Study methods for freshwater bryozoans

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Abstract: Bryozoans are found in a wide variety of aquatic habitats. For most species proper collection requires their removal along with the substratum to which they are attached. Specimens are narcotized in menthol or chloral hydrate, then fixed and preserved in 70 % ethanol. Statoblast valves may be separated with hot KOH for examination with light microscopy. For laboratory culture the colonies are grown upside down in water conditioned by the presence of fish. In situ culturing can be achieved on artificial substrata. Standard techniques are used for histological work and scanning electron microscopy. Practical methods for chromosome and molecular studies also are available. Several good reference books review the biology of freshwater bryozoans and offer identification keys.

Key words: Bryozoa, Phylactolaemata, culturing, methods.

Introduction

Bryozoans are among the most fascinating invertebrate animals in fresh water. Although similar in structure to their marine relatives, the freshwater species are larger, bolder, and easier to study. There is much to be learned about the entire group, since many aspects of their ecology, physiology, and development are still poorly understood. Moreover, like corals, bryozoans represent a fine example of modular organization, in which the entire animal is composed of many individual units capable carrying out basic functions of life. Studying these minute creatures offers rewarding opportunities to scientists, students, and other interested persons. This paper describes a range of techniques and procedures currently being used to investigate freshwater bryozoans.

Finding bryozoans

Bryozoan colonies are often seen but seldom recognized. Their name, meaning "moss animal," refers to the appearance of certain species: inert brown crusts and spindly tendrils that are easily mistaken for dead moss. Other species, however, are gelatinous blobs ranging from the size of a pea to that of a football.

All bryozoans grow on submerged surfaces, including plants, wood, rocks, glass, aluminum, and a wide range of synthetic materials such as automobile tires, plastics (including plastic bags) and fiberglass. Most species occur on the undersides of objects where they are protected from settling particles, although this is less true in turbulent water. Although cold adapted species can be found at great depths, all species normally occur in water less than 1 m deep, and so they are easily collected from shore. Warm, eutrophic waters generally support greater growth and species diversity than habitats were the water is clear and cold. Places where bryozoans are usually **not** found include:

- Oily, rotting, or actively corroding substrata;
- Habitats with low oxygen or a pH below 6;
- Sites lacking firm substrata;
- Rivers and streams where smooth, rounded rocks shift easily during high water.

Quiet backwaters of lakes and rivers often harbor bryozoan colonies. Other good places to look are the undersides of floating or dangling objects that are out of reach to benthic predators such as snails and flatworms. Floating buoys and docks, old dangling rope or branches, padded boat fenders

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Fig. 1: Basic tools for collecting bryozoans: 14x magnifying lens, fixed blade knife, and wide-mouth polyethylene jar. The knife is safely transported inside the jar, especially when the collector is clambering around submerged substrata.

even boat hulls are all good potential substrata. Additional good sites include streams below reservoir impoundments where there may be abundant suspended organic particles. Certain aquatic vegetation offers better substrata than others. For example, bryozoans grow well on floating leaves of American lotus (*Nelumbo lutea*), but are seldom seen on leaves of the white or yellow water lily (*Nymphaea virginiana*, *Nuphar lutea*).

In temperate regions of North America bryozoans are abundant in the late spring when water temperatures rise above 20 °C. There may be a period during July and August when colonies are scarce, but this is followed by renewed growth in the fall. Some species, such as *Fredericella* and *Lophopus* are well adapted to cold temperatures and may even be collected under the ice in winter.

One useful technique for finding bryozoans is to search first for their statoblasts. While this may not lead directly to bryozoan colonies, it can at least indicate which species are occurring in the general area. A simple method is to use a magnifying lens to examine the small particles that cling to floating objects at the waterline. Free statoblasts adhere particularly well to plastic foam (expanded polystyrene, or Styrofoam®), and they show up well against the white background. HILL & OKAMURA (2005) have detected the presence of the rare *Lophopus crystallinus* by examining flood and floating debris. JONES et al. (2000) describe a method for recovering statoblasts from lake sediments by passing material through a stack of standard sieves with mesh openings of 1.0 mm, 500 µm, and 150 µm. Organic particles are separated from heavier material by washing and swirling, and the final product is examined on P8 coarse filter paper.

