CHAPTER 2. Collection, Culture, Methodology

Early cryptogamic botanists seeking saprolegniaceous fungi paid scant heed to collecting methods: there was little need to do so since any discovery of watermolds simply involved picking up from aquatic habitats bits of vegetable and animal matter and examining them. Freshwater fish and their eggs, of course, were often invaded by the "fish molds" as they came to be known, and many reports in the pre-20th Century literature are of Saprolegniaceae collected on just such substrates. Purposely submerging organic substrates in bodies of water, or in samples of such -- the process of "baiting" -- eventually became standard practice, and concomitantly, closer attention was paid to methods and sites of collection. Several sources on methodology are extant. Among these, the publications by Durbin (1961), Dick (1976), T. W. Johnson (1956b), Seymour (1970), R. B. Stevens (1974), and Sparrow (1960) are useful, as is the thesis by D. L. McKay (1967). An especially succinct account of general methods applicable to watermolds as well as to other zoosporic fungi is that by Emerson (1958). Some concept of the discovery of saprolegniaceous fungi prior to Minden's baiting method (1916) may be had from Petersen's (1909a, 1910) study on Danish zoosporic fungi.

SAMPLING AND BAITING

There is no one sampling method that is necessarily superior if a diversity of species is to be had. Three general methods of collecting are practiced: (1) organic substrates (seeds, for example) are added to samples of soil or water brought into the laboratory, (2) baits are submerged in aquatic habitats, and (3) naturally submerged or floating organic debris is examined directly with or without prior incubation.

Containers for soil and water samples can be of any sterilizable (or sterilized) material of a convenient size. Plastic or glass wide mouth vials with a snap-cap or screw-cap closure are preferable to closeable plastic bags for water samples only with respect to durability. Small, disposable wax paper, plastic, or cellophane (T. W. Johnson, 1956b) containers are suitable for soil, but glass or plastic vials or bottles also may be used (Seymour, 1970; Scott, 1961a). For routine, large-scale collecting, G. C. Hughes (1959) used plastic-coated, eight-ounce (about 240 mL) paper food containers both for water and soil; he baited the samples directly in the containers (first adding water to collected samples). Strict attention to sample size is not necessary, but the tendency is to take larger amounts of material than can be properly cultured in Petri plates. About 10 g (net weight) of soil -- in rough measure a teaspoonful -- is sufficient for one plate. Twenty-five mL of water fills an ordinary Petri plate about $^1/_3$ full, and this amount if adequate for all gross culture work with water samples. Sparrow (1957) recommended placing soil samples into water already in the culture plates to prevent turbulence that would otherwise shift debris over any baits present.

Collected samples may, of course, be cultured in any glass or plastic dish (provided the container can be sterilized), but 100 x 20 mm Pyrex or Kimax brand Petri plates are the best for gross culture purposes. Standard plastic dishes are equally

suitable, although the amount of soil or water sample put into them must be about $^1/_4$ less than that put in the deeper glass dishes.

Water samples should be diluted with sterile water prior to baiting; a 1:1 ratio is adequate. Collections of soil are covered with water (the dish is filled to slightly over half-full), and the soil stirred well. If the Petri plate is tilted slightly as the stirred soil sample settles, and is then carefully placed horizontally, a slanted surface results on which bait can be laid directly (this is essential if species of *Geolegnia* are to be collected). Hempseed halves (or other substrates) then also can be floated on the water and be relatively free of adhering detritus. O'Sullivan (1965) found that there were greater yields of watermolds if baits were allowed to settle on the bottom of gross culture dishes rather than to float on the water. We have not observed a similar response in our cultures.

A novel method of suspending hempseed in dishes of water and soil was devised by Lowy (1958). Small glass spikes about 7 mm long are pulled up from the bottom of a Pyrex Petri plate (requires heating to about 900 ^{o}F). The gross culture of soil and water is then prepared in the dish, and halves of hempseed impaled on these spikes below the water surface. Colonies that develop are relatively free of entangling soil and silt.

Little attention has been paid to the source of water used in preparing gross cultures, although Moreau and Moreau (1936e) contended that this was an important consideration in terms of vigor in mycelium development. Common practice is to use distilled water filtered through powdered animal charcoal (W. A. Sherwood, 1966b, also used plant charcoal, but found it not as suitable) and then sterilized by autoclaving. There are two basic ways to prepare charcoal-filtered water. The first method was developed by J. N. Couch (1927) in an attempt to induce motility in spores of some Saprolegniaceae. Twenty grams of animal charcoal are added to one L of water; the mix is filtered and then autoclaved. The resulting pH is about 6.6 - 6.8. A second method is to stir about 1 teaspoonful of powdered charcoal into 1 L of distilled water, allow the particles to settle out over a one-hour period, then filter the water through coarse paper. The charcoal allegedly chelates metallic ions, some of which may be toxic to the fungi. It has been noted (J. N. Couch, 1927) that if charcoal-treated water is redistilled it no longer "supports" motility of spores.

