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Marine, Bethonic Nemerteans: How to Collect and Preserve Them

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INTRODUCTION

In recent years only a few zoologists have worked systematically on the Nemertini. The "proboscis worms" are thus often neglected during marine biological surveys, and, even when occasionally collected, are usually not treated in such a way that a specialist can undertake successful microscopical studies. No doubt collectors and field assistants have the best intentions when they place living nemerteans into a collecting jar with alcohol. Unfortunately, however, the result is broken (autotomized) specimens, with ejected or lost probosces, which soon show no color or pattern and which, furthermore, are not fixed properly for the histological examination necessary to identify the species. This maltreatment of material is due not so much to the shortage of time during cruises and field work as to the lack of easily available information for persons who are not familiar with the group. This lack of information is evidenced by an increasing number of inquiries from students who are interested in marine invertebrates and in methods of collecting nemerteans.

The following account covers the entire procedure—from collecting the living worms to preserving the specimens. It includes successfully proved methods for obtaining nemerteans from different types of substrate, ways of preparing the living animals for drawings, instructions

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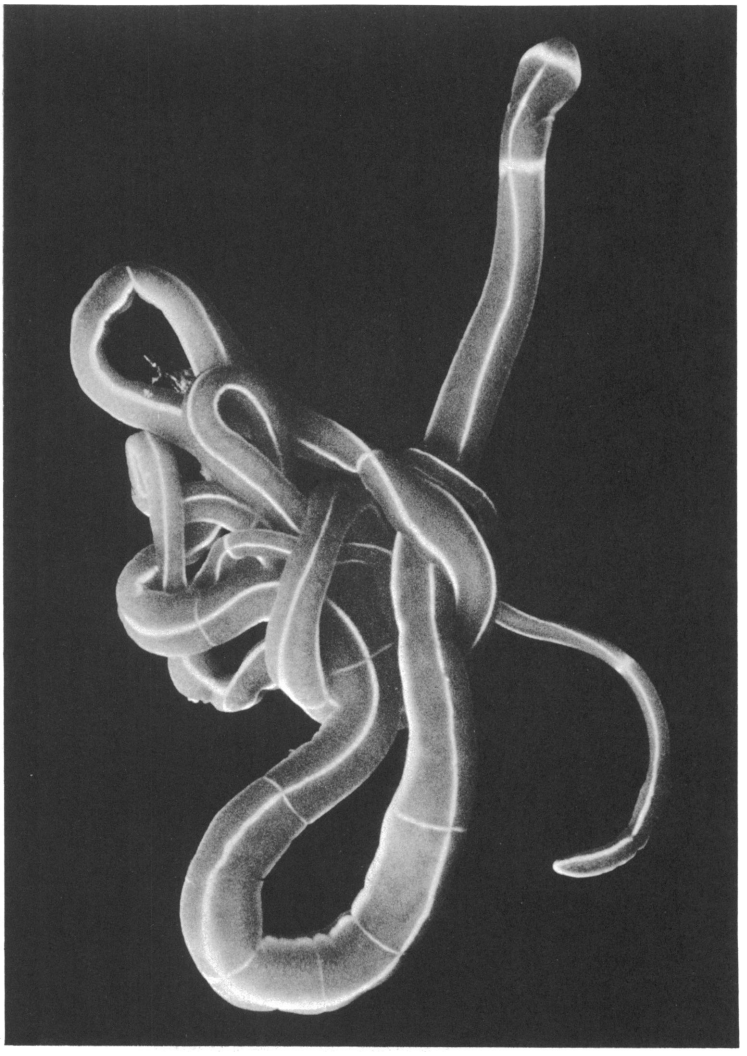

FIG. 1. *Tubulanus annulatus,* an example of a larger proboscis worm that can be found by examining the content of a dredging sample. This species is red, with white stripes and transverse bands. It reaches a length of 250 mm. Photograph by Maria Wimmer.

for the narcotization of nemerteans, and the proper fixing and preserving of the material for future histological investigations. It is hoped that the suggestions made here will be helpful for persons who want to collect nemertean materials of scientific value.