Collecting bryozoans

When collecting bryozoans it is desirable to keep the specimens intact. For branching colonies this can be done only by removing them together with the underlying substratum. With wood and other soft substrata this is easily done with a sturdy, sharp knife. For reasons of safety a fixed blade knife is recommended over a folding blade. For many years I have used a 9-inch Buck knife (El Cajon, California), which is perfect for almost any situation (Fig. 1).

Hard, brittle substrata, such as stone, require a hammer and cold chisel. With practice, it is possible to chip off the specimen intact and then trim away excess rock with the chisel or a small pair of pliers. Some rocks, such as granite or basalt, do not chip well and are best avoided.

Other useful equipment for collecting bryozoans (Fig. 1) includes:

Small magnifier (or loup), usually worn on a lanyard around the neck, magnifying 10x to 14x. Some people like an elasticized lanyard so the lens is not flopping about or dipping into the water. A Coddington-type magnifier with solid lens is preferred because it is always fully submersible. To use the lens properly, bring it to your eye and then raise the object into view. The hand holding the lens should reach out with the small finger to stabilize the object.

Specimen container, preferably one that is buoyant and unbreakable. I use a standard 1-liter widemouth polyethylene bottle. Both the bottle and the lid will float, and the bottle will also hold my knife snugly. (Note, however, that when the bottle contains the knife or heavy specimens it will sink unless some air is trapped inside).

Preparing specimens

Collected specimens will normally keep in a 1-liter jar for up to 12 hours, depending on temperature and the degree of crowding. Colonies are then sorted in a shallow tray and processed as soon as possible.

Normally the first step in processing is to anaesthetize the zooids so the lophophores will remain extended. Among the most effective and convenient narcotizing agents are menthol and chloral hydrate, both available in crystalline form from chemical supply companies. In tropical regions chloral hydrate has the advantage of a higher melting point, but it is often difficult to obtain. Crystals of menthol can be powdery and messy to work with, so you may want to reform them into larger chunks. Melt them together over low heat, pour into a shallow pan of aluminum foil and cool to make a small slab 1.5-3 mm thick. Break the slab into smaller pieces and store in a tight container in a cool place. To anaesthetize bryozoans place colonies in a small amount of water, add enough menthol to cover at least half the surface, and cover the container. Leave undisturbed for at least 45 minutes at room temperature. The menthol diffuses slowly into the water and relaxes the muscles. To achieve uniform effect you may swirl the water once **very gently** after about 30 minutes. This procedure is finished when zooids no longer respond to gentle probing. The more tentacles on the lophophore the more quickly menthol takes effect, so for example *Cristatella* is immobilized more quickly than *Fredericella* species. If left too long in menthol the tentacles start to curl, then slowly disintegrate.

Ethyl alcohol at 70 % is a good general fixative and preservative. Colonies containing significant amounts of water, such as *Pectinatella* or *Asajirella*, should have a least one change of alcohol after 24 hours. In the past, some workers injected full strength formalin over colonies to harden the lophophore and keep it nicely expanded. A 10 % solution of formalin is still sometimes used as a fixative and preservative, sometimes with sucrose added (BUSHNELL 1965). However, because of the toxic vapors formalin should be used only with excellent ventilation, and most workers choose to avoid it altogether. Bryozoan specimens to be used for DNA analysis require additional treatment and should be stored in 100 % ethyl alcohol (see Subcellular studies below).

Identifying specimens

Several good identification keys are available (see Further information below). Genera can often be determined from colony morphology alone, but identification to species almost always requires the presence of statoblasts. Searching for statoblasts is best done with a dissecting microscope and fine forceps. If statoblasts are not immediately apparent it may be necessary to dig into the colony, especially in older, central regions. Plumatellid sessoblasts are sometimes best found by peeling a portion of the colony away from the substrate. Try to disregard statoblasts found outside the colony since these may have come from a different source.