Because distilled water may have an inhibitory effect on some zoosporic fungi (Emerson, 1958), sterile tap water (Emerson, 1941) can be substituted. Emerson advocated using a mix of one part filtered pond water: two parts glass-distilled water, and Sorenson (1962) found this formulation to be most suitable for propagating watermolds. On the other hand, C. E. Miller and Ristanović (1969) concluded that hempseed cultures of saprolegniaceous fungi produced oogonia much sooner in glass-distilled water than in any mix of glass-distilled and lake water, and this appears to agree with Dick's (1965) observation. To circumvent possible problems with distilled water alone, Machlis (1953a) devised a very dilute salt solution adjusted to pH 7. In gross culture work, at least, the use of such a solution is not necessary. In the final

analysis, glass-distilled, ion-exchanged water may well be the best source, but there are acceptable substitutes for water of such exceptional purity.

Scott (1961a) baited gross cultures in sterile soil extract, but did not specify how it was prepared. For a number of years J. N. Couch and his associates used an oak leaf decoction in which to propagate watermolds; such a solution favored oosphere maturation and antheridial development. The oak decoction can be prepared by boiling a few dry leaves (*Quercus nigra*, and other species) in distilled water for 10 minutes or until a strong infusion is made. The extract is then diluted (to obtain particular pH levels) and used as the culture solution (A. W. Ziegler, 1950; A. W. Ziegler and Linthicum, 1950). We have used water from streams, bogs, ponds, or lakes in gross culture, but have not noticed that autoclaved "natural" water provides any advantage over more purified forms. If, for instance, pond or stream water is to be used, it should be filtered through several layers of cheesecloth and cotton, reconstituted to a given amount with tap water, and then autoclaved (15 minutes, 121 C). The sterilization process ordinarily precipitates some of the suspended material; care should be taken not to pour the precipitate into gross cultures. We prefer a cold steep method for preparing soil extract. About 100 grams of fertile soil (tilled or pasture land, preferably) is steeped for 6-8 hours (with occasional stirring) in one L of tap or pond water. The water is filtered through cheesecloth and cotton, reconstituted to a liter, and then autoclaved.

The use of potassium tellurite ($K_2Te\ O_3$) as a culture adjuvant to reduce bacterial populations has been criticized (Dick, 1976) because of its concomitant suppression of sporulation by watermolds. Some calculations by Padgett (communication) demonstrate, on the contrary, that there are fewer watermold spores in culture water lacking tellurite than when this chemical is present, Perhaps the adjuvant's chief function in water culture or media is to depress the competition that bacterial populations create, and this benefit may well outweigh any suppressive effect on planonts. The matter in any case needs extensive experimentation.

Gross cultures (baited soil or water) are ordinarily incubated at room temperature, but better growth of watermolds (coupled with reduced contamination) is obtained if the samples are incubated at about 18 ^{o}C. Cultures usually yield watermolds within 3-5 days after baiting, but should be kept for 3-4 weeks to permit the slower-growing species to develop. According to Klich (1980), the greatest diversity in yield of identifiable watermolds is obtained if gross cultures are incubated for several days in a lighted area, and then are moved to the dark. A temperature of 15 ^{o}C, she suggested, is most suitable for the establishment of colonies on the baits.

After the initial culturing period, soil samples may be set aside and simply allowed to dry out in the culture dishes with the covers in place. Once dry, these samples can then be again wetted and baited. Occasionally such "second growth" gross cultures yield species not recovered initially, but it is well to recognize that the longevity of some watermolds is drastically shortened by desiccation (Remy, 1950; Apinis, 1964).

If colonies (on hempseed) from gross cultures are to be stored temporarily before isolation, they should be kept in the original soil or water dishes rather than being transferred to clean water. When the contaminated colonies are put in fresh water (even after thorough washing), the populations of protozoans and rotifers accompanying the hyphae are quickly amplified. Subsequent attempts at isolation are made much more difficult because of the vast numbers of organisms that develop during storage.

A word on preventing (or delaying) mite infestation is essential since gross cultures of soil invariably introduce these troublesome creatures into the laboratory. Goldie-Smith (1956) battled mites by placing the seeded gross culture dishes in closed containers surface-sterilized with alcohol, but other chemical means (Curl, 1958; Jewson and Tattersfield, 1922; Shafik and Page, 1930) are also known to combat these pests. A satisfactory method is to prepare glycerin traps and place these below stacks of Petri plate cultures. A small hole is bored or burned into the center of the bottom of a standard plastic culture plate. Enough undiluted glycerin is then poured into the dish lid to cover it with a layer about 2 mm deep. The bottom is then put into place in the inverted cover and this creates a "moat" of glycerin in the space between the bottom and lid of the dish. Pedestrian mites are trapped in the moat if they crawl out of the gross cultures. Barnes (1933) used containers of water as barriers, and elevated culture dishes on tripods. Barriers of cigarette paper fastened across the mouth of culture tubes also are an effective deterrent according to Snyder and Hansen (1946).

BAIT TYPES

An early attempt at baiting samples -- as opposed to seeking aquatic fungi directly by examining organic matter from water -- was that by E. J. Butler, reported in 1907. He used slices of *Abutilon* root either without treatment or after being soaked in a decoction of flies -- reaching back, doubtless, to early published reports on the invasion of flies by watermolds. Since Butler's time, a wide variety of organic substrates has been used; some of these are listed in Table 2 (*see* also Sparrow, 1957, 1960). Half of a boiled, autoclaved achene of *Cannabis sativa* L. (hempseed) is the bait of choice for watermolds, although no one has as yet discovered why it is so effective. Preparation of hempseed is relatively simple. Emerson (1958) recommended boiling the seeds for 10-20 minutes to rupture the ovary wall and seed coat and to sterilize them. Goldie-Smith (1956), on the other hand, boiled the achenes for 5 minutes then split them with a sterile knife. We find that boiling hempseed in distilled water for 1-2 minutes is sufficient to soften the seed coat and the bait can then be cut transversely into two halves with a razor blade. Sterilization of the dried halves by autoclaving at 121 ^{o} C for 5-7 minutes follows. Neish (1976) noted, as had W. N. Tiffney (1939a), that a whole hempseed, punctured with a needle, seemed to enhance oogonial production in some isolates of the family from fish.

