Protocol for Sampling and Preservation of Marine Organisms

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The identification of many invertebrates requires morphological and histological examinations of preserved specimens and experience for correct interpretation of the observed features. Poorly preserved or damaged material resulting from improper sampling complicates examination and identification or even makes it impossible. In addition, many characteristics of the living specimens which could help with identification are lost during the preservation process. When correctly applied, digital photography and correct sampling methods allow the documentation of a lot of information on the living animal and its habitat and create optimum prerequisites for the identification process.

For a sound identification of a species, it is often necessary to collect and examine the specimens. In challenging groups such as Porifera, Cnidaria, Ectoprocta, etc., the collected specimens often have to be delivered to an expert, ideally with pictures of the same individuals and as much additional information as possible. In the following section, we describe general and specific protocols for the correct documentation, collection and preservation of marine organisms.

1) *In situ* Documentation

Except for well known species, pictures alone are not enough. For ecological studies, a few identified voucher specimens of every species should be deposited at a museum to make the identification verifiable for further studies.

At the collection site, carefully record all available biotic and abiotic information such as microhabitat, coordinates of sampling site (ideally with a GPS), substrate, depth, and time. Also write down who collected the specimen. Underwater *in situ* photography gives essential information concerning colour and shape of the specimen. Well focused and correctly exposed pictures of the whole animal as well as close-ups showing characteristic features should be taken (see chapters for characteristics of taxonomic importance).

2) Collection

Many species are slow growing and live up to many decades or more. Some are rare. Thus collection of specimens should not be done without necessity. In addition, be careful not to destroy or damage specimens in the vicinity of the specimen you want to sample; e.g. with your fins while diving. The interest of conservation should always be kept in mind.

Intertidal species can be collected at low tide; subtidal shallow-water species should preferably be collected by SCUBA diving. Sessile or sedentary species should be carefully detached from the substratum; mobile species are caught by hand or with nets. Buried species are often attached to stones or shells in the substrate; small unattached specimens living in soft substratum are best collected using a shovel or trowel and a sieve. Any interesting reactions should be noted. In some groups (such as sponges, zoanthids, gorgonians, or large ectoproct colonies) it is enough to collect a part of the specimen/colony, and to take a photo of the entire specimen/colony (to avoid damages of the population of slowly growing species). In some species (encrusting sponges and ectoprocts, sea anemones) it is useful to collect the specimen with the substratum to avoid destruction of important features. Avoid putting more than one specimen into one bag or jar, to prevent confusion and destruction of the specimens (if one feeds on the other, or one secretes substances that damage others, e.g. be careful with cnidarians, sponges, sea cucumbers, ascidians ...). The material should already be labelled in the field.

3) Observations in the Aquarium

The collected specimens (if complete, not heavily damaged and alive) should be transferred into an aquarium with well ventilated and/or regularly changed water. For photography, the ideal aquarium is long, narrow and low, thus allowing easy manipulation and pictures to be taken from different perspectives with a minimum of water between the animals and the camera

lens. One or better two strobe lights facilitate even illumination, keep exposure times short and enable small apertures of the diaphragm. Put a natural background (stones, algae), or, if not available, black velvet IN the aquarium for a good photo background. Putting background below the aquarium causes reflections from the flash. Some features can be seen more easily on living than on preserved specimens. Keeping specimens in an aquarium also allows for observations of behaviour and activity patterns. If the specimen is damaged, dead or you collected only a part of it (e.g. in sponges), you should preserve it as soon as possible (within a few hours for the latest) to avoid decomposition.

4) Relaxation

Before preservation, the specimens of many taxa need to be relaxed to prevent contraction and secretion of mucus during fixation. Strongly contracted specimens are difficult to examine and even preclude identification in some cases (see Tab.1). In addition, relaxation reduces unnecessary suffering of higher developed organisms. Relaxation protocols are numerous, but are not without difficulties. An effective protocol for many taxa is to put them in a dark and cool place (e.g. fridge) into a jar containing a small amount of freshly collected, well ventilated water, ideally with current. The choice of anaesthetic depends on the taxon (see Tab.1). Depending on the group or even species, specimens need less than one hour or up to a day until they are completely relaxed and cease to respond to tactile stimuli.