COLLECTING

A collector can find some of the larger species of nemerteans (fig. 1) by turning over stones in intertidal and shallow sublittoral waters, by examining pieces of coral heads, by inspecting the contents of dredging samples, or by sifting mud samples, but, of course, one cannot expect to collect a representative sample of the nemertean population of a particular biotope under survey by these methods. Such samples average only a small percentage of the actually existing nemertean fauna living in the particular collecting area. Many more animals can be collected and, according to the type of substratum, the following methods are suggested for obtaining most of the nemerteans from a sample:

MUD: Samples of mud, whether taken with a dredge from great depths or directly by the collector in estuaries or mangrove swamps, should be placed as soon as possible in wide trays. There the substrate must be gently stirred with sea water until no clumps remain. After the sediment has settled, the substrate layer in the tray should not exceed 15 cm. in thickness and should not be covered by more than 2 cm. of water. When the water is clear, the mud surface should be checked for nemerteans that by chance were not buried in the sediment. At the same time larger animals (mainly polychaetes, mollusks, Crustacea, and echinoderms) can be removed; otherwise they disturb the upper mud layer and are irritating to the nemerteans that are attempting to reach the surface. The smoother the substrate surface, the easier it is to find the nemerteans, which generally appear about 24 hours after the sample has been brought in. The worms can be picked up with a pipette, with the aid of the light of a lamp and a magnifying glass. If no more nemerteans are found in this way (normally after about two days), smaller samples should be taken from different parts of the mud surface. Sediment from the upper 3 mm. of the mud in the tray is transferred into glass dishes and examined under a dissecting microscope. If no additional nemerteans are found in these control samples, it is an indication that the main sample is exhausted.

The old but often-used method of sorting the fauna of mud samples through a set of sieves is not of any particular advantage because most of the soft and delicate nemerteans may be damaged or lost.

SAND: To obtain sand-living nemerteans different methods can be used: (1) Sand is placed in a pail and stirred fairly vigorously with about three times as much sea water as its own volume. After being stirred, when the sand has almost settled, the water with the still-suspended animals is poured into a plankton net, and from the net the animals are transferred into glass dishes and sorted. This procedure

should be repeated four to five times for each sample. (2) A sand sample can be treated in the same way as described above, but, instead of pure sea water, 6 per cent of MgCl (in tap water) is used. (3) Glass cylinders (2–3-liter capacity) can be filled with samples of sand and covered with sea water to a level of about 1 cm. above the sand surface. If the samples are left standing undisturbed, within three to five days all the animals appear on the upper layer of 1 cm. of sand, from which small samples can be examined under the dissecting microscope. A careful check is necessary, as psammobiontic nemerteans are generally rather small (1–10 mm.).

ROCKS, CRUSTS OF SEDENTARY ANIMALS, REEF CORALS, MARINE PLANTS: As far as the procuring of samples of the substrate is concerned, it is preferable to collect them by diving (skin- or SCUBA-diving). This method, of course, depends on the abilities of the collector, available diving equipment, and the depth of the water. The advantage of this method is that the sample material can be placed immediately, while still under water, in polyethylene bags or cloth sacks, thus eliminating the danger of washing the nemerteans out of the substrate.[1] Furthermore, quantitative samples can be taken and ecological data are obtained more easily. Later, on deck or in the field laboratory, the surface of hard substrates such as rocks, clumps of encrusting organisms from hard bottoms or submarine caves, and reef corals are examined first to remove the larger nemerteans. The removal must be done quickly to prevent the sample from drying out. If the pieces of the sample material are larger than the size of a fist, they should be broken up, the collector again looking for nemerteans (fig. 2). Next, the substrate and the remaining water in the collecting bags are placed in glass jars (most convenient are rectangular jars with a capacity of about 30 liters and higher than wide; avoid aquaria with steel frames). The glass jars are filled with sea water to a level of approximately 5 cm. above the substrate. These samples should be kept in a dim light or in the shade, only one corner of the jar being exposed to sunlight or to artificial light.

If one is dealing with marine plants, the material can be transferred immediately from the collecting bags to the glass jars. A desirable density of the sample material can be obtained if the jar is filled to two-

[1] Tests that were carried out by the author in the Mediterranean showed, for instance, that a sample of *Cystoseira barbata* (brown algae), which was collected from a boat and brought in from a depth of 2.5 meters with a rake, contained only 7 per cent of the nemertean fauna that was obtained from a similar sample taken from the same place by a diver.