Very little has been done to assess the relative efficiency of substrates used as bait. It is curious that Diaz (1976) did not recover one watermold in hempseed-baited cultures, evidently finding only a variety of aquatic imperfects and mucoraceous

species. To be sure, some watermolds are most readily (if not exclusively) "caught" on substrates other than hempseed. Huneycutt (1948) used horse hoof shavings for collecting and subsequently propagating *Aphanodictyon papillatum*; Dick (1966) reported that species of *Aphanomyces* were prominent on snakeskin bait, and we have found this to be the case in our numerous collections (T. W. Johnson, 1977c). We have used 5-6 mm^2 bits of roach wing and short segments of infant hair successfully in collecting species of this genus. It is not the nutrient composition of snakeskin, Dick contended, that provides a suitable (required?) medium for members of *Aphanomyces* since those species that grow on snakeskin may also be recovered on hempseed. He concluded that *Aphanomyces* species utilize snakeskin because of reduced competition (other water-molds grow only very sparingly on this substratum). Pretreated snakeskin (T. W. Johnson, 1977c) is useful in isolating species of *Aphanomyces,* but also is advantageous when used as bait in gross cultures. Bits of dry snakeskin (any convenient size up to 10 mm^2 are floated for 24-30 hours on the surface of freshly collected, untreated pond or lake water. The bait is cut, washed, and boiled for 3-5 minutes, and is then ready for immediate use.

Although he was unable to collect members of the Saprolegniaceae by either particle or dilution plating, Park (1972a, 1974) found the cotyledons of seeds of *Brassica* (sp.?) to be a suitable bait. By boiling the seeds he sterilized them, and during preparation the seeds also opened to release the four disc-like half-cotyledons. Small pieces of bait yielded a greater variety of watermolds, Park found, than did larger ones (whole hempseed).

The most extensive study made of seed types as substrates for the growth of a watermold, is that by Ristanović and Ritter-Studnicka (1970). Their investigation was not, to be sure, directly solely at finding suitable baits, but the results certainly apply to the subject of substrates for watermolds. They grew *Achlya klebsiana* (=*debaryana*) on seeds of 87 species of vascular plants [29 families; most representatives were in the Papilionaceae (Leguminosae) and Gramineae] in distilled water at 20 ^{o}C. On seeds of 15% of the species, the fungus grew only vegetatively: *Alnus glutinosa* (L.) Gärtn., *Centaurea scabiosa* L., *Rhamnus fallax* Boiss., and *Bromus inermis* Leyss., are examples. Mycelium with antheridia and oogonia grew on 35 species, 26 of which supported colonies that produced oospores; among these species were *Evonymus europaea* L., *Trifolium pratense* L., *Bryonia alba* L., *Carpinus betulus* L., *Sambucus racemosa* L., *Ostry carpinifolia* Scop., *Gossypium herbaceum* L., and *Cicer sanguinea* L. Abnormal oogonia appeared on the mycelium propagated on seeds of such species as *Vicia pannonica* Cr., *Agrostis alba* L., *Onobrychis sativa* Lam., and *Ceratonia siliqua* L. The only seed on which *A. klebsiana* would not grow was that of *Punica granatum* L. The authors of this study concluded that the seeds of *Evonymus europaea*, *Cicer arietinum* L., *Trifolium pratense,* and *Bryonia alba* are as favorable for the development of this fungus as are hempseed.

Although her study of bait types was not extensive, Klich (1980) found that sweet clover seeds (*Melilotus alba* Desr.) were superior to those of alfalfa (*Medicago sativa* L.) or hempseed for the propagation of *Aphanomyces* species. Although her tests showed that

hempseed was a more suitable bait for recovering Achlyas, sweet clover seeds could be substituted acceptably for the collection of species of *Dictyuchus* and *Saprolegnia.*

SPECIAL METHODS FOR COLLECTING AND BAITING

Not all successful methods of collecting watermolds require baiting samples brought to the laboratory. Several methods have been developed for *in situ* baiting or for collecting watermold populations by various mechanical means; a representative sampling of such special methods follows.

It appears that Minden (1916) was the first employed *in situ* baiting. He placed fruits and various other plant parts in gauze bags and submerged these "traps" for 8-14 days in about 0.5 meter of water. Small envelopes constructed of wire screening were used by Kanouse (1925) as containers for bits of bait; Padgett (1978a) enclosed halves of hempseed in folded pieces (12.5 x 7.5 cm) of fiberglass screening which were then stitched shut with monofilament line and autoclaved prior to submergence. Similar methods employ mesh bags (or miller's cloth, Höhnk and Bock, 1954), loosely woven nylon (W. B. Cooke and Bartsch, 1960; W. B. Cooke, 1970a), or bags made of mosquito netting (S. B. Saksena and Rajagopalan, 1958). The small metal containers known as "tea balls" were used by W. B. Cooke (1970b), W. B. Cooke and Bartsch (1960) and Seymour (1970); the latter found these to be ideally suited containers for submerging substrates. Perrott (1960) immersed fruits and twigs (as bait) in perforated galvanized containers.

