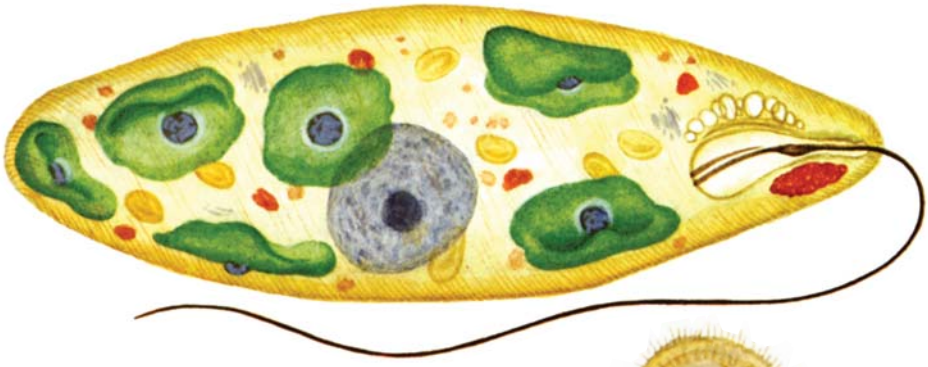
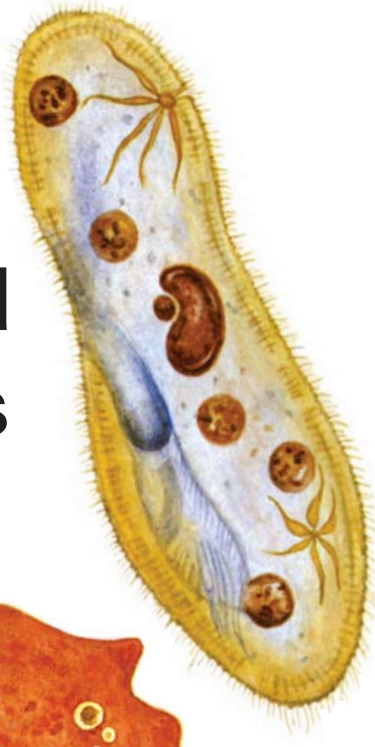
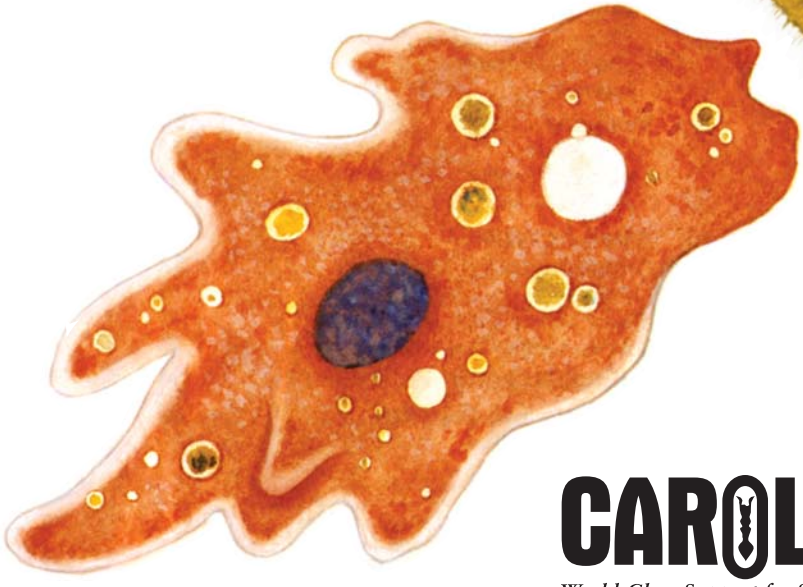


13-1065

Carolina™ Protozoa and Invertebrates Manual





CAROLINA®

World-Class Support for Science & Math

Carolina™ Protozoa and Invertebrates Manual

For your convenience, we have listed throughout this manual the catalog item numbers of products available from Carolina Biological Supply Company. For pricing and culture information, please refer to the most recent *Carolina™ Science* catalog, call toll free 800-334-5551, or visit the Carolina Biological Supply Company Web site at www.carolina.com.

Additional copies of this publication (13-1065) are also available.

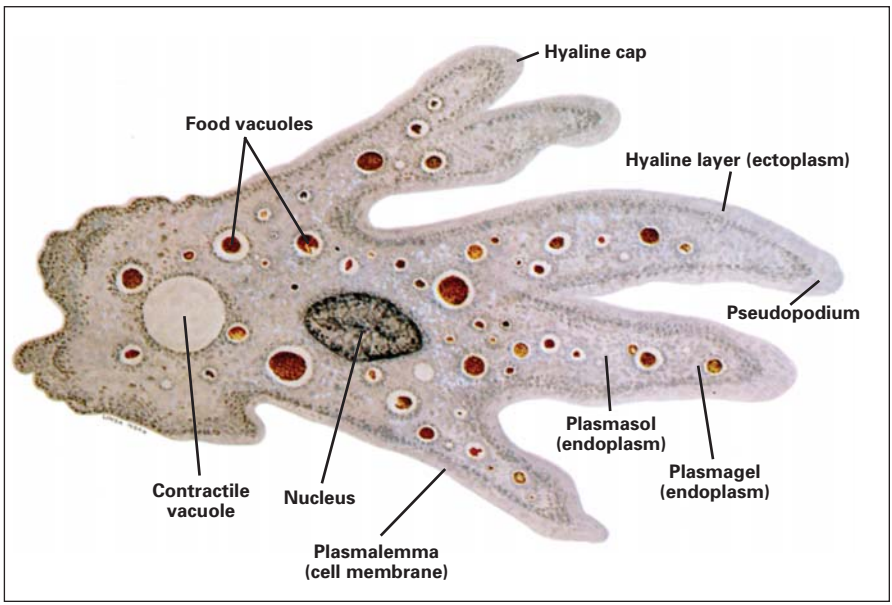
Introduction

Carolina's cultures are maintained under conditions that have proven effective for many years. Our culture service has set the standards for producing clone cultures. These cultures are always available for immediate shipment. Specimens leaving our laboratories are healthy and vigorous, and our experience has shown that living animals can travel successfully to any point in the United States—and to many other countries—within a reasonable time and despite most transit conditions. However, because we have no control over shipping circumstances, the cultures may have been traumatized during shipment. *Please give them your immediate attention.*

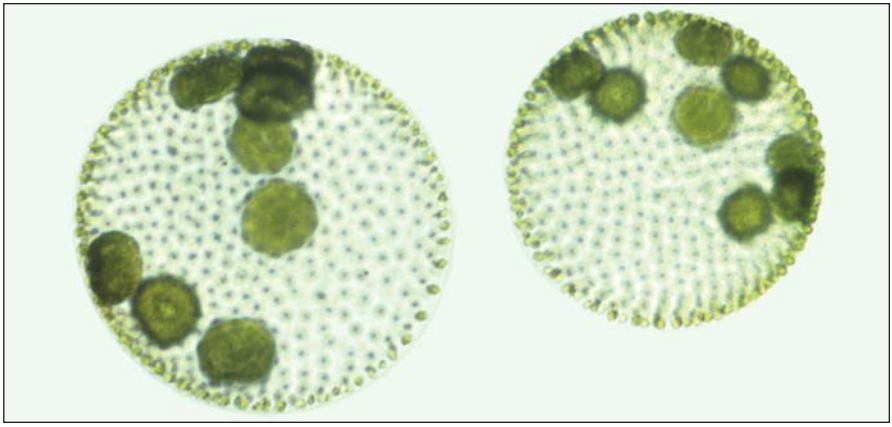
Protozoa

The protozoan body consists of a single cell, but it is a mistake to think of these organisms as simple. Without benefit of multicellular tissues or organs, many protozoa achieve structural complexity that rivals that of some multicellular animals. For this reason, some biologists prefer to think of protozoan organization as "acellular" rather than "unicellular." Whichever way you look at it, the protozoa are well worth studying.

Most protozoans are microscopic, but certain amoebae reach 4 to 5 mm in diameter, and the shells of their cousins the foraminifera may be 10 cm across. Such shells accumulate on the ocean bottom, forming limestone. Incredibly enough, the Egyptian pyramids were largely built of such foraminiferan limestone; yet, this is only a relatively minor example of the



Anatomy of *Amoeba proteus* (13-1306).