Species identification in the large genus *Plumatella* may involve measuring various parts of the floatoblast using an ocular micrometer. It is useful to know that each floatoblast is composed of two halves, or valves, which normally separate at the time of germination. By convention, the valve designated as "dorsal" is the one with the smallest central area covered by large chambers of the annulus (Fig. 2). If the two valves are unequal in size the dorsal valve is always the smaller one.

Separating and cleaning the two valves is a simple process devised by WIEBACH (1974) to reveal some of the pattern and texture of the central fenestra. Statoblasts are placed in a 1 M solution of potassium hydroxide and heated in a spoon over a flame for 30-60 seconds. Care should be taken not to let the solution boil and spatter statoblasts in all directions. Valves may separate immediately in the KOH solution, or they may open spontaneously after being transferred back to clean water. In some species the capsule can be isolated from the outer periblast. If the statoblast fails to open it may be given additional time in hot KOH. Roughened or scalloped edges are indications of overcooking.

Culturing bryozoans

Growing bryozoans in the laboratory is seldom easy, and for *Cristatella* and *Pectinatella* species it has never been achieved for more than a few days. ODA (1980) was able to maintain colonies of *Lophopodella carteri* on pure cultures of *Chlamydomonas reinhardtii*. WAYSS (1968) reported using various unicellular green algae in 1:1 Knop's solution and soil extract. Mukai (pers. comm.) simply employed frequent changes of water from a eutrophic lake. However, one of the most effective and rrouble-free culture system uses aged water conditioned by the presence of fish. In my lab we keep a number of large goldfish (*Carassius auratus* L.) in water that is never filtered and seldom changed. Their 200-liter aquaria are well lit and the fish are well fed. Water from these tanks is circulated continuously through smaller, darkened chambers in which bryozoans grow (Fig. 3).

It is important that the bryozoans be protected from settling particles. The easiest way to achieve this is to grow the colonies on the underside of artificial substrata, such as inverted dishes. For example, individual plastic petri plates containing bryozoans can have plastic toothpicks applied with thermal glue and inserted into glass tubes attached to the inside wall of the culture chamber (Fig. 4). Or, glass petri plates containing colonies can be inverted on racks. Yet a third possibility, good for mass culturing, is to grow colonies on the lower sides of glass plates that incline in parallel series like honeycomb in a beehive (Fig. 3).

It is relatively easy to transfer colonies of *Lophopodella*, *Lophopus*, and *Cristatella*. They are simply nudged off their original substratum and placed directly onto a new one where they should attach within 3-12 hours. Branching colonies, however, will attach to new substrata only by the actively growing tips. Free branches of *Fredericella* and certain *Plumatella* species may be left undisturbed for 1-2 days so the branch tips are in contact with the desired substratum; or the branches may be gently weighted with short lengths of fine glass rods until they adhere. In general, branches that are firmly attached throughout their length are seldom successfully transferred to a new location. However, if a portion of the substratum can be positioned against a glass plate with wedges or rubber bands the colony may grow out onto the glass. At that point the old substratum can be removed.

Bryozoans may also be cultured in situ on artificial substrata. WÖSS (1996, 2000, 2002) achieved recruitment of colonies on panels of wood and plexiglass hung verrically below a floating raft. Others have successfully used glass and corrugated polyethylene substrata, but found little colonization on vinyl or rubber sheeting (WOOD 1973; Mahuchariyawong, pers. comm.). For mosr studies I prefer growing colonies on inverted glass petri plate lids held inside customized sections of corrugated HDPE drainage pipe (Fig. 5). Suspended in the water under tethered, floating logs or other support the black plastic devices are almost inconspicuous. The inverted Petri plate covers are easily removed without disturbing the attached colonies, and with transmitted illumination the transparent substratum affords an excellent microscopic view. To make the plastic holders, a pipe (diameter 4 in or 110 mm) is cut into 8 cm lengths. Four tabs, 2 cm long and 2 cm wide, are cut into one end of each unit and bent 90° inwards to support the petri lid. To hold their shape, the tabs are heated at their base with a flame, then held in bent position while cooling water is applied.