To submerge substrates at given depths, and to insure that the bait is first exposed only at that depth, Paterson's (1967) method is perhaps the most useful. Hempseed is placed in wide mouth jars covered with nylon screening (1 x 1.5 mm mesh). The individual jar is then sealed temporarily with a thin sheet of polyethylene to which a cord is tied. When the "trap" is lowered to the desired depth, the cord is pulled, ripping the plastic sheet, and allowing water to enter.

The length of submergence time when using hempseed as the substratum varies from 72 hours (Padgett, 1978a) to 3-5 days (W. B. Cooke and Bartsch, 1960; Höhnk and Bock, 1954). Substrates such as fruits and twigs are submerged up to four weeks (Perrott, 1960), although longer periods may be required. In some of the cold, deep, slowly moving streams in Iceland, for example, we have collected watermolds only after submerging rosaceous fruits and hempseed for 2 $^{1}/_{2}$ months. In the tropics, 2 to 3 weeks is sufficient.

Methods of concentrating water samples to obtain fungal propagules, and then plating out the concentrate have also been substituted for baiting. Fuller and Poyton (1964) and Maestres (1977) centrifuged samples, then mixed the resulting particulate matter with water and plated it on a suitable nutrient medium. Resuspended residue collected on Millipore filter discs also provides inoculum (C. E. Miller, 1967); the suspension is streaked onto a suitable medium (for example glucose-peptone-yeast extract agar) containing antibiotics.

Additional methods for exposing organic substrates to water have been devised, but these seem not to have been any more practical than baiting field samples brought

to the laboratory. Stoll (1936), and later (1957) Johannes, fastened ant pupae to bits of paper with wax, and submerged them. Hoshina (1963) impaled hempseeds on fishhooks, and immersed these in water. A more elaborate method was devised by Alabi (1967). A 3.5 cm^2 sheet of glass is covered with a thin film of 2% malt agar, and a tablet of Bacitracin secured to the center of this sheet by the solidifying agar. A second glass plate is placed over the first (before the agar hardens), and the two sheets of glass -- separated by the agar film and the antibiotic tablet -- are secured with rubber bands. The resulting apparatus is submerged horizontally in the water for 24 hours then removed. The film of agar is peeled off, and the saprolegniaceous hyphae or germinating spores attached to the film can then be isolated. A serious problem with this technique, of course, is that fungi other than watermolds infest the agar film as well (Alabi, 1967). Seamless dialysis tubing (regenerated cellulose) partially filled with an agar medium, then tied off into sausage-like lengths, was used by Bandoni *et al.* (1975) as bait. The tube (30 x 2.6 cm) was filled partially with a sterile agar medium, tied off, placed in 12 cm^3 galvanized mesh baskets and submerged for 2-4 weeks. These cylindrical tubes were then examined directly for fungi, and some portions plated out. Species of *Achlya* and *Saprolegnia* were collected in this fashion. One problem with such cylinders, of course, is decomposition of the dialysis casing by cellulolytic organisms.

In passing, it is well to mention that watermolds themselves have been used as bait. Register (1959) immersed colonies of *Achlya flagellata* (=*debaryana*) in boiling water for 30 seconds, then put these in gross cultures and successfully secured growth of a chytrid.

ISOLATION AND CULTURE

Following the recovery of members of the Saprolegniaceae from water or soil, it is mandatory that they be isolated prior to identification. It is quite evident in the early taxonomic literature that little if any attention was paid to isolating these fungi. Indeed, the first species of *Achlya* and *Saprolegnia* (Nees von Esenbeck, 1823) probably were described from mixed colonies in which, fortunately for later studies, one species predominated. Had Pringsheim (1882a, b), for example, isolated and cultured his watermold material, it is unlikely that he would have described motile sperm cells in the specimens he examined. It is even less likely that the papers by Leidy (1850), Bail (1860), De-La-Rue (1869), H. Hoffman (1867), and Fückel (1869) would have appeared. Bail attempted to show, by following the development of fungi on flies and midges, that *Empusa muscae* (*Entomophthora* Fresenius is the correct generic name, as D. M. MacLeod, 1963, has properly stated), *Mucor mucedo* (L.) Fres. and *Achlya prolifera* were merely stages in the growth of one fungus. In a moist habitat, he wrote, the *Empusa* was transformed into *Mucor*, and in water it became *A. prolifera.* Culturing surely would have shown the absurdity of this conclusion, and might also have demonstrated that the watermold supposedly involved in this miraculous transformation was a species of *Saprolegnia.* In 1867, H. Hoffman alleged that a *Saprolegnia* he cultured from a fish developed into a species of *Mucor* -- an unparalleled case, he thought, of extreme

polymorphism. De-La-Rue took exception to H. Hoffman's proposal, and concluded (from observations on some filaments on flies in water) that a *Saprolegnia,* which he named *S. dioica-ramosa* DLR, did not transform into *Mucor* but that *Empusa muscae* was a stage in the development of *Saprolegnia.* About the same time, Fückel (1869) was declaring that *Empusa* (included by him in the group Saprolegniei de Bary) developed *Achlya* (species?) sporangia in water.

