it dissolves better in water. In this way it is also used in the field to drive out stomatopods from burrows, or to knock out crustacean or fish communities so that they can be collected from intricate habitats like branching corals (see Boyer et al 2009). Note we had poor luck with cheaper clove oil prepared from leaves; so use the classic clove oil prepared from clove buds.

Tricaine (MS-222): this is a fish anesthetic licensed in the US for use on fish destined for human consumption, so pretty benign for humans. It is sensitive to light and high temp, so keep it in dark/dark bottle in cool area, and only take as much as you need per trip, then discard remainder. A pinch added to water is excellent anesthetic for peracarids (which tend not to respond well to alternates listed here) and ascidians. Also great for fish and likely other groups.

Cooling / freezing: Prevents autotomization in many and is a relatively humane way of killing animals, but will not commonly lead to expansion of contracted animals. Ice crystals can make tissues unsuitable for fine-scale morphology or histology, so do not freeze when such preparations may be desired. Good general method for crabs though, and defrosted crabs make great photos.

Propylene phenoxitol: Excellent relaxant for bivalves and some other inverts. A couple of drops added to a bowl go slowly into solution and rapidly knock out clams. Can be hard to obtain because there are few suppliers.

Chloretone = chlorobutanol. Chloretone is not readily miscible in water, so it needs to be prepared in a concentrated ethanol solution (a large amount of the chloretone can be dissolved in a volume of alcohol). Add a couple of drops

to a bowl or a pipette-full to a bucket. While this compound works well for echinoderms, especially holothurians, $MgCl_2$ and menthol works even better and does not have the potential problem of toxicity of chlorinated organics.

**Fixation:** The purpose of fixation is to fix tissues for long-term storage and study. Formalin or similar fixatives (Bouin's fixative, glutaraldehyde, Osmium tetroxide) are necessary for histological quality fixation and for most groups where detailed anatomy or histology is desired, or needed for identification (e.g. ascidians, most worms, cephalopods, opisthobranchs, etc.). Formalin makes tissue difficult to use for DNA sequencing however. Ethanol is fine as a fixative for groups where only external characters or gross anatomical features are used in taxonomy (e.g. most crustaceans and sponges), and is more pleasant and less hazardous to work with. It is also preferred for groups (like holothurians) where the greater acidity introduced by formalin may etch or destroy tiny calcareous sclerites useful in taxonomy. Ethanol provides adequate fixation for DNA sequencing, although reasonable specimen vs. DNA fixation are not entirely compatible. Placing specimens straight into high concentrations of ethanol dehydrates them, thus they shrink and can collapse, and also extracts lipids, etc.. These decreases utility of specimen for morphological work, but increases its utility for genetic work, as it is rapid dehydration that preserves DNA. Thus while 70-80% ethanol is better for morphological specimens, 95-100% ethanol is best for genetic samples. For formalin-fixed animals it is useful to take a small tissue sample and fix it in 95-100% ethanol to allow future genetic study.

When fixing large animal (such as a large sea cucumber, sponge, soft coral) in ethanol, it is best to use 95% ethanol to counteract the water content of the animal - you can eyeball this volumetrically. Avoid using 100% ethanol for non-DNA fixes as it is much more expensive then 95% ethanol. You should always use plenty of fixative - at least 3X volume of specimen, to make sure that the final concentration of fixative is adequately high to do the job (70% for ethanol, 5-10% for formalin). If you are fixing larger animals (>2-4 cm in all dimensions) it is important to make sure that the fixative penetrates. This is best achieved by injection with a hypodermic needle into the body / body cavity, or by cutting the animal open. Be careful not to overly distend the animal or unduly destroy anatomy when doing this. Some fixatives, like Bouin's, have chemical agents to facilitate tissue penetration.

Formalin: used generally at 5-10% strength of the industrial "formalin" mixture - which itself is ca. 38% formaldehyde gas dissolved in water. Thus 10% "formalin" is 3.8% formaldehyde. For sea animals, mix formalin with sea water to make it isoosmotic; for freshwater animals mix with freshwater. For any group with calcareous parts you need to buffer formalin, as the liquid turns acidic (forms formic acid) with age. Buffering can be done with fine $CaCO_3$ powder or with a variety of buffers such as borax (sodium borate). For good histological / anatomical fixation you may want to use special buffer recipes, or use special fixatives like Bouin's. Most animals fixed in formalin are stored in 70-75% ethanol after fixation; exception are some fragile taxa like medusae. When replacing formalin with ethanol it is useful to rinse the specimen in freshwater for a day or two to wash away not only the formalin, but also the salt derived from the seawater. If the specimen is transferred directly from seawater formalin to alcohol, salt can precipitate on it, obscuring features and potentially damaging the specimen.