5) Fixation and Preservation

The relaxed animals should be carefully transferred into the preservative, which is generally a solution of 4–15% seawater: formalin (concentration depending on specimen size) in the case of soft-bodied specimens (high concentrations of ethanol harden and dry soft tissue), and 70% ethanol for species with hard structures used for identification or for species containing $CaCO_3$ (formalin dissolves calcium carbonate unless it is buffered. Borax $Na_2B_4O_7$is the most commonly used buffer). Larger, soft specimens can be prevented from further contraction by injecting formalin. This also avoids decomposition of internal structures due to low concentrations of formalin. Large specimens may cause too much dilution and the preservative should be changed after 1–3 days. Normally 70% EtOH is used as long-term storage. In the case of formalin-preserved material, the transfer to 70% ethanol should not be carried out before the material has been stored at least 24 hours to some weeks (depending on taxon) in formalin. Put the specimens in a sieve and wash once or twice with tap water before transferring into ethanol.

IMPORTANT: When diluting ethanol (sold in concentrations of 96%), use water from the tap; when diluting formalin (sold in concentrations of 37%), use seawater. An easy method to dilute highly concentrated liquids is the following: to make 70% ethanol from 96% ethanol, put 70 ml of 96% ethanol in a measuring cup, and fill up with tap water to 96 ml (to make higher amounts, use multiples of the values). To make 10% formalin from 37% formalin, put 10 ml of 37% formalin into a measuring cup, and fill up with seawater to 37 ml (to make higher amounts, use multiples of the values).

Be CAREFUL, formalin is corrosive and potentially carcinogenic unless used under a fume hood. No equipment used with formalin should be used with specimens preserved by other means. The slightest trace of formalin in a sample can degrade the proteins, making it useless for molecular work. Always mark pots/equipment with "F" to be sure. DO NOT dump used formalin, bring it to a University which should have containers to collect it and safely dispose of it (there are companies collecting old formalin in larger amounts).

6) Tissue for Molecular Work

Ideally preserve at least 3 to 5 individuals (if small) or pieces of individuals (depending on the group, different regions of the specimen are used) per sample for future molecular work. For isoenzyme analysis, frozen tissue is required. Tissue for molecular work should be preserved either in a solution of dimethylsulfoxid and sodium chloride (DMSO–NaCl) or in 90–100% ethanol (the higher the concentration, the better). Although the degenerate ethanol sold in pharmacies in most cases leads to useful results, absolute ethanol (laboratory quality) is preferable.