Numerous ways have been devised to isolate and culture members of the Saprolegniaceae. The methods have had one of two purposes, namely, the isolation of a single species of fungus, that is, growth of a unifungal culture, or propagation of axenic cultures ("pure", in the various perturbations in its application has lost its original meaning). Some investigators were content to satisfy the first of these purposes, while others developed and worked exclusively with axenic material. A sampling of the extensive literature that touches in part or entirely on isolation and culture techniques follows.

In general, the various methods of isolation and propagation involve either some mechanical manipulation (or barrier) or the incorporation of a chemical adjuvant to suppress contaminants. There were exceptions, of course, and the method employed by Horn (1904) is an example. He reported isolating *Achlya polyandra* Hildebrand simply by growing it on 1% peptone agar; the hyphal tips, he maintained, were bacteria-free because of their rapid growth on this medium.

It is not surprising that it should be de Bary (1884) who was among the first to realize the need for pure cultures. He mentions methods only briefly, and not clearly enough that they could be duplicated precisely, but what he does record suggests that he used techniques much advanced for the time. Inasmuch as mycologists of the 19th Century studied saprolegniaceous fungi by collecting them on dead flies (among other invertebrates) the first attempts at culturing simply employed such carcasses. Trow's method (1895) is illustrative. He obtained what would now be recognized as a unifungal culture by placing a single hypha-bearing oogonium in a drop of boiled water and adding a fly leg. He also secured fungal growth by placing a sporangium with spore initials directly on a fly leg in water. Trow recognized that the resulting colonies were not without bacteria, infusorians, and the like (which, he said, were troublesome in warm weather), but could be made free of such organisms by using sterile water and sterile instruments. He also maintained that culture purity could be determined simply by microscopic observation. Since by purity he meant free of bacteria, his contention is suspect. Obel (1910a) used a method nearly identical to that of Trow. Single spore cultures of *Aphanomyces laevis* were developed by Trow although he seems not to have recognized the significance of his method. He spread a drop of beaten egg white onto a coverslip, and immersed the preparation in boiling water. When the water had cooled, the coverslip was floated on the surface in a jar containing spores from a mixture of watermolds. Spores became attached to the coagulated albumen, and by trial and error he located isolated ones that, when further incubated, produced mycelium of the *Aphanomyces.* Later, P.-A. Dangeard (1931) modified Trow's method by coating the coverslips with nutrient gelatin or agar.

In a paper on a sexually sterile *Achlya,* Weston (1917) described a technique that provided the basic manipulation for development of single spore cultures. Using an atomizer, he sprayed a spore suspension onto beef extract agar, then located single, germinated spores, and transferred these to new media. Although not all subsequent students of the family isolated their fungi by atomizing, Weston's method of mechanically separating individual spores is still practiced widely and successfully. He also obtained unifungal and contaminant-free cultures by serially diluting suspensions of gemmae, and Afanasiev (1948a) achieved the same result with spore suspensions. Somewhat earlier (1901) Schouten devised a rudimentary micromanipulator involving a microscope slide, moveable needles, and drops of spore suspension and nutrients suspended from a coverslip. His explanation of the method is not entirely clear (and is without illustration), but the technique involved pushing one spore from the mixed or contaminated suspension into the nutrient medium. This cumbersome method has no advantage over streaking or spraying a spore suspension onto an agar surface. Beverwijk (1948) maintained that spreading spores over an agar surface with a loop was the only reliable method for freeing watermolds from bacteria. Slifkin (1964) recommended mechanically pushing or rolling individual spores over and into plated agar to free them at least partially from bacteria.

Single spore isolation -- as well as some other manipulations of watermold material -- requires extremely fine-pointed (yet rigid) dissecting needles. Such instruments should resist wear, withstand flaming, and be free of jagged edges or other imperfections. Insect pins (000 grade) or other fine metal wire can be used -- and be variously shaped for specific purposes -- but no matter how carefully sharpened, these are inferior to tungsten wire shaped in hot sodium nitrite. Pantin (1946) first made brief mention of such needles, stating that they were suited for use with a dissecting machine devised by Harding (1939). The details of the method for preparing tungsten needles follow.

Enough crystalline, reagent grade sodium nitrite is put into a nickel metal crucible (one with a cover is essential, and porcelain or glass crucibles are not suitable substitutes) to fill it about three-fourths full. The uncovered crucible is heated over a Bunsen burner to melt the nitrite, which then must be kept hot while in use. A short length (1.5-2.0 cm) of 0.010 gauge tungsten wire is fastened into an ordinary needle holder. When the sodium nitrite is hot, the end of the wire (about 0.5 cm) is dipped into the liquid at an angle to the surface. If the nitrite is at the proper temperature, the area immediately around the submerged end of the wire will show obvious bubbling, indicating that the tungsten is eroding. The amount of time required for the chemical reaction to erode the wire to a point can be determined only by periodically removing the wire and examining it under a dissecting microscope. The wire is dipped into tap water after it is taken from the hot nitrite; this will remove the residue, and leave the dipped end clean. The end of the tungsten can be repeatedly dipped into the hot nitrite (and then into water) until a point of the desired thinness is obtained, It is possible to erode the tungsten to a point too slender to use, because it bends easily. The point can be blunted by dipping the end of the wire perpendicularly into the nitrite for a few

seconds. The amount of submergence time required to blunt a point must also be determined by trial and error.