FIG. 2. Large pieces of the sample material are broken up. The substrate then is transferred to the rectangular glass jars.

thirds of its capacity with plants, and sea water is added almost to the top of the jar. Floating plants can be lowered from the water surface by arranging two wooden sticks diagonally in the jars. The sticks should be weighted with a clean, smooth object, such as a stone, which is heavy enough to keep the uppermost plants about 5 cm. under water. The sample is otherwise treated the same way as is mentioned above for hard-substrate samples.

In a few hours the first nemerteans will appear in the lighted corner of the jar. They are, together with many other animals, forced to leave their hiding places in and on the substrate by the change in the environmental conditions. The respiration of all the animals in the sample continues and is even intensified by the rising water temperature, but the plants have almost completely stopped assimilation because of the dimness of light. The result is a decreasing oxygen supply and an increasing carbon dioxide content in the water of the sample. These unsuitable conditions apparently exert a strong influence on the behavior of nemerteans. Most nemerteans are positively geotactic and negatively phototactic under normal circumstances, but, under the conditions above described, they migrate to the water surface (negative geotaxis) in the

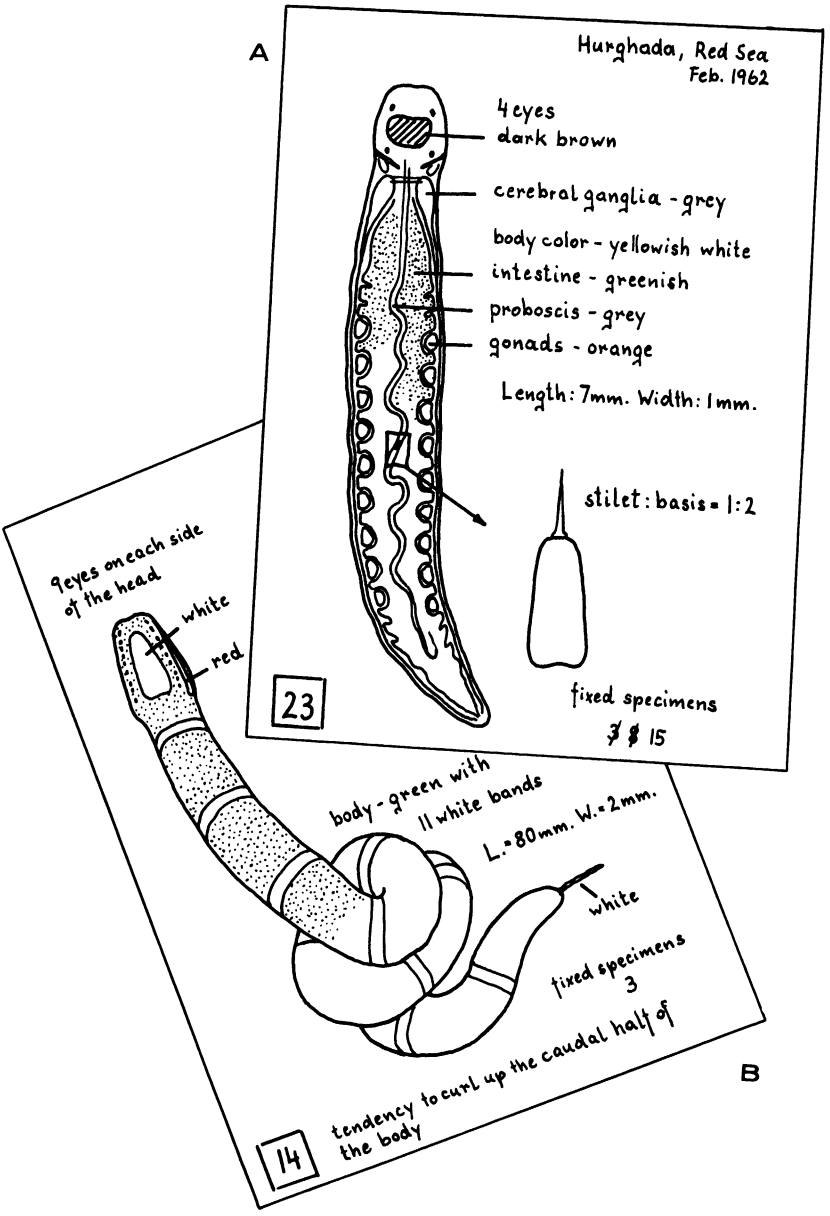

FIG. 3. Samples of sketches made from living animals. A. A small and semi-transparent nemertean with details obtained from a squeezing preparation. B. A large and intensively colored worm from which only external features were recorded.