Phylum	Taxon	Preservative	Relaxant	Note
Porifera	Calcarea & Demospongiae	96% ETOH	No	Take only a piece; preserve shortly after collection to avoid decomposition
Cnidaria	Octocorallia (soft corals, gorgonians, sea pansies)	70% ETOH (in formalin spicules will dissolve); small pat in 96% ETOH for molecular work	Menthol crystals (polyps should ideally be expanded)	A piece of a larger colony is enough when the entire colony is photographed
Cnidaria	Actiniaria, Corallimorpharia, Zoantharia & Ceriantharia (soft-bodied hexacorals)	4–10% formalin (the larger the specimens, the higher the concentration); if more specimens available, 3 specimens/pieces of pedal disc in 96% ETOH; in Zoantharia: 96% ethanol specimens are crucial	Menthol crystals for formalin samples (1h–1 day; tentacles should be expanded and unresponsive to touch)	Strongly contracted specimens, and specimens preserved in 96% ETOH are not -or only on a very limited scale- useful for morphological identification
Cnidaria	Scleractinia (stony corals)	70–96% ETOH, or dried (in formalin skeleton will dissolve)	No	When cnidae will be examined, formalin should be used as preservative (in which case relaxing the specimens is necessary)
Cnidaria	Antipatharia (black corals)	96% ETOH (if very big, a large part of the colony can be preserved dry)	No	In case of large colonies, a piece is enough; colony should be photographed
Cnidaria	Hydrozoa, Stylasteridae (hydrocorals)	96% ETOH (if very big, a large part of the colony can be preserved dry)	No	In case of large colonies, a piece is enough; colony should be photographed
Cnidaria	Hydrozoa, all others	2–5% formalin; small sample in 96% ETOH	Slowly add concentrated solution of $MgCl_2$ (stock solution) to animals until they are unresponsive to touch (if not available, use menthol crystals); if histological studies are necessary, use Bouin's fixative	
Cnidaria	Scyphozoa & Staurozoa (jellies)	Entire animal in 5–10% formalin; small piece in 96% ETOH	5 min. in seawater with 1% $MgCl_2$	
Platyhelminthes	Polycladida	Fixation in Bouin's or 10% formalin (at least 3–6 h); 96% ETOH for molecular studies	7% $MgCl_2$ solution or menthol crystals	
Nemertea		Cut the worm in 2 pieces behind the head+foregut (~4–5x the head, often more cylindrical); cut off 0.5 mm^3 from anterior end of posterior fragment and preserve in 96% ETOH for molecular studies; then fix the remainder as straight as possible in 10% formalin (cut in pieces if long)	7.5% $MgCl_2$: seawater (1:1) or MS–222 (Tricaine-Methane Sulphonate) (add powder); small worm 0.5–2h; large worm 2–8h	Without proper treatment, the specimens most probably cannot be identified!
Sipuncula & Echiura		5% formalin; 96% ETOH for molecular studies	5% ethyl ether: seawater or menthol crystals (much slower; several hours) until unresponsive to touch; then 1 h in freshwater	
Annelida	Polychaeta	10% buffered formalin (at least 24 h); 96% ETOH for molecular studies	7% $MgCl_2$ solution or sparkling water (add over several h); recommendable to put in or 10–30 % ETOH before preservation so that proboscis is expanded.	To avoid ripping parts off, use feather steal forceps. Proboscis should be expanded.

Mollusca	Polyplacophora (chitons)	phosphate-buffered formalin, cacodylate-buffered glutaraldehyde* in combination with osmium-tetroxide* postfixation, or 80% ETOH; 96% ETOH for molecular studies	7% MCl_2 or menthol crystals	Press flat when put into preservative so that they don't roll in.
Mollusca	Opistobranchia (sea slugs)	70% ETOH; 4% formalin, or buffered and isotonic 3.7% glutaraldehyde solution*; 96% ETOH for molecular studies	Isotonic $MgCl_2$ solution (~7%) (or menthol crystals) until unresponsive to touch; a few min. until more than 1 h	
Mollusca	Other Gastropoda (snails) & Bivalvia (mussels)	96% ETOH, or dry	No	
Mollusca	Cephalopoda (squid, octopods)	15% formalin; a piece in 96 % ethanol for molecular studies	Menthol crystals	Unrelaxed (and thus contracted) animals are very difficult to identify!
Arthropoda	Pycnogonida	4% formalin; 96% ETOH for molecular studies	Menthol crystals	
Arthropoda	Peracarida	5% formalin; 96% ETOH for molecular studies	No	
Arthropoda	Cirripedia (barnacles)	90% ETOH; 96% ETOH for molecular studies	No	
Arthropoda	Decapoda (crabs, shrimps, lobsters)	4% formalin (24 h), then transfer to 70% ETOH through series (i.e. 30%, 50% to 70%); 96% ETOH for molecular studies	Clove oil is better than menthol crystals; takes a long time	Careful, legs can fall off!
Ectoprocta (Bryozoa; moss animals)		70% ETOH; 96 % ETOH for molecular studies	No	In case of large colonies, a piece is enough; colony should be photographed
Brachiopoda (lamp shells)		96% ETOH	No	
Echinodermata	Crinoidea (feather stars)	70% ETOH; 96% ETOH for molecular studies	7% $MgCl_2$ or menthol crystals	Careful, can throw off arms!
Echinodermata	Echinoidea (sea urchins)	70% ETOH; 96% ETOH for molecular studies	No	
Echinodermata	Ophiuroidea (brittle stars)	80–90% ETOH; 96% ETOH for molecular studies	Not absolutely necessary; menthol crystals	Careful, can throw off arms!
Echinodermata	Asteroidea (starfish)	10% buffered formalin or dry, but 70–96% ETOH also ok; 96% ETOH for molecular studies	Not absolutely necessary; warm freshwater or other relaxative	
Echinodermata	Holothuroidea (sea cucumbers)	10% buffered formalin, but 70–96% ETOH also ok, can be stored dry if climate is not wet; 96% ETOH for molecular studies	0.015% propylene phenoxetol:tap water (stock solution 15 ml/l), used 1:9; $MgCl_2$, $MgSO_4$, or menthol crystals (tentacle crown should be open)	
Chordata	Ascidiacea (ascidians)	5–10% formalin; a piece in 96% ETOH for molecular work	Menthol crystals; need a long time, (several h to overnight)	Strongly contracted specimens (preserved without relaxation) can often not be identified!
Chordata	Petromyzontida & Gnathostomata	10% formalin; a piece in 96% ETOH for molecular work	Ideally 50–75 mg/l MS–222 (Tricaine-Methane Sulphonate)	Relaxation with MS–222 avoids unnecessary suffering of the fish!