Ethanol: Bulk alcohol usually comes at 95% concentration; absolute (100%) ethanol is considerably more expensive as the final dehydration has to be chemical. We use 75% ethanol for routine fixation, 95% for routine bulk genetic fixation, and 95-100% for fixing small genetic subsamples. Mix ethanol with distilled (deionized, whatever) water for the former, as precipitates can form when mixed with hard water. In field situations undenatured alcohol can be difficult to get, but denatured spirits (~95% ethanol + methanol + odor and sometimes color) are available in most places and reasonable for fixation. For genetic fixation remove excess water from the specimen as much as possible, add ethanol equal to 5-10X the tissue (not counting shell) volume of the sample, and change at least once in the field, and again back in the lab.

**Subsampling for genetics:**

Whenever time allows, it is useful to prepare a separate subsample of the specimen for genetic (DNA) work; such subsamples need to be clearly labeled to be associated with the voucher they came from. Subsampling is useful because you get better DNA preservation in small tissue pieces than in larger, whole specimens, because it allows more appropriate fixation of the voucher, and because it facilitates sample storage and processing for the genetic subsamples. If size permits, ideal genetic subsamples are a couple of cubic millimeter, in ~1 mm sized pieces. This much allows for multiple DNA extractions. When size is limiting, substantially less (even single cells under specific protocols) can work. To take a subsample, slice off a small piece of the animal (end of foot in a gastropod, tube foot from a sea star), and place directly in 95-100% ethanol in a small tube. An alternative is to fix tissue samples in

DMSO-EDTO-NaCl buffer. This works much better for animals with a lot of body fluid: sponges, cnidarians, ascidians, but will dissolve the tissue creating a frothy mixture that requires different handling. It also dissolves carbonate, so is not useful as a general fixative. A scalpel or razor blade is a good way to slice off a tiny end piece from a moving mollusk or worm. Use at least 10X the volume of ethanol to fix the sample. Larger tissue pieces do not allow sufficient fixative penetration, while high tissue:fixative ration will not allow proper tissue fixation. With small animals or those that deteriorate rapidly (sponges, flatworms, etc) it is important to fix a subsample (or the sample) before, or quickly after death as DNA can deteriorate rapidly. Ideal subsample vials are 1.2-2.0 mm plastic tissue vials that seal well. There is a lot of variation in vial quality – some allow rapid fluid evaporation. We found GeneMate tissue vials & caps to work well (VWR, catalog numbers 490003-494, 490003-344). After subsampling, store tissue vials in freezer or refrigertator. While DNA keeps well in alcohol, cold temperatures greatly improve long-term DNA quality.

**Preservation:** Once a bug is fixed (takes a day or so for small (<1cm) animals to a week for big ones), the animal should be transferred into appropriate medium for long-term preservation. This most commonly means that you move samples from formalin to alcohol. To do this, you need to soak the formalin out a bit, so let specimen sit for couple of hours to days, depending on size, in water / seawater, then transfer to alcohol. One additional change of alcohol is useful to remove residual formalin. For formalin-fixed specimens that possess fine calcareous structures it is important to buffer the alcohol, as residual formalin may make it go acidic. Add 1 ml of concentrated solution of borax per 1 l of alcohol to do this. Initial alcohol fix should also be replaced with fresh alcohol after a couple of days, to remove bring ethanol concentration closer to target and remove debris and solutes from the jar. We generally preserve in 75% EtOH for most groups, with a few exceptions. Genetic samples also remain in 95-100% ethanol, but again the ethanol is replaced at least once after a day or two, to facilitate dehydration. For final storage, put animals in smallest jar / vial they will fit into without getting contorted, and fill that jar/vial to the very top. We fill to the top for two reasons: 1) more preservative takes longer to evaporate, thus we have more time to discover a problem seal, and 2) this sets a standard, so that evaporation can be immediately noticed and addressed by replacing lid or jar. How much alcohol you have relative to specimen volume is unimportant at this stage.

**Procedures by taxon:**

**Porifera:** No relaxation needed, fix in ample volume of 80% or 95% alcohol depending on size, transfer to clean alcohol in a week+. In addition to basic field data, you should record color (external and internal, if possible), texture, surface feel, odor, mucus production, and any other obvious live character of the sponge. *In situ* photos are very useful for sponges and should be associated with the exact specimen pickled.

**Cnidaria:**

**Soft corals** are not usually relaxed, and can be fixed in alcohol or buffered formalin (former fine for taxonomy, latter preferred if wish to look at histology like gonad development) and are stored in alcohol after thorough wash (if fixed in formalin). In situ photos are very useful.

**Gorgonians** should be fixed in alcohol, or fixed in buffered formalin quickly then dried (if they are too large to easily keep wet in a jar), with a reasonable portion of the colony either fixed and stored in alcohol, or fixed in formalin and stored in alcohol. In situ photos are useful.