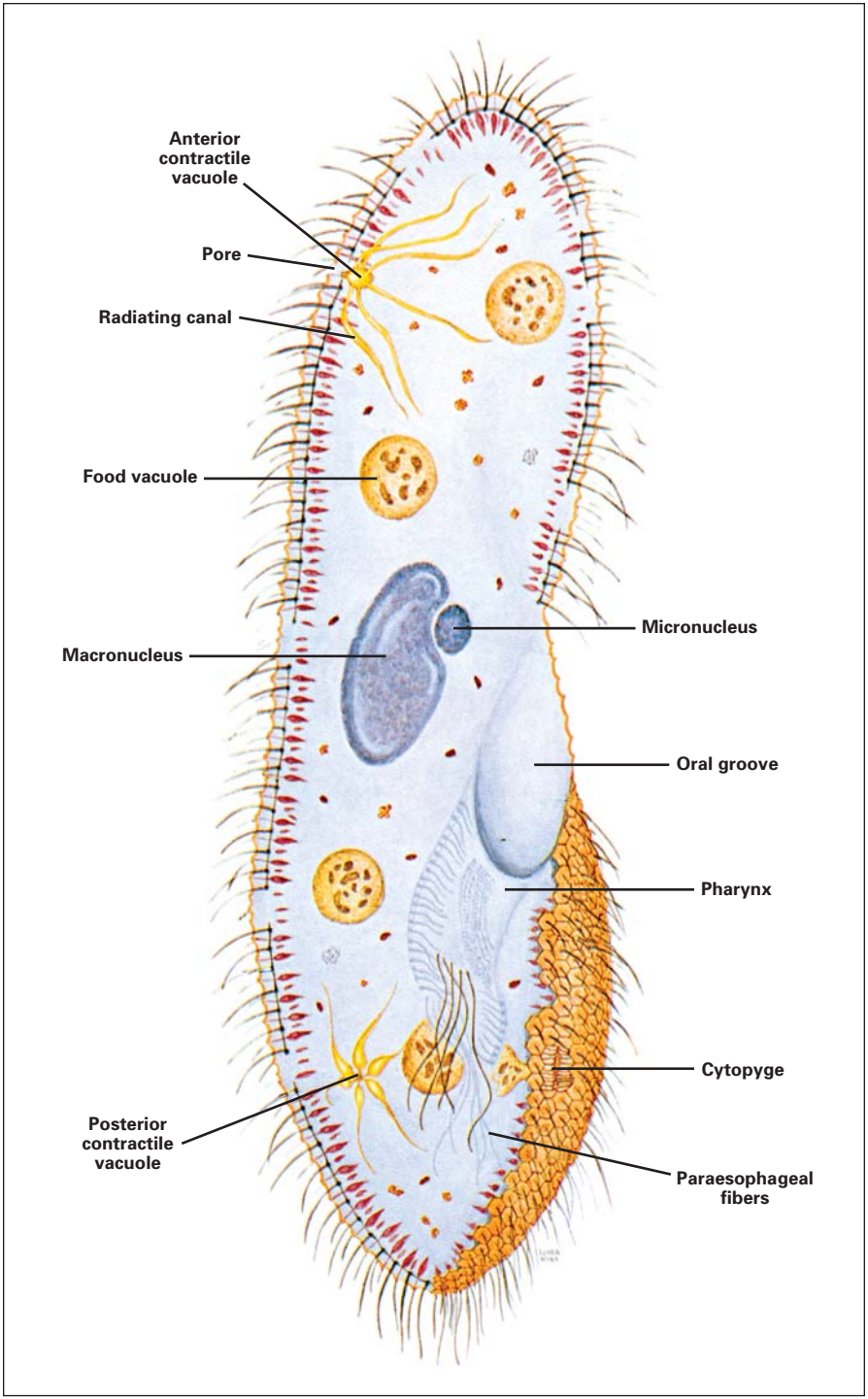
Volvox sp., a colonial flagellate. The dark green spheres are asexual daughter colonies.

important role that protozoans play in the life of mankind. It would be difficult to overestimate their importance in the food chains of soils, oceans, and fresh waters, upon which people depend. Many protozoans are agents of disease; malaria, for example is caused by the protozoan *Plasmodium vivax.*

The protozoans have traditionally been classified according to their means of locomotion. The shape of *Amoeba proteus*, a sarcodinian, constantly changes as it extends false feet (pseudopodia) from any part of its body. The pseudopodia engulf particles of food, a process known as phagocytosis. The flagellates, including *Euglena* and *Volvox*, move by means of one or more whiplike structures, flagella, which propel them through the water. *Euglena* moves in a spiral path. Ciliates, such as *Stentor*, *Vorticella*, and *Paramecium*, possess short, hairlike cilia which beat in unison, moving them rapidly through the water. Paramecia can swim either forward or backward while simultaneously rotating on their long axes.

Caring for Your Cultures

1. Open your shipment *at once*. Carefully open the jars and aerate the cultures using the pipets supplied (use only new or biologically clean pipets). To avoid cross-contamination, use a different pipet for each culture.
2. Never place the cultures in a refrigerator or in direct sunlight. They should be kept cool (20 to 22°C) with the lids placed lightly over the jars.
3. Allow 15 to 20 minutes after aeration for the animals to settle, and then inspect the contents using a stereomicroscope at low light level.
4. Instruct all laboratory assistants in the proper care and study of living specimens, with specific directions for locating and mounting each species.



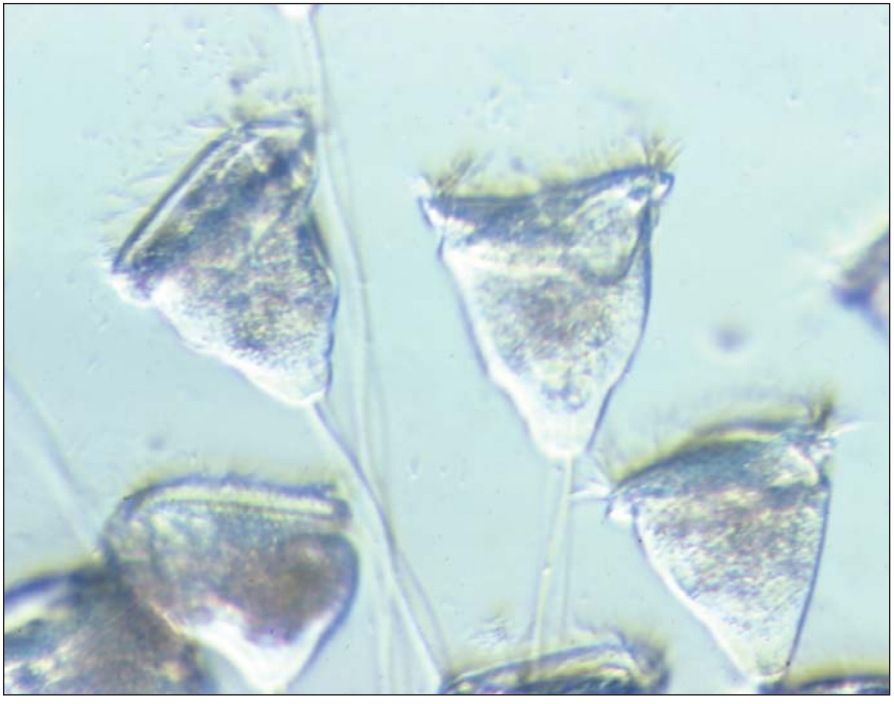
Anatomy of *Paramecium.*

5. Warn students against contaminating or accidentally killing a culture. Students should use the provided pipets.
6. Do not experiment with or subculture the organisms until the regular laboratory exercises have been completed.

Examining Your Cultures

Some protozoan cultures react noticeably to environmental changes. If the temperature suddenly drops, such forms as *Amoeba* and *Pelomyxa* become sluggish. Animals that were actively feeding or undergoing mitosis tend to ball up. Do not be alarmed, for they are not dead or dying; dead protozoans disintegrate, leaving no visible remains. The organisms may arrive partially covered with metabolic debris, but this, too, is normal. We provide all protozoan cultures with an ample food source, some of which may not have been consumed. This is not contamination. Careful examination of the cultures using a stereomicroscope should reassure you that they are still healthy.

While searching for the various organisms, remember that paramecia often congregate in masses around the perimeter of the jars; gently shaking the jar may help you locate them. By contrast, *Vorticella* is a sessile (stalked) form; you may have to search the water surface as well as the bottom and sides of the jars to find this bell-shaped ciliate.



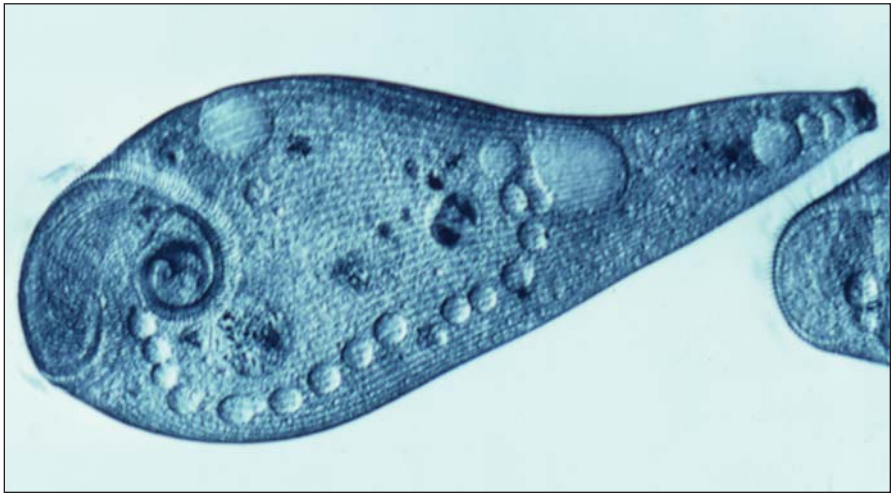
Vorticella (13-1660). This sessile ciliate has a contractile stalk.

Use the pipet provided with each culture to carefully remove the organisms from their shipping jar.

Students may prepare slides directly from the shipping jar; however, we recommend pouring its contents into a culture or petri dish. In any case, allow 15 to 20 minutes for the organisms to settle. Even during slide preparation, be careful not to agitate the container.

Using a stereomicroscope and a clean pipet, you can easily pick up a single specimen (or group of specimens) for a student's slide. If a stereomicroscope is not available, *very* carefully draw into a pipet a small amount of material from the bottom of the jar. One drop should contain more than enough organisms for one good slide mount.

Removing the sessile *Vorticella* requires special attention. Shake the jar and pour the contents into a culture dish. After a few minutes, carefully draw up the specimens with a clean pipet and place them on a slide for examination.



A whorl of beating cilia draws food into the cell mouth of the stalked ciliate *Stentor* (13-1598).