Germinating statoblasts

Bryozoan statoblasts undergo an obligate period of dormancy that normally lasts 3-5 weeks or more. In principle, it is possible to germinate many statoblasts in a predictable time by first storing them under unfavorable conditions until the dormancy period is exceeded. Then, upon the return of favorable conditions they should germinate simultaneously with 2-7 days. In practice, the germination of statoblasts can often seem capricious, and it is obvious we still have much to learn about this process.

Statoblasts from temperate regions easily survive desiccation and freezing in their natural habitat. ROGICK (1940) offers a summary of data on the viability of dried statoblasts. However I find that statoblasts ger-

minate more quickly and reliably after being stored in water at 4 °C. Statoblasts from tropical species seldom tolerate refrigeration and are best kept at room temperature in 1-2 % saline solution.

The following points are relevant to statoblast germination:

1. Deionized water or rainwater enhances germination in all species.
2. *Plumatella* statoblasts crowded together germinate faster than isolated statoblasts (OWEN, personal communication).
3. Lophopodid statoblasts may be stored at room temperature if they are kept in complete darkness. Exposure to strong light will then trigger germination (ODA 1959).
4. *Cristatella* statoblasts appear to have a longer obligate dormancy than other species.
5. *Fredericella* statoblasts stored at cold temperatures may rupture if warmed too rapidly.

Here is a convenient methods for germinating buoyant statoblasts: A glass Petri dish is inverted in a pan of water with edges resting on supports so the rim is about 1 cm above the bottom of the pan. (Alternatively, an inverted plastic Petri dish can be floated from the surface film of water). All trapped air is removed. Buoyant floatoblasts to be germinated are picked up with an eyedropper pipet and propelled with a squirt of water under the petri dish. Care is taken not to overshoot the dish or to insert air bubbles under the dish. It takes a little practice to jostle the floatoblasts inside the pipet so they will exit with the jet of water. Once the floatoblasts have been placed, a Petri lid can be gently positioned under the inverted dish and the entire unit can then be lifted from the water. In this way the germinating statoblasts can be examined with a microscope without being disturbed. However, evaporation works quickly, and the petri dish should not be kept out of the pan of water for more than a few hours.

Microscopy

Standard methods are sufficient for histological work. For their studies on non-branching colonies, for example, MUKAI & ODA (1980) embedded tissue in paraffin, stained with Delafield's hematoxylin, counterstained with eosin and sectioned at 7 µm. Methods for preparing chromosomes have been nicely worked out by BACKUS (1989; see also BACKUS & MUKAI 1987).

To prepare statoblasts for scanning electron microsçopy it may first be necessary to remove a closely applied outer membrane. If present, this membrane is visible with transmitted illumination as a thin halo around the statoblast. Usually it is easily removed with fine forceps and needles. TATICCHI et al. (2004) accomplish the same thing with a 30 second exposure to 40 % KOH. To avoid distortion of the fenestra upon drying the statoblast can be freeze dried or frozen in a small drop of water and the ice allowed to sublimate in a household freezer. Critical point drying is also an obvious option.

Subcellular studies

Available descriptions of subcellular procedures include RADP analysis (OKAMURA et al. 1993), identification of polymorphic microsatellite loci (FREELAND et al. 1999) and analysis of 18S ribosomal DNA (WOOD & LORE 2005). Whenever a very clean specimen is required without contamination from other organisms it is important to clear the gut. This is accomplished by holding living colonies in filtered or uncarbonated bottled water for at least 10-12 hours. Although seldom necessary, a very small amount of unscented talcum powder suspended in the water will speed this process, with inert talc replacing the original gut contents. Colonies are then narcotized as usual and fixed in 100 % ethanol. Tissue for DNA analysis should include only the polypides (lophophore, gut, and other tissues) plucked from the colony and stored in 100 % ethanol.

Further information

The following is a selection of useful resources for additional information on freshwater bryozoans:

Brien P. (1960): Classe des Bryozoaires. — In: Grassé P. (Ed.): Traité de Zoologie **5**(2): 1053-1335.

An outstanding summary of bryozoan biology, with emphasis on anatomy, physiology, and development, well illustrated with exceptionally clear drawings.