A tungsten needle sharpened by this method can be flame-sterilized repeatedly without losing its point. When the point is dulled, the end can be resharpened by dipping it again in hot nitrite. Flaming the tungsten point repeatedly eventually induces charring. The residue is removed simply by dipping the point in hot nitrite for a few seconds, then "washing" it in water. The crucible containing the nitrite must be kept covered when not in use because the chemical hydrates readily, and loses its effectiveness. We have found it best to replace the sodium nitrite entirely when hydration occurs rather than simply to add more chemical.

After individual spores have been isolated on the surface of an agar medium they can then be transferred to fresh medium for germination and growth. A funnel-like metal cutter screwed into a microscope nosepiece in place of an objective was devised by LaRue (1920) and later modified by Keyworth (1959) for ease in sterilizing the cutting edge. In use the LaRue device simply was lowered into the agar to cut a small, cylindrical plug of the medium bearing a single spore. The plug could then be removed from the plated agar medium with a sterilized, spatulate instrument. One of the chief disadvantages of the LaRue cutter is that the object to be removed from the medium must first be located with an ordinary brightfield objective, and then that objective must be removed and the cutting device inserted in its place. Messner's (1980) modification provides the cutting capability of the LaRue instrument, but positions the cutter (spring-loaded) directly on the objective itself. Hence, in use, Messner's cutter is simply lowered into the agar surrounding the object once the specimen has been located. The plug-cutting devices offer no substantial advantage over simply digging the germling from the agar surface by means of a simple spatula made by flattening the end of a 00 or 000 insect pin. Indeed, the tungsten needle described in the foregoing paragraph is quite adequate by itself for removing single spores from an isolation plate.

Hyphal tips (as well as spores) have been used in attempts to secure unifungal or bacteria-free cultures. This technique may be traced to Rothert (1888, 1890), who discovered that severed hyphae would continue to grow in hanging-drop preparations, Lechmere (1910) seeded washed hyphae onto a beef extract-gelatin medium, and made a successive series of transfers of hyphal tips from resulting colonies. After two weeks of subculturing an isolated fungus Lechmere reported that he had succeeded in growing specimens free of bacteria and infusorians. A year later (1911a) he described an improvement to his method. The agar plates on which the hyphae were growing were tilted to a 60° angle. He reasoned that the bacteria were carried along by those hyphae growing toward gravity (the "bottom" of the slanted dish), but not by those "negatively geotropic" filaments growing toward the top of the dish. Employing a much more elaborate method, Volkonsky (1933c) also prepared cultures of watermolds (possibly they were not unifungal) using detached hyphal tips. He poured a weak nutrient agar medium into a Petri plate, and then inoculated the center of the agar surface with a bit of mycelium. Liquefied nutrient agar was then dropped onto the center of the inside surface of the cover of another plate until a pellet of solidified agar

was built up. He placed this cover over the dish on which the mycelium had been planted, and inverted the plate. During incubation, aerial hyphae grew out from the inoculation site, contacted the mound of agar and grew onto it. Portions of this agar could then be removed easily with hyphal tips intact.

Somewhat less intricate methods than that of Volkonsky have been devised to encourage hyphal tips to grow into a semisolid medium, the assumption being that the filaments outgrow the bacteria which are unable to penetrate the medium, These methods have not utilized any mechanical barrier to bacterial growth other than the gel medium itself.

The technique employed by W. Brown (1924) utilized water agar in Petri plates. He inoculated the center of the surface of plated nutrient agar. In 5-6 days, the medium was cut in advance of the hyphal tips, and the agar turned over. Hyphae growing into the agar below the colony could then be picked out along with adherent medium. Instead of turning over inoculated agar, C. D. McKeen (1952) placed agar slabs over hyphae that had been wetted and then seeded on an agar medium (see also W. E. McKeen, 1949). Modifications of this technique were used by T. W. Johnson (1956b), and Schmitthenner and Hilty (1962). Johnson cut plates of 2% water agar into triangular sections and placed a bit of inoculum under each section. Hyphae (presumably free of contaminants) grew into the agar and subsequently could be transferred to water cultures. Whole plates of a suitable agar were used by Schmitthenner and Hilty (1962) to free *Aphanomyces cochlioides* from contaminants. They seeded the agar surface with a spore suspension, lifted out the entire circular slab of medium and inverted it in the bottom of the dish. After the hyphae had grown from germinating spores, shallow plugs of agar containing hyphal tips were taken from the surface. Working with rice seeds and seedlings infested by *Achlya klebsiana* (=*debaryana*) R. K. Webster *et al.* (1970) simply put infested plant material under water agar in Petri plates; the hyphal tips subsequently grew up through the agar. The same result was achieved by Oláh and Farkas (1978) by a different means. In their technique a 2 cm wide strip of agar (in an inoculated Petri plate) is removed in advance of hyphae. The filaments of the watermold grow along the glass, across the gap, and then are usually free of bacteria when they commence growing on agar on the opposing side.

In any method where inoculum is inserted under agar to reduce or inhibit bacteria it is mandatory that the agar surface on which the inoculum initially adheres be pressed flat against the glass of the dish to minimize air entrapment. Pockets of air around the inoculum favor bacterial growth, and contamination is not reduced but enhanced.