Tab. 1. Preservatives and relaxants sorted systematically by taxa. ETOH: ethanol (dilluted with tap water); *: highly toxic. **Note:** Preferably use buffered formalin (dilluted with seawater), especially important when specimens have calcareous parts. Most taxa that are recommended to be preserved in 70% ETOH with a small part in 96% ETOH for molecular studies, can also be preserved completely in 96% ETOH when time is restricted. Menthol crystals can be bought in crystal form in a pharmacy; some crystals are scattered on the surface of the water (they can be dried and re-used later).

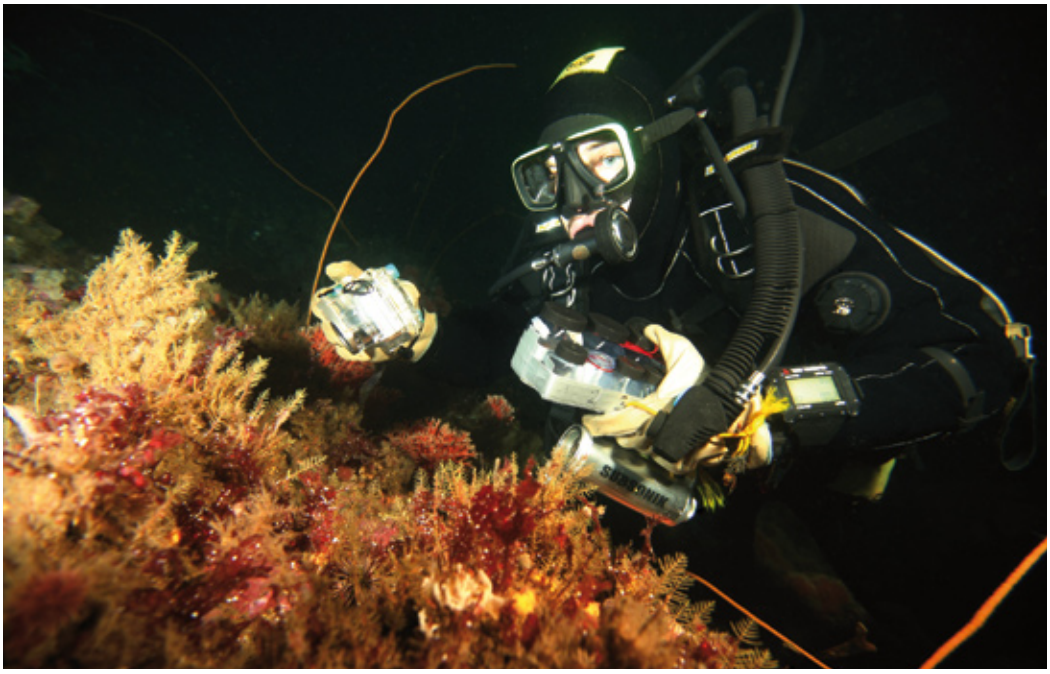

Fig.1. Organisms should be thoroughly photo-documented *in situ* before sampling.

Fig. 2. It is recommendable to transfer sampled specimens into an aquarium for further photo documentation and relaxation.