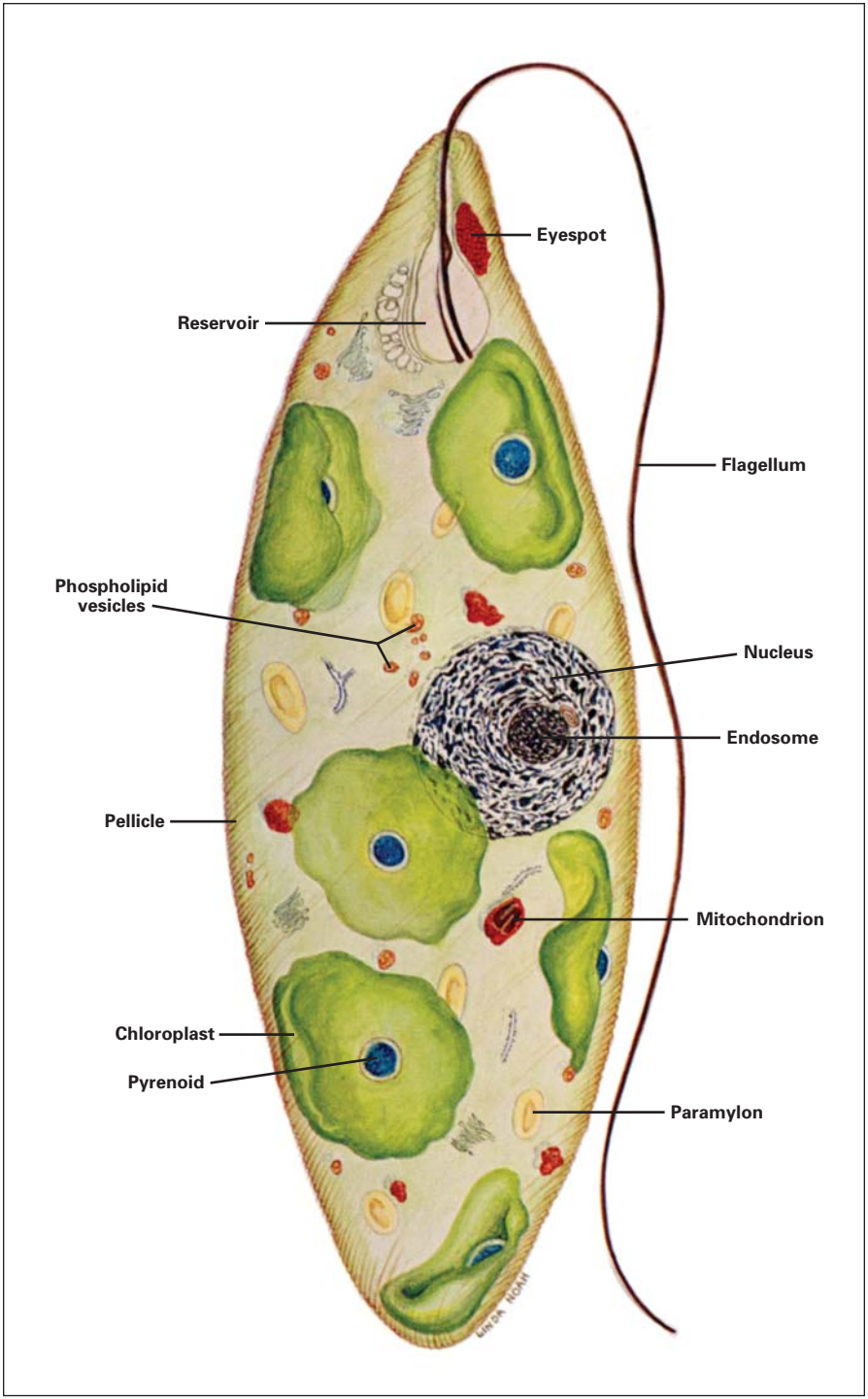
To dislodge *Stentor*, agitate the contents of the jar with a pipet. Leave the jar near a 75-W lamp for five minutes. *Stentor* will move away from the light, exhibiting negative phototaxis. Now you can pipet the protozoans from their concentrated gathering on the opposite side of the jar.

To concentrate *Euglena*, place a cardboard cylinder with a 2- to 3-mm wide slit in one side over the jar so the slit faces a 75-W lamp. After 10 minutes, carefully lift the cylinder and peek at the jar. The *Euglena* exhibit positive phototaxis, and should be concentrated in a thin, green line along the side of the container, where you can pick them up with a pipet.

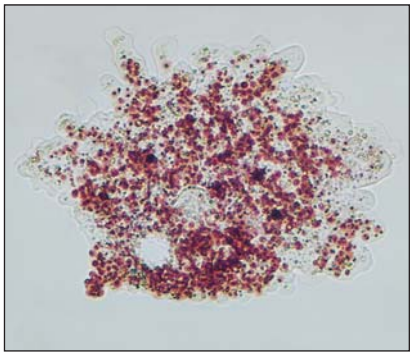
To slow fast-moving ciliates, use a quieting agent such as Protoslo® Quieting Solution (88-5141).

Slowing Ciliates

Moving ciliates are difficult to see under the microscope. A quieting agent such as Protoslo® Quieting Solution (88-5141) slows the ciliates' movement without killing them. Simply place a drop of Protoslo® on a slide, add a drop of concentrated *Paramecium* or other ciliate, mix, and watch the show.



Anatomy of *Euglena* sp.



Vitachrome® *Amoeba proteus* (13-1308). Carolina offers living, prestained Vitachrome® cultures to facilitate the study of protozoan structures.

Vital Staining

You can observe certain protozoan structures much more easily after vital staining. Unfortunately, most "vital" stains soon kill the protozoans. Carolina offers live, healthy *Amoeba proteus* (13-1308), *Pelomyxa* (13-1326), and *Paramecium* (13-1542) stained by our Vitachrome® process. The sharp, clear staining of these cultures facilitates finding the organisms and studying their structures. These prestained living organisms are excellent for teaching purposes and for photomicrography.

Culturing Protozoans

Protozoans, except for the photosynthetic forms that can manufacture their own food, must prey on other organisms to provide their energy. To culture protozoans successfully, you must provide them with food by setting up a short food chain with the protozoan of interest at its top. For example, when a new culture is inoculated with *Paramecium*, small amounts of bacteria and *Chilomonas* (a tiny flagellate) are included in the inoculum. The bacteria multiply around wheat grains placed in the medium, and the *Chilomonas* feed on the bacteria. *Paramecium* feed on the increased numbers of *Chilomonas*, but as they have no predators in the culture dish, the *Paramecium* increase rapidly and soon exhaust their food supply. Therefore, *you* must maintain the food chain or the *Paramecium* cannot survive for long.

The maintenance of any organism for a few weeks is quite different from culturing it over a period of years. Media that give excellent results at the start may not be satisfactory for continuous cultures over long periods. You may find the following hints helpful even for brief culture periods:

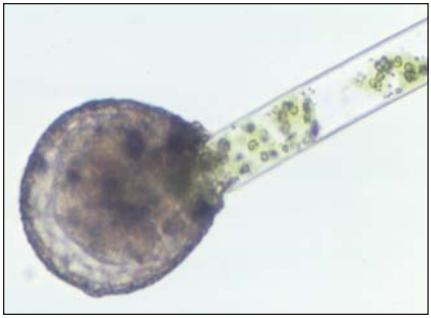
1. Choose water with good biological properties. Tap water is usually not suitable, because most municipal water supplies contain chlorine, fluorine, and other chemicals. If you use store-bought springwater, make certain that it is free of chemical additives. **Note:** Some tap water now contains chloramines, which are not "gassed out" by aging tap water. It will be necessary to treat such tap water with a dechlorinator such as ACE™ Eliminator (67-1939) or AmQuel® Water Treatment (67-1985). Carolina™ Springwater (13-2450) is ideal for making protozoan media and for maintaining cultures.
2. Pasteurize all media ingredients and allow them to cool to room temperature before inoculation.
3. Keep cultures covered to exclude dust, but not air.

4. Be sure culture vessels are clean. Traces of chemicals, soap, and soap powders may be toxic to organisms.
5. Always subculture just before the parent culture reaches maximum population. Inoculate heavily using biologically clean dropping pipets.

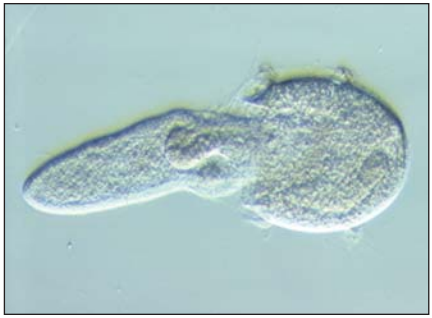
Protozoa are best cultured under conditions of dim to moderate light, a neutral or slightly alkaline pH, and temperatures of 20 to 21°C. Amoebae are especially prone to lose their vitality or die at higher temperatures. We maintain our cultures in 4½ × 2-inch dishes stacked on top of each other. The top dish of each stack is left empty and serves as a cover.

Recommended Media for Protozoans

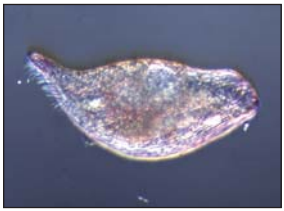
Protozoan	Recommended Protozoan Medium
Sarcodinids	
Actinosphaerium	Wheat Medium
Amoeba	Wheat Medium
Arcella	Hay-Wheat Medium
Centropyxis	Hay-Wheat Medium
Difflugia	Sand-*Spirogyra* Medium
Pelomyxa	Concentrated *Paramecium*
Flagellates	
Algae	Alga-Gro® Freshwater Medium (15-3752) for freshwater algae and dinoflagellates
Chilomonas	Wheat Medium
Dinoflagellates	Alga-Gro® Seawater Medium (15-3754) for marine algae and dinoflagellates
Euglena	*Euglena* Medium
Peranema	Wheat Medium
Ciliates	
Blepharisma	Wheat Medium
Bursaria	Concentrated *Paramecium*
Colpidium	Wheat Medium; Protozoan Pellets
Didinium	Concentrated *Paramecium*
Dileptus	Wheat Medium; Protozoan Pellets
Euplotes	Wheat Medium; Protozoan Pellets
Paramecium	Double Wheat Medium; Protozoan Pellets
Spirostomum	Hay-Wheat Medium; Protozoan Pellets
Stentor	Wheat Medium; Protozoan Pellets
Tetrahymena	Wheat Medium
Vorticella	Wheat Medium



Difflugia sp. (13-1334), a shelled relative of *Amoeba*, thrives on the green alga *Spirogyra* (15-2525).