light corner (positive phototaxis). When the lighted corner is checked, at intervals of 15 to 30 minutes, all animals should be removed so that later-arriving nemerteans are not irritated by other invertebrates, such as polychaetes, Crustacea, or mollusks. When animals no longer occur in this "physiological trap," the sample is exhausted, normally after one to four days, depending mainly on the number of organisms in the sample and the temperature of the water, which is, of course, influenced by the air temperature.

DRAWINGS

The systematics of nemerteans is based on differences in the internal organization of the species, primarily because the descriptions of many species were originally based on preserved materials brought back from expeditions, but from which no external features of the living animals were noted. Unfortunately such is still the procedure. A species description containing only external characters is of little value because the final decision concerning the identity of a species must be made after examination and comparison of the anatomy. On the other hand, a purely anatomical account is also insufficient, because a species so described cannot be readily recognized if it is subsequently found alive in the field by another person, thus increasing the already existing difficulties which are encountered while one is doing ecological investigations on nemerteans in the field. Therefore, it is most desirable to combine the anatomical account with a detailed description of the external features of the animal. The drawing of living specimens is, therefore, a very important step in the course of nemertean collecting.

Neither artistic ambition nor much time is necessary to make such drawings (fig. 3). A simple pencil sketch (on a sheet of paper about 14 by 20 cm.) is sufficient, if it includes the following details: outline of the animal, especially showing the shape of the head lobe and the caudal end of the body which, in certain species, can also bear a little tail called a caudal cirrus (fig. 3B); eyes, with their number and arrangement if they are present and discernible; other differentiations in the head region, i.e., cephalic slits (running lengthwise along the lateral sides of the head; see fig. 3B) and cephalic grooves (running transversely in the head region; see fig. 3A). If color patterns are found (longitudinal stripes, cross bars, or similar markings), they should be outlined in the drawing, and their color as well as the general body color should be described briefly. In some light-colored and semitransparent species, internal organs (proboscis, brain, intestine, gonads) are often seen through the dorsal body surface, so their outlines can be added to the drawing

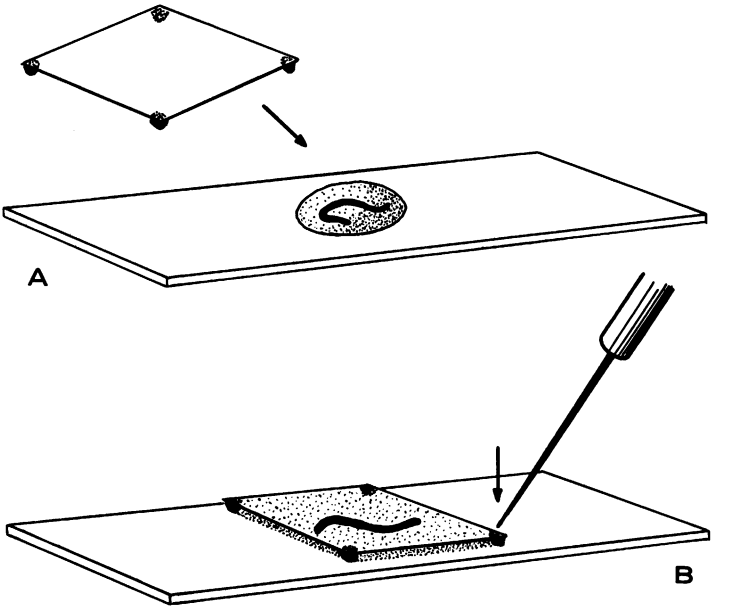

FIG. 4. The squeezing preparation. A. A small and lightly pigmented specimen of Hoplonemertini Monostilifera (or an everted proboscis) is placed with a few drops of sea water on a microslide. A coverglass with tiny pieces of beeswax on each corner is placed over the animal. B. The wax between the slide and coverglass must be compressed to flatten the animal (or proboscis) and in order to make it translucent.