Chaze (1924, 1925) is widely cited as a pioneer in the development of pure culture methods for Saprolegniaceae. He reported, contrary to the opinion of contemporaries, that isolation of *Saprolegnia* (sp.?) from fish was quite easy to effect. Hyphae on infested fish were washed in running water, then dipped into a solution (strength?) of lactic acid (exposure time?). The hyphae were then transferred to moist sterile bread in an Erlenmeyer flask. After several days of growth, some of the whitish mycelium, alleged to be free of bacteria because of its rapid growth, was seeded onto

soy agar. According to Chaze, such cultures were absolutely pure, by which he meant bacteria-free. Somewhat later, he (1933) modified the earlier method, transferring hyphae into a weak acid solution (one drop of lactic acid in 20 mL of sterile water; exposure for 30 seconds) then onto nutrient media, Weak acids were also employed by other investigators. Dop (1905a), for example, used three ppt citric acid directly in the medium; E. J. Butler (1907) did likewise, as did Moreau and Moreau (1938) and Moruzi and Toma (1968) with lactic acid. Küster (1913) pointed out, however, that some members of the Saprolegniaceae were sensitive even to small concentrations of acid, as Klebs had noted. Various chemical additives other than common organic acids have been used to combat bacterial contamination in agar cultures of watermolds (*see* Table 3).

Plant pathogenic watermolds may demand special handling to be isolated. Small bits of infected tissue are washed thoroughly and repeatedly, and during this process (if extended over 1-2 days) extramatrical hyphae will appear. These washed pieces of plant material are then blotted on sterile filter paper, and transferred to a suitable medium or freed of bacteria by using W. Brown's method (Drechsler, 1929). Since it is possible to induce sporulation in the *Aphanomyces* species that are the chief plant pathogens, isolation of these watermolds can be achieved simply by diluting and dispersing spore suspensions.

Perhaps the method of obtaining axenic isolates that has received the most attention was devised by J. R. Raper (1936, 1937), combining a mechanical barrier to the spread of contaminating organisms and inducing growth of hyphae into an agar medium. Seymour's (1970) modification is the best of several that have been proposed (Table 3). A glucose-glutamate medium (Table 5) is poured aseptically into a sterile Petri plate such that it is about half full. A van Tieghem cell (glass cylinder about 12 mm in diameter and 12 mm deep) is dipped into 95% alcohol, and touched to a flame to burn off the alcohol. While still hot, the ring is placed on the semisolid surface and gently pushed about half way into the medium. Spores or hyphal tips are then placed in the ring, and the seeded plate incubated at about 18 ^{o}C. Hyphae grow into the agar, under the ring, and through the agar outside the ring. Plugs or small blocks of medium containing hyphal tips then can be transferred to dishes of sterile water, sterile hempseed halves added, and water cultures propagated. Although Raper fastened small beads to the bottom edge of van Tieghem cells to hold them off the bottom of the Petri dish (Ark and Dickey, 1950, used small pellets of clay), this is unnecessary if the plated agar is deep. While glass rings are most often used, Powell and Tenney (1964) silver-coated such rings, and Powell *et al*. (1972) used sterling silver rings 1.5 cm in diameter, and one cm deep. Presumably through oligodynamic action the metal further reduced microbial populations around the inoculum.

Inasmuch as some cultures of watermolds are troublesome to free from bacterial contaminants even by the Raper ring method and meticulous attention to asepsis, various other methods have been devised. Glass rings combined with culture adjuvants have been used (Table 3), and the isolation medium itself treated, but even these techniques are not always successful.

One of the most extensive studies of isolation media is that by Ho (1975b). His goal was to develop a selective medium (Table 4) useful in the quantitative study of populations of watermolds. He tested several antimicrobial agents for their effect on the growth of a candidate organism, *Saprolegnia* sp., and devised a medium that, when seeded with one mL of raw stream water, provided a substratum on which almost all of the fungi first to appear were species of *Saprolegnia.* A medium somewhat simpler than that of Ho, but having the same purpose was devised by Maestres and Nolan (1978). Their concoction contained (per L) Emerson's YpSs ingredients (40.5 g), 200,000 units of penicillin, 200,000 µg of streptomycin, and 400 mg of pentachloronitrobenzene. Seymour (1970) also used a selective formulation (modified from Scott *et al.,* 1963) as an isolation and subsequently a culture medium (Table 5). In some cases, he diluted the resulting solution 1:4 to encourage growth of those *Saprolegnia* species that develop better on nutrient-poor media.

Blank and Tiffney (1936) irradiated plated media with a mercury vapor lamp (UV) for three hours before inoculating it, and noted that bacterial populations around the inoculum of hyphae of *Saprolegnia* sp. from an infested frog were substantially reduced. Extensive testing of eleven isolation methods led D. L. McKay (1967) to conclude that using an irradiated medium plus glass rings -- with repeated subculturing if necessary -- was the method of choice to eliminate bacterial contaminants, although even this multiple technique was not always successful. In connection with methods involving the illumination of media, it is well to recall that Weinhold and Hendrix (1963) found plated media exposed to light prior to seeding with fungi to be inhibitory to growth. Their observation is in need of further exploration, although certainly Lee's (1965) work on oogonial production by some watermolds is not entirely supportive of their findings.