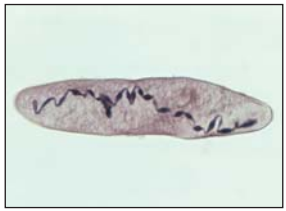


The predatory ciliate *Didinium* (13-1460) feeds voraciously on *Paramecium*.



Blepharisma (13-1430), a pear-shaped, rose-colored ciliate with an undulating membrane.

Bursaria truncatella (13-1434), one of the largest ciliates.



Spirostomum (13-1590), a ciliate with a conspicuous helical macronucleus.

Wheat Medium. For most protozoa, place three of four grains of previously boiled wheat (13-2425) in each culture dish. For paramecia, use six to eight grains (double wheat medium). Pasteurize springwater and while it is hot pour 200 mL into each dish. Cool to room temperature and inoculate.

Hay-Wheat Medium. Pasteurize springwater and while it is hot pour approximately 200 mL into each culture dish. Add two grains of wheat and two 3-cm stems of timothy hay (13-2385) that have been previously boiled. When cool, inoculate.

Euglena *Medium.* Combine 1 liter of springwater, 40 wheat grains, 35 rice grains, and 5 cm^3 of dry skim milk. Boil for five minutes. Let stand overnight and inoculate. Deep culture vessels or wide-mouthed gallon jugs work well. Keep *Euglena* cultures in a well-lighted area, but out of direct sunlight because high temperatures are harmful. Artificial illumination is adequate.

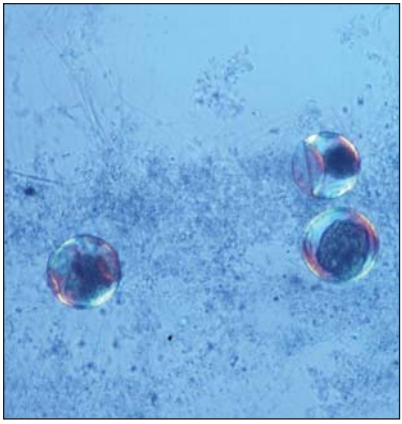
Concentrated **Paramecium.** Thick cultures of *Paramecium caudatum* (13-1554) are required. With a dropping pipet, remove a large number of paramecia without getting too much fluid. Place in fresh springwater. Strain through a cloth handkerchief to remove debris and large masses of bacteria. Let the filtrate stand until paramecia congregate in white masses on the bottom of the dish. With a dropping pipet, transfer the concentrated paramecia to fresh

Culturing protozoa in wheat medium. (a) Place three or four grains of previously boiled wheat in each culture dish. (b) Add approximately 200 mL of hot, freshly pasteurized springwater to each dish. (c) After the medium has cooled to room temperature, inoculate the dishes with protozoa. (d) Stack the culture dishes, placing an empty dish on top to serve as a cover.

springwater in another dish and inoculate with *Didinium* or *Bursaria*. More paramecia must be added every few days, and subcultures should be prepared every two weeks.

Sand*-Spirogyra *Medium. Place 0.5 g of clean sand in a culture dish containing 200 mL of heated springwater. After the water cools, add a generous portion of healthy *Spirogyra*, preferably a single-chloroplast species. Inoculate.

Protozoan Pellets. Add one Protozoan Pellet (13-2360) to 1 liter of previously boiled water and let it dissolve. Do not filter or attempt to remove residue. Divide liquid into culture dishes in 150- to 200-mL amounts. Add two to four boiled wheat grains to each bowl. Inoculate bowls with the protozoan to be cultured and maintain at approximately 22°C. Begin new cultures every two to four weeks, depending on the protozoan to be maintained.



Didinium cysts.

Protozoan Cysts

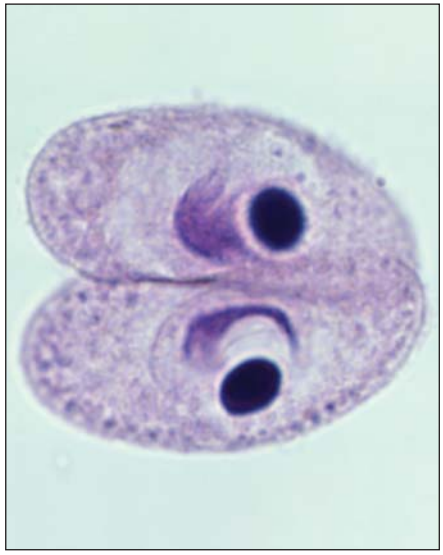
Many protozoans form protective cysts which allow them to survive in hostile environments (e.g., lack of water). *Didinium* (13-1460) is an ideal organism for demonstrating cyst formation. The cultures of *Didinium* you receive contain numerous paramecia as a food source, but if you pour the liquid into a culture dish and do not add paramecia, the *Didinium* will eventually starve. They will reduce in size to 20 to 25% of their "well-fed" diameter and will form tiny black spheres on the bottom of the dish.

The cysts remain viable for months. To revive them, prepare "hay infusion" medium by heating dry grass or hay in springwater or rainwater for 10 minutes, to boiling. Pour some of the yellowish water and a few hay straws into a clean culture dish and let cool to room temperature. Add a thick concentration of *Paramecium caudatum* or *P. multimicronucleatum* and pipet in a number of *Didinium* cysts. Within 24 hours, most of the cysts will open and tiny *Didinium* will swim about, preying on the paramecia.

Conjugation in *Paramecium*

The strains of *Paramecium* that we provide are checked for a conjugating reaction before they are shipped. *P. multimicronucleatum* (13-1558) conjugates two or three weeks after subculturing. Noon is the best time of day for mixing the strains. For best results, mix by numbers of paramecia rather than by volume, as the two separate strains supplied are not of equal concentration.

Conjugation should take place within 30 minutes. If you watch for more than one hour, cover the culture vessel to prevent evaporation. After 24 hours, you can observe pairs separating. After 48 hours, few if any pairs remain.



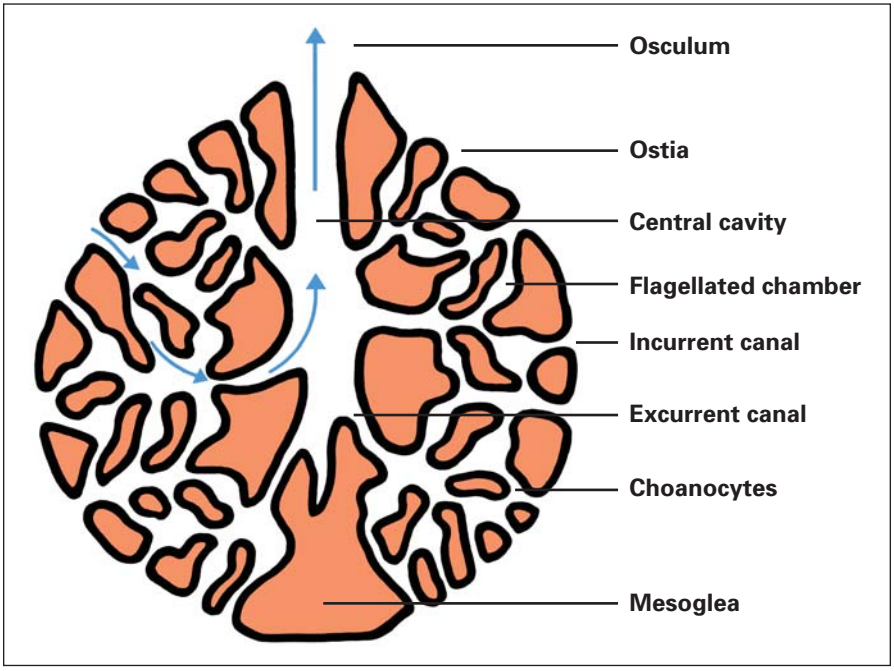
Paramecium bursaria conjugation, stained.

Spongilla

The sponges are among the simplest multicellular animals, with relatively unspecialized tissues and no organs. They are of special interest because they are probably *not* on the main line of evolution of the other multicellular animals.

The body of a sponge is saclike, with a single large opening called an osculum. The many tiny pores called ostia which perforate the body wall give the sponge phylum its name: Porifera (pore-bearing). The pores lead into flagellated chambers where special cells, choanocytes, wave their flagella to produce weak water currents. The water then enters the central cavity and is expelled through the osculum. The water currents moving through the sponge's body bring food particles to its cells and carry away wastes.

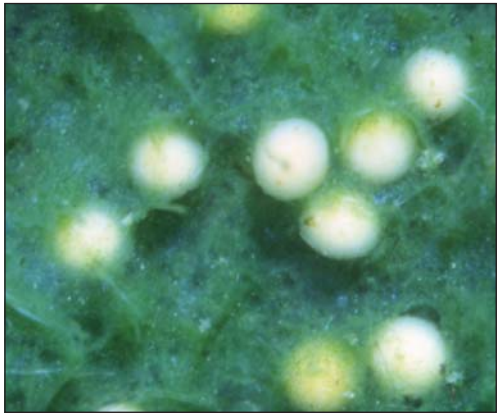
Spongilla belongs to the only freshwater family of sponges. It grows attached to submerged logs and rocks in ponds, lakes, and slow-moving streams. Freshwater sponges produce highly resistant internal "buds" called gemmules. You can observe these asexual gemmules most easily in autumn, when the sponge's superficial tissues disintegrate.