(fig. 3A). It is necessary to take the measurements (length and width) of the specimen when it is not irritated and therefore not contracted, and some remarks on the behavior of the animal (showing a strong tendency to curl up; posterior portion of the body always bent to one side; animal swims from time to time by undulation of the body; and so on) are very useful if they supplement the drawing. A dissecting microscope is always necessary for the observation of the animals. Each drawing must show the number or letter under which the species is filed in the collecting-data records.

For collectors with enough time, with a particular interest in nemerteans, with some familiarity with the group, and with a compound microscope, a more complicated method of obtaining further details about the inner organization of Hoplonemertini Monostilifera is described below. The purpose of this method is to prepare the living animals in a way that permits the observation and drawing of the proboscis arma-

ture which, in this suborder of the Nemertini, consists of a central stilet resting on a special base and two or more pouches containing several accessory stilets. As it is rather difficult to reconstruct the shape of the stilet and base from microsections, drawings made from living animals are very helpful (fig. 3A). If possible the ratio of stilet length to base length (1/3, for example) should be noted beside the drawing. Small (1 to 10 mm.), lightly pigmented specimens are placed with one or two drops of sea water on a microslide and covered with a coverglass which is supported on all four corners with tiny pieces of soft beeswax (fig. 4A). When the slide is under the microscope, the wax between coverglass and slide can be compressed (with a dissecting needle) just sufficiently so that the coverglass prevents the animal from moving and squeezes it enough to be translucent (fig. 4B). The drawing can begin, but it should not continue for more than approximately 10 minutes because otherwise the animal will die and disintegrate. After the observation is finished, more sea water is added to the preparation, and, by a careful lifting of the coverglass, the animal can be freed and transferred to the glass dish from which it was taken.

For larger species and those of the smaller ones that are dark colored, only the proboscis is used for a squeezing preparation. The animal is separated from the rest of the specimens and a few drops of 70 per cent ethyl alcohol are added to the sea water, thus irritating the animal so that it everts its proboscis. The proboscis then is treated in the same way as described for the whole specimen in the foregoing paragraph. It must be mentioned that this proboscis examination should not be performed unless at least three undamaged specimens of the same species have been preserved in the proper way. Nevertheless the examined animal and its ejected proboscis should be fixed and preserved if both are still alive at the end of the observation, but the animal and its proboscis should be kept separate from the other specimens in another vial.

NARCOTIZATION

For many of the marine invertebrates, narcotics must be used prior to the fixation in order to kill the animals in expanded condition. The importance of this procedure should not be underestimated, especially for nemerteans, as these proboscis worms are capable of high contractability, and most of the larger specimens will break into pieces (caused by overly strong contraction of circular muscle portions in the body wall) if they are directly transferred to the fixative. Furthermore they will evert the proboscis, which should be avoided whenever possible.

As far as nemerteans are concerned, urethane and chloral hydrate are

very satisfactory for narcotizing. A specimen may be kept in a glass dish large enough to contain sufficient sea water (for a specimen 5 cm. in length, about 300 cc. of sea water should be used); a few crystals of either of the two narcotics are added gradually at intervals of about 10 minutes. The required time for complete narcotization differs with the size of the specimen, but generally after 30 minutes to two hours the animal will be motionless and expanded. If, during this process, an animal contracts and remains in that position or the proboscis is partially everted, too much narcotic has been added and the animal must be removed to fresh and clean sea water. The narcotization can be repeated after the animal revives.

FIXING AND PRESERVING

As soon as the animal under narcotization is completely relaxed and expanded, it should be transferred with a pipette to the fixative. One of the best fixatives for nemerteans is Bouin's fluid (75 parts picric acid in saturated aqueous solution, 25 parts formalin, 5 parts glacial acetic acid), which will kill the animal, fix the structure of the tissues in a very satisfactory condition for future histological examination, and provide good staining for the microsections. Small animals (up to 10 mm. in length) should be fixed three to five hours; larger ones, 12 to 24 hours. The fixed specimens are then preserved in 70 per cent alcohol which must be renewed at intervals of a few days, as long as it turns yellow from the picric acid washed out from the specimens. Animals that ejected the proboscis during fixing, though they were narcotized, should be separated from the other specimens of the same species and stored in another vial. Every vial should contain a label with the number or letter under which the preserved species was labeled on the drawing and registered in the collecting-data records.