Geimer G. & J.A. Massard (1986): Les Bryozoaires du *Grand-Duché de Luxembourg et des Régions* Limitrophes. — Musée de l'Histoire Naturel. Marché-aux-Poissons, Luxembourg: 1-188.

An excellent treatment of most European species, including *Paludicella articulate*, with descriptions and scanning electron micrographs, good ecological information, and more.

Lacourt A.W. (1968): A Monograph of the Freshwater Bryozoa – Phylactolaemata. — Zoologische Verhandelingen **93**, Leiden, E.J. Brill: 1-159.

A good but aging compilation of information with emphasis on systematics, including an exhaustive bibliography through about 1964. Species descriptions and distribution data beyond Europe and North America are unreliable, and the identification key is useless.

Nielsen C. (1989): Entoprocts: Keys and notes for the identification of the species. — Synopses of the British Fauna (New Ser.), Leiden, E.J. Brill **41**: 1-131.

An excellent taxonomic treatment of entoproct bryozoans, all of which are marine except *Urnatella gracilis*, including basic information about the phylum.

Prenent M. & G. Bobin (1956): Bryozoaires, première partie, Entoprotes, Phylactolémates, Cténostomes.— In: Lechevalier P. (Ed.): Faune de France **60**: 1-398.

A detailed but somewhat dated review of bryozoan biology illustrated with many line drawings, with descriptions of major taxonomic groups and a limited identification key.

Wood T.S. (2001): Bryozoans. — In: Thorp J. & A. Covich (Eds.): Ecology and Classification of North American Freshwater Invertebrates. Academic Press, San Diego: 505-525.

A good summary of bryozoan biology, including Entoprocta, with a well illustrated key for most North American species.

Wood T.S. & B. Okamura (2005): A new key to the freshwater bryozoans of Britain, Ireland and Continental Europe, with notes on their ecology. — Freshwater Biol. Ass. Publ., Ambleside, UK **63**: 1-111.

The newest regional monograph, including species descriptions, many drawings and photographs, a good taxonomic key, and the most complete background information to date on the ecology of freshwater bryozoans.

International Bryozoology Association. www.nhm.ac.uk/hosted_sites/iba, and www.civgeo.rmit.edu.au/bryozoa/iba.html. Founded in 1965, the IBA is composed of scientists and students from nearly 40 countries. They include paleontologists, marine and freshwater biologists, and specialists in systematics, ecology, genetics, physiology, embryology, and other aspects of bryozoology. A conference is held every three years, and published proceedings are widely distributed. New members are always welcome.

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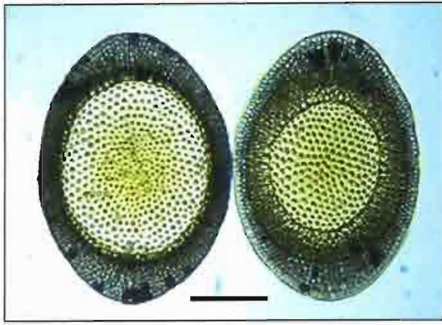

Fig. 2: Paired valves of the floatoblast of *Plumatella fungosa,* separated with hot KOH, showing the pattern of tubercles in the central "fenestra" area. The valve with the smaller fenestra (right) is considered dorsal. Scale bar = 100 µm.

Fig. 3: Aquaria for culturing freshwater bryozoans. The larger aquarium holds only goldfish; the smaller one, normally shielded from light, has bryozoans (not seen) growing on the lower side of inclined glass plates. The foreground tubing is an airlift pump: bubbles of air released at the inside bottom of the long vertical shaft carry siphoned water from the small aquarium into the larger one (Wood 1996).

Fig. 4: Culturing bryozoans in the laboratory. Inverted plastic petri dishes with plastic half-toothpicks attached are hung from pieces of glass tubing cemented to the inside wall of a culture aquarium. The bryozoans are *Plumatella vaihiriae* on the left and *P. fungosa* on the right. Photo is taken from outside the aquarium. Scale bar = 2 cm.

Fig. 5: Culturing bryozoans in situ. Plastic holder for glass petri dish is made from HDPE drainage pipe. The petri plate is inverted with bryozoans facing down. The device is normally suspended below a fixed or tethered floating object.

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