Anatomy of *Spongilla*. The body wall of this leuconoid sponge is highly folded, increasing the absorptive surface area.

Care

Our freshwater sponges are collected just prior to shipment because they do not keep well in the laboratory for long periods. As soon as the shipment arrives, carefully remove the sponges with forceps and transfer them to an 8-inch diameter culture dish or to a shallow tray containing 5 cm of cold (10 to 16°C) springwater. Keep them in a semi-dark area and aerate frequently. Change the water every other day.



Spongilla with gemmules. The gemmule is an aggregate of food-filled cells surrounded by a hard covering studded with spicules.

Hydra

Hydra is a freshwater member of the largely marine phylum Coelenterata (or Cnidaria). Hydras are the simplest animals with definite tissues. The tubelike body has two cell layers, with a layer of jelly and wandering cells between. A single opening, the mouth, leads into a gastrovascular cavity. The mouth is surrounded by tentacles armed with stinging cells. When a prey organism, such as a small crustacean, brushes against a hydra's tentacles, the hydra first harpoons it with stinging nematocyst threads; it then uses its tentacles to guide the meal into its mouth.

Hydra with bud. Hydras commonly reproduce by forming asexual buds on the parent animal.

Hydras living on the undersides of lily pads and other leaves in cool ponds and streams look like tiny pieces of frayed string. They can contract and extend their flexible bodies and tentacles. Extended, they may reach 25 mm or more in length. Hydras can move from place to place by somersaulting on their tentacles or by sliding along measuring-worm (inchworm) style. Sometimes called the "eternal animal," *Hydra* has an amazing ability to regenerate lost body parts. A hydra cut in half will form two complete animals within a few days. Be certain to change the water after 24 hours to prevent bacterial buildup.

Maintaining Cultures

Despite its regenerative powers, *Hydra* can be quite difficult to maintain over long periods, although short-term culturing is relatively easy. Hydras are voracious eaters, feeding on small, motile forms, particularly crustaceans. To keep your hydras alive and healthy will require a continuous food supply. Hydras thrive on *Daphnia* and brine shrimp. Provide several animals for each hydra and increase the number of food animals daily as the hydra population grows.

Use aquarium tanks up to 20 liters (5 gal) or large glass culture dishes to culture your hydras. Fill the containers almost to the top with springwater, filtered pond water, well water, or rain water. *Do not use distilled water or tap water.* Keep the containers at approximately 21°C in dim light (except for green hydras, see "Special Problems" below). Too much light favors the development of algae, which can be detrimental to the cultures. Use a glass pipet to transfer a dozen or more hydras to each container. *Use caution: hydras are extremely delicate.*

Change the springwater in the cultures every day, or, if this is not practical, siphon off at least one-third of the old culture water and replace it with fresh springwater.

Special Problems

Hydra is difficult to culture during the seasonal period in which spermaries and ovaries are formed. In North Carolina, *H. littoralis* commonly undergoes sexual cycles in March and April, and occasionally in November. At these times, the animals appear sickly, and the tentacles and body column contract. During this period, the animals are most susceptible to attack by *Hydramoeba*, which eat their way down the tentacles and body column. The flatworm *Microstomum* may also attack and completely eliminate a culture. The best way to combat these problems is to maintain a "clean" culture, frequently changing the springwater and immediately removing any worms found in the culture.

Hydra with spermaries or male gonads. Mature sperm escape through the nipple of the conical spermary.

Green hydras (*Chlorohydra viridissima*) must be exposed to light, but not so much that the culture water is heated. Eight to ten hours per day of window light or artificial light should be sufficient. Keep the containers clean and avoid algal buildup. A few snails added to the container can help prevent algal buildup.

Planaria

Planarians belong to the phylum Platyhelminthes (flatworms). These seemingly simple animals represent a level of organization far more complex than that achieved by *Hydra*. Their body plan features bilateral symmetry; a permanent, internal reproductive system; various layers and bundles of muscles; a branching, blind gut that reaches throughout the body; an excretory system composed of specialized "flame cells;" and a simple nervous system with an enlarged "brain" in the front end. Research indicates that planarians can master a two-choice maze, that their responses to stimuli can be conditioned, and that memory of training can be artificially transferred from one planarian to another. Thus, they (and other flatworms) are considered by animal behaviorists to be the simplest animals that exhibit an ability to learn in response to simple conditioning.

Unlike their parasitic cousins the flukes and tapeworms, planarians are free-living. Freshwater planarians live on the undersides of rocks and debris in streams, ponds, and springs. They avoid strong light and are much more active at night than during the day.

Care and Maintenance

We ship recently collected planarians which need not be fed for at least a week. However, as planarians foul their water rapidly, you should transfer them to fresh springwater immediately upon their arrival. *Use care to avoid damaging the animals.* Move them individually with a glass pipet or, after carefully pouring out the shipping water, use your finger to dislodge the planarians from the walls and bottom of the jar. Do not use deep containers. Planarians keep best for extended periods in a large, shallow, enameled or stainless steel tray.

Extreme heat or cold will harm the planarians. Maintain a temperature of 21 to 23°C for most species shipped from North Carolina.

Planarian stained to show digestive tract.

Keep the water clean and change it daily. Use only chemical-free springwater or unpolluted pond water. Tap water may contain metals or chlorine, which will kill the planarians. Wash the old culture bowl free of slime and film, but do not use soap or detergents. Simply run your finger around the sides and bottom to dislodge the slime, and then pour it off with the fouled water.

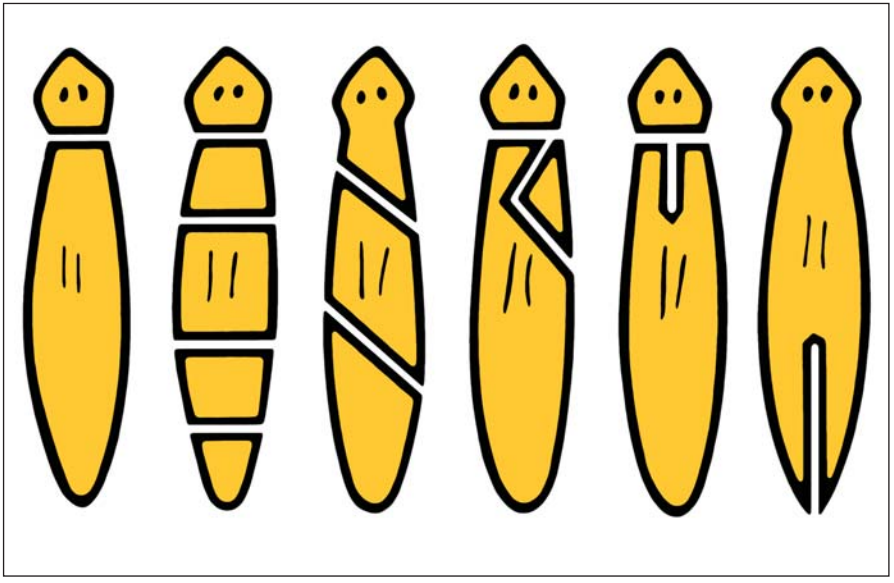
Planarians in the laboratory thrive on fresh beef liver, hard-boiled egg yolk, *Lumbriculus* worms (14-1720), pieces of earthworm, crushed aquarium snails, etc. We suggest hard-boiled egg yolk for feeding brown planarians and fresh beef liver for feeding black and white planarians. Feeding once a week is usually sufficient, but you may need to vary this routine according to the number of worms being maintained.

The amount of food depends upon the number of animals and the size of the culture vessel. For example, for 50 planarians in an 8-inch culture dish half-filled with springwater, feed a pea-sized portion; for 500 animals provide half an egg yolk or a piece of liver the size of a bottle cap. Do not overfeed the animals.

Allow the planarians to eat for 30 minutes, then transfer the worms to a bowl of fresh water. If you must retain the same bowl, remove the food particles, clean the container, and top it off with fresh springwater to prevent a detrimental increase in bacteria.

Regeneration

Planarians exhibit remarkable powers of restoring lost body parts. Of the various common freshwater flatworms, members of the family Planariidae



Suggested operations to demonstrate regeneration in planaria (*Dugesia tigrina*).

(such as *Dugesia* and *Phagocata*) usually perform quite well in regeneration studies. In most cases, pieces of moderate size, regardless of which part of the worm you cut, form complete animals. Smaller pieces may not be able to regenerate perfect heads, however. As a rule, the degree of regeneration of the head region depends on the level from which you take the cutting: the more posterior the cutting, the less likely it is that a normal head will regenerate.

Dugesia tigrina and *D. dorotocephala* are dependable species for experimentation because they are easily maintained in the laboratory. Regeneration is slower in the white planaria (13-2960) than in either the brown planaria (13-2954) or black planaria (13-2956). A few operations you might try are indicated in the figure. Keep an accurate record of the various operations you choose to perform.

Procedure

First, place the worm on a moistened cork, ice cube, or piece of cold glass, where it should extend itself. While observing through a stereomicroscope or other magnifier, make the cuts using a razor blade or very sharp scalpel. After the operation, place the individual pieces in separate culture dishes partially filled with clean springwater or pond water. Cover the dishes to reduce evaporation, and keep them in a cool place with subdued light.

Do not feed the planarians; they are hardly in a position to begin eating right away. One or two days after the operation, renew any cuts that are intended to separate only and not to detach; otherwise, the parts will fuse back together. Complete regeneration should take 2 to 2 1/2 weeks.

Sexual Cycle and Cocoons

The black planarian and the brown planarian we supply undergo a sexual cycle in late February or early March, depending on the temperature of the water in the collecting pools. During this period the worms are easily torn and will die from excessive handling. Do not feed them during this time. Cover the culture bowls and change the springwater every three days.