**Black corals** should be fixed like gorgos, with at least a good portion of the colony pickled. Ethanol is an adequate fixative, formalin is required for histology only. Color notes and in situ photos, especially of the expanded polyps are very useful for taxonomy.

**Anemones (all orders)** need to be relaxed well, so they are expanded, fixed in formalin, and stored in alcohol. Photo of live animal is very useful. Menthol is a good relaxant.

**Stony corals** are usually bleached and skeleton stored dry. A small colony fragment or tissue scrape in alcohol provides genetic subsample. Photos are important.

**Hydrocorals** can be dried or bleached then dried. A small colony fragment or tissue scrape in alcohol provides genetic subsample. Photos are useful.

**Jellies** relax in MgCl2, fix and store in formalin. Need to guard against physical damage, so in shipping do not drain or allow much bubbles in container.

**Flatworms:** Take snippet (use razor blade to slice posterior end off) for DNA work fixed in ethanol. Place gently onto frozen formalin (kept in freezer) to fix; preserve in ethanol. This is easiest to do by placing animal on wet paper, than carrying animal on paper onto frozen formalin surface. Allow formalin to thaw to fix worm. Photos of all colorful species are essential.

**Other worms:** Relax (usually with with $MgCl_2$), fix in formalin, preserve in EtOH. Snippet in ethanol for DNA is useful. Photos useful.

**Crustaceans:** Kill big decapods by freezing, fix and preserve in EtOH. Can also relax with clove oil for decapods or tricaine for peracardids. Color photos useful: for crabs it is best to photograph freshly killed animals with legs spread; for shrimp and other translucent species living photos are much better. A tissue sample extracted from a leg, pleopod in shrimp, or abdomen and fixed in concentrated ethanol provides a good additional genetic sample. Fix more fragile crustaceans in 70-80% alcohol, as legs, especially, become brittle and easily break if fixed in alcohol of higher concentration.

**Mollusks:** For routine diversity work shells are adequate, but the scarcity of available anatomical and genetic material means that soft body fixation is very important. For anatomy (and for all softies like opisthobranchs) relax carefully ($MgCl_2$, propylene phenoxitol, etc – varies with taxon) and fix in formalin or Bouin's, with a subsample in ethanol if possible. For Genetic samples (and poor anatomy), fix in 95% ethanol or compromise with 70-80% ethanol. Cephalopods have to be fixed in formalin, stored in EtOH, an ethanol subsample is very important. Photos are most useful for opisthobranchs and cephalopods, but also for soft body of any species. A good technique for shelled mollusks where shell and tissue are desired is to microwave them for 0.5-2 minutes in a high power microwave; this blows the animal out of its shell and then the body fixes well and is accessible without breaking the shell. Microwaving does not destroy the DNA.

**Asteroids and ophiuroids:** Relax with $MgCl_2$ or chloretone in flat pan so animal spread out flat, then add or transfer to formalin or alcohol. Asteroids can be fixed without relaxing if flattened out in seawater pan. Best asteroid fixes are achieved by letting bug flatten out in seawater then adding enough concentrated formalin to bring solution to 5-10%. Fix for several days then wash in freshwater to get salt out (or fix initially in freshwater formalin), then air dry in shade. Smaller animals are better fixed and preserved in ethanol. Associated field photos and genetic ethanol subsamples important. For large star this can be snipped tube feet, or hepatic caeca/gonad extracted from body prior to fixation.

**Crinoids:** Rapidly push down face first into 95% EtOH, spreading arms out as pushing animal into fixative by hand; animal will die in 30-90 seconds in a spread-out position. Preserve in alcohol. Field photos useful; keep associated commensals.

**Holothuroids:** Relax in $MgCl_2$ with a squirt of menthol on top, wait until animal does not contract when touched. Place in ethanol until it dies, then inject with 80-95% EtOH depending on size; preserve in EtOH. Field /live photos extremely useful. A subsample of tentacle, tube feet, or internal organs useful for DNA.

**Echinoids:** Can relax in fridge or by chemicals or fix straight; fix in EtOH or formalin and preserve in EtOH, or fix in formalin, wash, then dry if big and bulky. See under asteroids for drying. A subsample of tube feet, or gonad/gut, taken with forceps inserted through a cut in the peristomial membrane provides a useful tissue sample.

**Ascidians:** Relax with tricaine (or menthol if tricaine is not available), fix in formalin, store in EtOH. Didemnids need neutralized formalin as they have calcareous ossicles. Take snippet of inner part of siphons in ethanol of larger solitary species or of chopped up bit of colony for DNA. Best to fix a good piece of colonial species for DNA, so zooids can be pulled for extraction later, so use a larger vial for that. Photos very useful.