If you maintain the planarians as suggested, the sexually mature worms will deposit cocoons (first observed as swellings on the ventral sides) in the culture dishes. Carefully remove the cocoons and place them in small glass culture dishes of springwater maintained at room temperature. Change the water every two or three days.

After two or three weeks, the outer cover of the cocoon will crack and two to six tiny planarians will emerge. The culture procedure is the same as for full-grown planarians, except that the pieces of food given should be approximately the size of pencil lead. With care, you should be able to raise the juveniles to full size.

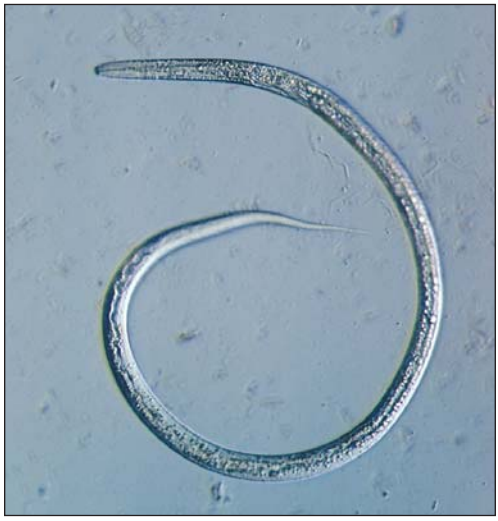
Nematodes

Nematodes are roundworms. They are sometimes placed in their own phylum, the Nematoda, and sometimes, together with the rotifers, in the phylum Aschelminthes. The slender roundworms are among the lowest animals to possess a very important feature: a complete digestive tract with not one but *two* openings, mouth and anus. This makes possible the efficient one-way "assembly-line" processing of food.

Parasitic nematodes such as intestinal roundworms probably infest the bodies of two-thirds or more of the world's human population. One species, *Trichinella*, causes the serious disease trichinosis. Free-living nematodes occur in enormous numbers in the sea, fresh water, and soil: several billion may exist in a single hectare (slightly less than 3 acres) of farmland.

Culturing Vinegar Eels

Many vinegar eels can withstand a pH as low as 1.5. To culture vinegar eels (*Anguillula*; 13-3266), fill a 4-L (1-gal) jar three-fourths full with apple cider vinegar, or use Vinegar Eel Medium (13-3270). Vinegar Eel Medium is undistilled, pure, apple cider which has turned into vinegar. We add to it a generous clump of "mother of vinegar." Peel and core an apple, cut it into eight parts, and add it to the liquid. Inoculate with vinegar eels and cover the jar loosely with a lid or a glass plate. Make certain that air can get into the jar by putting a piece of paper between the glass plate and the top of the jar. Keep the culture at room temperature (22 to 24°C), away from direct sunlight.



The vinegar eel probably feeds on the bacteria and fungi that grow in the sediment of unpasteurized vinegar.

For a smaller culture, pour 200 mL of apple cider vinegar or Vinegar Eel Medium into a 4-inch culture dish, add a 2-cm cube of apple, and inoculate with vinegar eels.

The population of vinegar eels will gradually increase; they should be abundant after two months. The eels will congregate near the surface of the medium. Do not attempt to clean out the debris that accumulates in the bottom of the bottle. As the medium is used up, simply add more apple and vinegar.

Potato Plug Medium

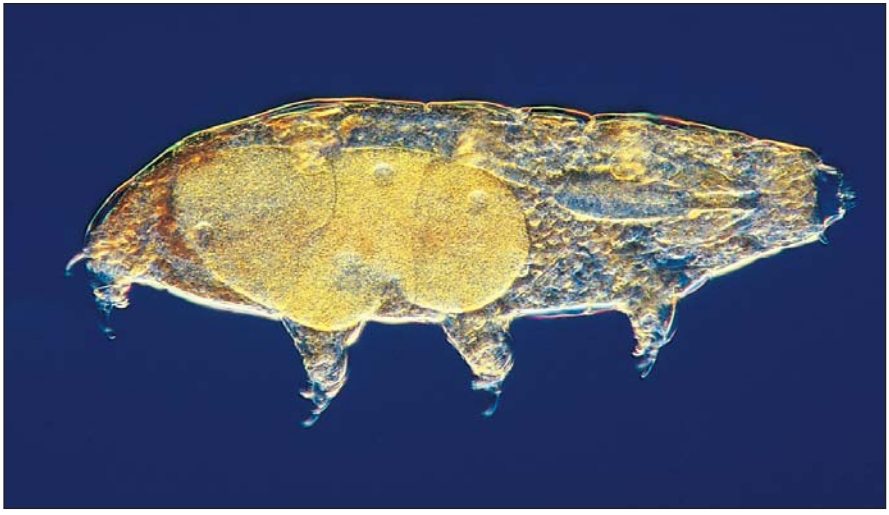
Nematodes such as *Rhabditis* (13-3258) and *Cephalobus* (13-3250) can be cultured in potato plug medium. You can make your own medium or you can purchase Potato Plug Medium (13-3262). To make your own, use a cork borer to cut plugs of Irish potato (approximately 4 cm long) to fit standard bacteriological culture tubes. Remove any potato skin, wash the plugs, and insert them into the tubes. Add springwater to fill the spaces between the potatoes and the walls of the tubes, and plug the tubes with nonabsorbent cotton. Autoclave at 15 pounds pressure for 15 minutes on liquid cycle. When the medium is cool, use a wire loop to inoculate it with worms. Some mold may develop, but this apparently does little harm.

You can collect nematodes by burying a piece of potato 5 to 8 cm below the soil surface. After one week, the potato usually contains enough nematodes to culture in the laboratory.

Rotifers

The rotifers may be placed in their own phylum (Rotifera) or in the Aschelminthes. They derive their name from their crowns of beating cilia, which resemble rotating wheels. Most species are freshwater inhabitants that creep or swim around the vegetation of ponds and lakes. Probably the most frequently studied rotifer is *Philodina* (13-3172), which has around its trunk a cuticle whose rings simulate segmentation. The cuticle is thicker and creates an outer case (lorica) in *Monostyla* (13-3170).

Rotifers can be cultured on wheat medium as described under "Culturing Protozoans."



Tardigrade, or Water Bear (13-3960); photographed with Rheinberg/oblique illumination.

Tardigrades

Tardigrades, otherwise known as Water Bears for the pawing motion by which they move, are fascinating animals that exhibit a number of characteristics well suited for laboratory exercises. Their relatively large size (most are between 0.3 mm and 0.5 mm) compared to other micrometazoans makes them ideal subjects for light microscopy.

The typical tardigrade has a short, barrel-shaped body that exhibits distinct cephalization and four less well-defined body segments. From each body segment extends a pair of short, ventrolateral legs. Each leg bears four claws or two double claws, used chiefly for locomotion and for clinging to plants or other suitable substrates. Tardigrades lack cilia, although some species have cirri located anteriorly, laterally, or both.

The entire tardigrade body is covered by a protective, proteinaceous cuticle that is secreted by the underlying epidermis. Cuticle textures range from smooth to highly sculptured and granular. The number of cells that comprise the epidermis is constant within a species. This species-specific trait is invaluable in differentiating tardigrades taxonomically.

Coloration among tardigrades varies from a bluish-gray to yellow or reddish-brown. Young animals are frequently transparent, especially just after emerging from an egg case, while older, mature individuals are usually dark and opaque.

Dimorphism between the sexes occurs, but it is not the general rule. Differences in shape of the last pair of appendages, claw formation, and size sometimes indicate sex.

Feeding

Most tardigrades feed exclusively on plants or algae. Two long, sharp stylets located in the buccal apparatus pierce the walls of moss and algal cells. The liquid contents of the cells are then ingested by powerful pharyngeal pumping action. Some tardigrades occasionally consume the body fluids of small metazoans, and *Milnesium tardigradum* appears to be exclusively carnivorous. Contact with suitable prey such as the rotifer *Philodina* apparently occurs at random, although papillae surrounding the mouth may aid in food detection. The tardigrade grasps the rotifer near its base or pedal gland, the stylets puncture the cuticle, and the pharynx sucks the pseudocoelic fluids of the rotifer into the gut of the tardigrade. The piercing action of the stylets and pharyngeal pumping are clearly visible at low-power light microscope magnification.

Life Cycle

Sexes are separate in most tardigrade species. Reproduction among species where males are unknown is almost certainly by parthenogenesis only. The bulk of a population is always composed of females; the number of males peaks in the winter or early spring. Most tardigrade reproduction occurs from late fall to early spring, but egg-bearing females can usually be observed year-round.

Tardigrade eggs are deposited freely in groups or singly, depending upon the genus. Eggs may also be left behind in the safety of a spent cuticle following molting. Eggs deposited freely are either highly sculptured or have a sticky surface to aid in attachment to the substrate. Eggs deposited in an old cuticle are usually smooth.

Two separate egg types, thin-shelled and thick-shelled, have been observed among several genera. These two egg types may be analogous to summer and winter eggs produced seasonably by rotifers. Undoubtedly, the egg types correspond to favorable and unfavorable environmental conditions.

The rate of development of the embryo is usually dependent upon the type of egg produced. Thin-shelled eggs hatch in 3 to 12 days and thick-shelled eggs in 10 to 14 days. The young tardigrade ruptures its surrounding egg case with the aid of its stylets. Upon emergence, immature tardigrades are one-fourth to one-third adult size.

A typical tardigrade will undergo four to twelve ecdyses during its life span, increasing in size until reaching maturity sometime after the second or third molt. Prior to ecdysis, the animal shrinks slightly, becoming completely detached from its surrounding cuticle. The anterior end of the old cuticle ruptures from the thrashings of the contained tardigrade. The animal crawls out, leaving the old cuticle and possibly a new clutch of eggs behind. The claws and mouth or buccal apparatus as well as the lining of the rectum are left behind as the animal exits the old cuticle. Once these discarded tissues and structures are replaced, feeding resumes.

Cryptobiosis

Perhaps the most fascinating characteristic of the water bears is that they are able to enter a state of suspended animation, termed the *tun state* or *cryptobiosis*. The tardigrade inhabits aquatic or semiaquatic environments, and is active in such a setting. But if their environment should become arid, they are able to bring their metabolism to a virtual standstill for years at a time. The animal will tuck in its legs and curl up into a protective structure called a tun. This particular type of cryptobiosis is termed *anhydrobiosis*. In this state, the animal's body water percentage will decrease to less than 1%.

Tardigrades in their cryptobiotic state (tuns) are able to survive very extreme conditions. Temperature ranges from –253°C to 151°C can be tolerated. It has been reported that tuns can survive pressure as high as 600 megapascals (while most living things would perish at half that pressure), exposure to X-ray radiation, and even being placed in a total vacuum. Despite their toughness in the tun state, reanimation may be accomplished by simply rehydrating the tardigrades.

In its animated state, the typical tardigrade has an expected life span of between two months and two and one-half years.

Annelids

Members of the phylum Annelida, the segmented worms, have bodies that are divided into many similar rings or segments, a feature called metamerism. Metamerism occurs in the reproductive, circulatory, excretory, nervous, and muscular organs, as well as in the external body features. The most familiar annelid is the common earthworm, found in garden soil and on sidewalks and streets after a heavy rain. Earthworms require moist, rich soil for their burrows, and they actually enrich the dirt by bringing subsoil to the surface that is rich in phosphorus and potassium. Charles Darwin estimated that the earthworms in one acre of land pass through their intestines from 10 to 18 tons of earth and leaves every year.

The Oligochaeta, the class to which the earthworm belongs, includes many other inhabitants of fresh waters and damp soils. White worms (*Enchytraeus* sp.) have the surprising ability to reproduce by fragmentation. *Tubifex* sp. live in tubes and eat the bottom muck in polluted waters.

Another familiar class of annelids, the Hirudinea, includes the leeches. Small, aquatic, bloodsucking leeches are a nuisance to human beings in many tropical countries. The medicinal leech was used in bloodletting for centuries. In fact, *Hirudo medicinalis* (14-1772) is still used by physicians to alleviate venous congestion and restore blood circulation in microsurgical procedures such as finger reattachment.

Earthworms

When your earthworms (*Lumbricus terrestris*; 14-1620) arrive, immediately discard any dead or sick animals. The remaining worms can be retained in the shipping material if you keep them cool and moist. Earthworms are large

Lumbricus terrestris. Large, mature specimens (14-1620) are suitable for laboratory study and dissection. Medium-sized specimens (14-1626) are useful for making slides and for feeding snakes and large salamanders.

specimens and are suitable for laboratory study and dissection; however, temperatures above 15°C are harmful to earthworms, so they may be difficult to maintain in a classroom setting.

For best results, place the worms in a light, loamy soil. *Do not use soils high in clay or sand content.* Spread moist breadcrumbs or cornmeal sparingly over the top of the soil and cover with 3 cm of loam. Feed the earthworms every two to three weeks.

Earthworm cocoons can be kept in a small wooden box filled 3 cm deep with loamy soil. Again, keep the soil moist and cool (15°C). The young worms should emerge in a few weeks.

Redworms

Redworms (*Eisenia foetida*; 14-1650) are smaller than earthworms, and are suitable for feeding animals such as fish, amphibians, and reptiles. These worms are ideal for composting and culturing. Also, the redworm does not require the cool temperatures that an earthworm does; in fact, they will thrive at room temperature.

Before the worms arrive, prepare a large dishpan or other deep-sided pan or tray. Make bedding by spreading 8 to 13 cm (3 to 5 in) of shredded newspaper in the pan or tray. Dampen the newspaper with aged water—water that has sat out overnight to allow the chlorine to escape.

The worm's skin must be kept moist at all times for respiration, but not too wet. Occasionally add some water to the newspaper. If the container becomes too wet, add more shredded newspaper. When the worms arrive, put them and the soil from their shipping container on the newspaper bedding. If they were shipped without soil, obtain some garden soil and spread a layer at least 3 cm (1 in) deep over the newspaper.

There are several different materials you can use to cover the container, among them a damp towel, a damp piece of burlap or dark cloth, a large piece of black paper, or a piece of black plastic trash bag. If you choose a towel, burlap, or cloth, sprinkle it with water so it stays slightly damp but not dripping wet. If you obtain burlap from a feed dealer or mill, wash it well—it might contain traces of substances that could harm the worms. You might also place a sheet of dry cardboard over the burlap to keep out light. This permits the worms to crawl around in a darkened area not just at night but also during the day.

Redworms require food with a much higher organic content than do earthworms (which is why they are so useful in composting). Redworms can be fed cornmeal or oatmeal, and each worm may eat its own weight in food every day. Place the food on top of the bedding or just under the soil layer, but do not push it too far into the bedding. Uneaten food may mold. If this occurs, scrape the mold off with a spoon. An easy alternative is Magic Worm Food (14-1670), which contains everything necessary to produce fast-growing, healthy worms.

Lumbriculus variegatus (14-1720) is quite easy to maintain as a long-term culture.

Lumbriculus

Otherwise known as the California Blackworm, the Freshwater Blackworm, and the Mudworm, *Lumbriculus variegatus* (14-1720) is quite easy to maintain as a long-term culture. To maintain a culture of *Lumbriculus*, you will need a deep pan or dish, springwater or aged tap water, a plastic pipet, brown paper towels, and sinking (pelleted) fish food. Fill the pan with 2 to 3 inches of springwater, and transfer the worms into the water with a plastic pipet. Cover the bottom of the pan with strips of brown paper towel, which will serve as a substrate for the worms.

If possible, maintain the *Lumbriculus* culture at 15°C. Temperatures higher than 21°C will cause disintegration. Gentle aeration is recommended but not required. The culture water should be replaced every 2 to 3 weeks as the paper towel disintegrates and waste accumulates. Decant the culture slowly, taking care not to pour out the worms and substrate at the bottom of the pan. Rinse the substrate and the worms, decant, refill the pan to the original water level, and add new paper towels. If storage temperatures exceed 15°C, change the culture water every 2 to 3 days, as opposed to every 2 to 3 weeks if stored at 15°C.

Sinking fish food is a fine primary food source for *Lumbriculus*. Add one or two pellets initially. After a few days, one or two pellets may be added if the original pellets are consumed. Do not overfeed, as decomposing food can contaminate the culture. Irregular feedings or even weeks of starving will not harm the worms.

Occasional harvesting of surplus worms is advised, and maintenance of at least one duplicate colony is strongly suggested. Under these conditions, *Lumbriculus* will reproduce asexually and cultures may be sustained for years.

Note: You can find an in-depth discussion of the anatomy and classroom utility of *Lumbriculus* in the August 1996 *Carolina Tips*® (Vol. 59, No. 3) article, "Those Wonderful Worms," available in the *Carolina Tips*® online archive at www.carolina.com, or by calling 800-334-5551.

Other Oligochaetes

The small, freshwater *Aeolosoma* (14-1748), the reddish *Dero* (14-1750), and *Stylaria* (14-1810) can be cultured on wheat medium as described under "Culturing Protozoans."

Leech (14-1762). The segments at both ends of the flattened body are modified to form suckers.

Leeches

Leeches are very sensitive to chlorine and traces of metals such as copper, so use only clean springwater or rain water to culture them. Change the water daily, if possible, or at the first sign of fouling. Use glass or plastic vessels kept at room temperature or lower, away from direct sunlight. Leeches should not be crowded, and vessels must be securely covered to prevent their escape. Leeches can thrive for months on a single meal, but you might feed them a few live water snails once a week.

Mystery snail, *Ampularia cuprina* (14-1190).

Aquarium snail, *Physa* sp. (14-1212).

Mollusks

The distinctive structural feature of the mollusks is a soft unsegmented body. Most members of the phylum possess a ventral muscular foot; a thin sheath of tissue called the mantle surrounding the soft parts of the body; a limy shell; and a tongue-like, rasping organ called the radula. Mollusks are quite a diverse group, however, and one or more of these features may be missing. Clams, oysters, and mussels have no radula, for instance, and the squid, octopus, and cuttlefish do not have external shells. Mollusks are found in both marine and freshwater habitats, and a few, such as snails and slugs, are terrestrial. Many people are unfamiliar with terrestrial mollusks such as land snails, because the animals usually forage for green vegetation at night, sliding about on their slime trails. During the day, particularly if the weather is dry, snails often remain out of sight under leaves or in burrows.

Land Snails

Carolina offers both the Texas Land Snail (14-1144) and the California Land Snail (14-1147). When your shipment of land snails arrives, put them in a terrarium in indirect light. Maintain a temperature of 20 to 22°C. If a terrarium is not available, place the snails in a glass jar with 5 cm of sand on the bottom. Include pieces of damp tree bark in the jar, and punch air holes in the lid. Feed land snails lettuce or cabbage leaves and slices of apple.

The snails will probably stay in their shells most of the time and may appear lifeless when they are cool. However, when they are warm they will climb up the sides of the container.

Due to USDA restrictions, land snails may not be available for shipment. Please inquire upon ordering to confirm availability.

Aquarium Snails

Carolina offers several varieties of aquarium snails. These include the ramshorn snail (*Planorbis* sp.; 14-1230), the pond snail (*Physa* sp.; 14-1212), and the mystery snail (*Ampularia cuprina*; 14-1190).

Check the condition of the snails as soon as they arrive. Rinse them in water to remove the packing material, and place them in an aquarium (they should be approximately the same temperature as the aquarium water). Snails can be held for a week in a pan of water 2 cm deep. Change the water daily and provide aquatic plants for them to crawl on and eat.

The best holding facility is a well-established aquarium with a supply of algae, other plants, and waste food materials for the snails to scavenge. If you must use a newly established aquarium, provide pelleted or flaked fish food. Contrary to popular belief, snails do not live on waste products from fish. In fact, they eat whatever food is left over by fish or other animals.

It is important to furnish adequate food because otherwise, *Physa* and ramshorn snails will eat aquarium plants. Boiled spinach or lettuce and fish food, if offered weekly, usually keeps snails satisfied. Do not overfeed the snails, however, and remove all uneaten foods to prevent fouling the water.

Mystery snails eat essentially the same food as other snails, and they do very little damage to plants. Keep the aquarium covered, however, since they will crawl out if given the opportunity.

Remember that many fish, especially larger ones, will eat snails, so you should separate the aquarium animals by size. Particularly vulnerable are young snails that hatch from eggs laid on plants and on the bottom of the aquarium. The eggs of *Physa* and ramshorns are laid in jelly capsules. The mystery snail lays its eggs in a pink capsule above the water line. The tough membrane of the capsule protects the eggs from drying out; when they hatch, the young snails simply drop into the water and go on their way.

Due to USDA restrictions, aquarium snails may not be available for shipment. Please inquire upon ordering to confirm availability.

Freshwater Mussels

Carolina offers both 1- to 2-inch mussels (14-1278) and larger mussels (14-1274). If practical, keep large freshwater mussels in big outdoor containers or in natural water areas; however, you may find it more convenient to store the mussels in a large aquarium (allow a 40-L capacity for one large mussel or two small mussels). Spread coarse sand or fine gravel approximately 8 cm deep. The aquarium should be aerated and filtered. Use only clear springwater, rainwater, or pond water, not tap water. Maintain a water temperature of 10 to 16°C. Warmer water will quickly kill the animals. Change fouled water immediately. Be aware that mussels may uproot bottom-rooted plants.

Freshwater mussel, a bivalve mollusk.

Small Aquatic Crustaceans

Of the 1.25 million kinds of animals, over 800,000 are arthropods (joint-footed animals). Of these, over 30,000 are crustaceans—arthropods with two pairs of antennae. Most live in fresh or marine waters and possess gills. The head and part of the thorax are often covered by a shieldlike carapace. Many large crustaceans, including crabs, lobsters, and shrimp, are familiar food sources for humans. Smaller, less-familiar crustaceans such as *Daphnia* (water fleas), brine shrimp, and copepods, provide abundant food for fish and form an essential link in aquatic food chains.

Daphnia

Daphnia pulex *Culture Instructions*

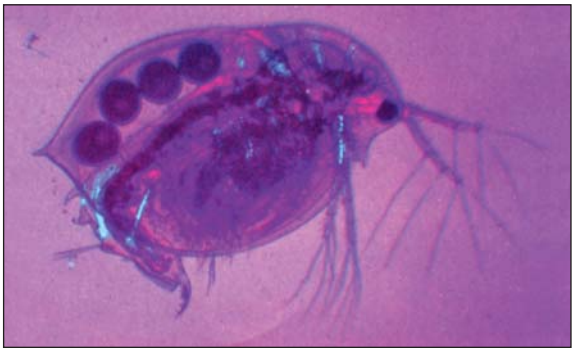
The following instructions are appropriate for use with the *Daphnia pulex* Culture Kit (14-2304). Upon the arrival of your *Daphnia pulex* (14-2314), pour the gallon of culture water into a plastic aquarium. Remove the lid from the jar of daphnia and slowly immerse the jar in the culture water until the jar is completely filled. While the jar is submerged, "pour" the culture into the aquarium. Introducing the daphnia into their new home this way prevents air bubbles from becoming trapped under their carapaces, which would lift them to the surface where they would probably not survive.

Daphia pulex (14-2314).

Next, add 1/16 teaspoon (a pinch) of Daphnia Food (14-2316) to the aquarium. **Note:** It is important that no more than 1/16 tsp of food be added initially. Adding additional food does not increase the reproductive rate of the daphnia, but instead causes the bacteria in the aquarium to reproduce faster than the daphnia, lowering the dissolved oxygen level in the aquarium to the point that the daphnia are displaced.

Once the bacteria begin to break down the food pellets and their population increases, the culture will become cloudy or turbid. The daphnia will begin to "graze" upon the bacteria suspended in the water and, in 7 to 10 days, will reduce the amount of bacteria. Once the water in the aquarium begins to clear, add another 1/16 tsp of food to the aquarium. As the population of *Daphnia pulex* in the aquarium increases, the animals will consume the bacteria at a faster rate. At this point, it may become necessary to feed the daphnia more frequently.

Over the life span of the culture, debris will accumulate on the bottom of the aquarium. Do not remove this debris, as it will contain ephippial eggs



Daphnia with eggs in brood pouch; photographed with polarized light.

(fertilized, resting eggs) that will hatch later. Algae may also begin to grow on the sides of the aquarium. Adding several small snails will help to reduce or eliminate algal buildup and will promote a healthier culture.

Daphnia do need light, but keep the aquarium out of direct sunlight, because the ultraviolet light will have an adverse effect on the culture. Ambient light from overhead fluorescent lights will suffice. Maintain the culture at 68 to 72°F.

Daphnia magna *Culture Instructions*

The following instructions are appropriate for use with the *Daphnia magna* Culture Kit (14-2326). Immediately upon receiving the culture, examine the jar of *Daphnia magna* (14-2330) to determine if the culture arrived in satisfactory condition. Look for individuals that are actively swimming. A culture of daphnia will normally experience a 10 to 15% mortality rate during transit. As these animals perish, they settle to the bottom along with the discarded carapaces from daphnia that have graduated from one instar to another. Living daphnia may also settle to the bottom of the shipping container, but they will be active.

If the culture arrived in satisfactory condition, loosen the lid to permit air exchange. Place the culture in an undisturbed location that is shielded from direct sunlight. Maintain the culture at or below 72°F. (**Note:** If your culture is not satisfactory, call Carolina's Customer Service Department at 800-334-5551 for a replacement.)

Fill a plastic aquarium approximately 80% full of hot tap water. Immediately add approximately 3 oz of water conditioner (the entire jar, if using the *Daphnia magna* Culture Kit). Allow the aquarium to cool overnight, or until the temperature of the water in the aquarium matches the ambient room temperature. In addition to preventing the daphnia from experiencing thermal shock, this cooling period allows most of the chlorine commonly found in municipal drinking water to dissipate.

Once the water temperature in the aquarium is close to the ambient room temperature, remove the lid from the jar of *Daphnia magna* and slowly submerge it in the aquarium. Once the jar is completely full and underwater, gently "pour" the culture of daphnia into the aquarium. Introducing the daphnia into their new home this way prevents air bubbles from getting trapped under their carapaces, which would lift the daphnia to the surface where they would probably not survive.

After you remove the empty jar, sprinkle a level 1/8 tsp of dried Daphnia Food (14-2316) into the aquarium. The food will float on the surface, but as it begins to absorb water it will settle and be consumed by the daphnia. (**Note:** Adding more than the prescribed amount of food will not increase the rate that the daphnia reproduce, but will promote bacterial growth that may harm the culture.) As the daphnia feed, they will begin to exhibit a dark, dorsal streak; this is food accumulating in the gut. Feed the daphnia every other day.

Your culture of *Daphnia magna* should remain viable for approximately 10 weeks. Ultimately, metabolic waste will begin to accumulate in the culture, causing adverse pH changes in the water and other harmful effects. At this time, it will be necessary to begin a new culture.

Brine Shrimp

Brine shrimp are easy to hatch and to raise in the classroom. The newly hatched animals are excellent food sources for hydra, small aquarium fish, and planarian worms; in addition, the mature brine shrimp are useful laboratory specimens.

The brine shrimp (14-2230) is found in salt lakes and ponds throughout the world.

To culture brine shrimp, select a container with a large surface area, such as a plastic pan, an enameled bucket, or an 8-inch culture dish. Fill the container to within 3 cm of the top with a 1% solution of noniodized table salt. Sprinkle the eggs evenly over the surface of the solution (approximately 1 g of eggs per liter of solution). Keep the culture at 21°C, and hatching should occur in 24 to 36 hours.

When you are ready to draw off the hatched shrimp, place a light source close to one side of the container. The shrimp will concentrate near the light and can be removed with a pipet. Before you feed them to a freshwater predator, pour fresh springwater over the brine shrimp several times to remove all traces of salt.

Other Aquatic Crustaceans

Copepods and ostracods can be cultured on wheat medium as described under "Culturing Protozoans." Larger crustaceans, such as aquatic isopods (14-2360) and amphipods (14-2355), should thrive in plastic dishpans covered with cardboard to provide darkness and minimize evaporation. Include several partially decayed leaves in the container for the animals to hide under. A good food source is the freshwater plant *Elodea densa* (16-2101).

Resources

You can access Carolina's *Living Materials Care and Handling Guide* online at www.carolina.com/.

Printed Materials

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Morholt, E., et al. 1966. *A Sourcebook for the Biological Sciences.* Harcourt, Brace, and World, Inc., New York.

Orlans, F.B. 1977. *Animal Care from Protozoa to Small Mammals.* Addison-Wesley Publishing Co., Inc., Reading, Massachusetts.

Carolina Biological Supply Company
2700 York Road, Burlington, North Carolina 27215
Phone: 800.334.5551 • Fax: 800.222.7112
Technical Support: 800.227.1150 • www.carolina.com
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