Where contamination of inoculum proved to be a recurring problem (with species of *Pythium,* largely), Machacek (1934) used another application of a mechanical barrier to bacterial growth. He poured plates of cornmeal agar (for watermolds), and before the agar solidified, inoculated the medium. He then placed a circular, alcohol-flamed coverslip over the inoculum and pushed the glass firmly onto the warm but still liquid medium. The coverslip prevented lateral spread of the bacteria, but the hyphae grew into the agar past the coverslip, and emerged free of contaminating organisms.

In conclusion it is of passing interest to record an observation by Graff (1918). He prepared bacteria-free cultures of *Achlya apiculata,* but found that the fungus failed to reproduce sexually. Only when he introduced bacteria into the cultures did the organism develop antheridial branches and oogonia!

AQUATIC AXENIC CULTURES

Although some members of the Saprolegniaceae reproduce on various semisolid nutrient media, not all will do so. It is therefore necessary to transfer axenic cultures on agar to water. Two methods may be employed.

A block of agar (about 1 cm^2) supporting hyphal growth of the fungus is cut from the edge of a colony. The block is transferred, inoculated surface uppermost, to a sterile Petri plate, and sterile charcoal-filtered, distilled water sufficient to cover the block with a free-standing film is added. A sterile hempseed half is placed cut surface down on the agar block, and 2-3 additional halves floated in the surrounding water. About 24 hours later, additional sterile water is added to bring the plate approximately half full. Planonts from sporangia produced by hyphae growing on the seed half in contact with the agar block inoculate the free-floating hempseeds within one or two days of incubation. An alternative procedure (Seymour, 1970) requiring less manipulation is that of placing a sterilized hempseed half, cut surface down, directly on the advancing edge of a watermold colony growing on a semisolid medium. After 2-3 days, the infested hempseed is removed aseptically and put in sterile water. Additional hempseed halves then may be added to duplicate the culture.

GROWTH MEDIA

Investigators of the watermolds have been noticeably independent in their choice of media on which to propagate isolates, Accordingly, there exists in the literature a good deal of information on an array of concoctions and formulations for media. The following is an illustrative selection of accounts from that literature.

Although Oscar Brefeld (Westerdijk, 1947) ushered in the use of natural substrates for media -- fruit juices, for example -- it was Klebs (1899) who was a pioneer in exploring the growth of watermolds on a variety of substrates selected for particular purposes. Using pea broth as a base, he added various concentrations of such compounds as peptone, gelatin, hemoglobin, asparagine, alanine, oxalic acid, acetic acid, glucosamine, and a variety of sugars [dextrose, maltose, levulose (= fructose)], inorganic salts, and plant alkaloids. His work was swiftly and widely emulated (Pieters, 1915a; Kanouse, 1932; Kauffman, 1908). Coker's (1923) account of the watermolds he studied clearly show the Klebsian influence because he also used a considerable assortment of substances singly or in combination on which to grow his fungi: egg yolk, mushroom grubs, ants (termites) in water from various sources, egg yolk plus ants (in water), hemoglobin, various minerals alone and in combination with a maltose and peptone solution, and corn meal-egg yolk agar.

Chaze (1925) propagated a culture of a *Saprolegnia* (sp.) that he alleged was bacteria-free on a number of natural solid or broth substrates: fish bullion, bread, potato, carrot, rice, wort, and soybeans, for example. His goal was to determine which of the various media supported vegetative growth or sporangium production, and which ones stimulated gemma formation. Crooks (1937) also tested a series of media for their ability to aid growth and reproduction: carrageenan (as carragheen) agar, sugars in combination with peptone, peptone-leucine-maltose broth, and saccharose plus cubes of egg albumen. Earlier (1932), Höhnk had devised carrageenan agar for the propagation of watermolds: dextrose, citric acid, and mineral salts, with 8-10 g agar and 5 g carrageenan as solidifying agents. For supporting the production of oogonia on the

mycelium of species of *Aphanomyces* Satour (1967) recommended an agar-solidified extract of rape seed (*Brassica napus* L.).

Natural product extracts of various types support the growth of watermolds but two seem to have the widest acceptance. Cornmeal agar (Difco, dehydrated) is an excellent growth medium, but it does not necessarily support sexually reproductive mycelium. The second general-purpose medium is Emerson's (1941, 1958) YpSs agar (Table 6). It has an additional advantage over simple plant extract media (such as cornmeal agar) in that substitutes can easily be made for one or more of the ingredients.

Media have been concocted to provide particular nutrient or growth requirements for specific watermolds. Two examples are illustrative. An isolation medium for fungi in polluted waters was devised by W. B. Cooke (1954). We find his formulation (Table 7) to be routinely useful for recovering and culturing saprolegniaceous species from mud samples. To propagate keratinophilic forms of *Aphanomyces,* Huneycutt's (1955) soil water-cow horn medium is satisfactory. This medium is prepared by boiling for one hour 500 grams of barnyard soil in one liter of water, filtering the extract and reconstituting it to one L. Ten grams each of agar and cow horn filings are added and the medium is autoclaved.

With the advent of highly refined studies on genetic, physiological, nutritional, and biochemical activities of members of the Saprolegniaceae came the need for chemically defined media. Many formulations were developed; the following examples show their diversity and complexity. There is a thread of commonality in nearly all chemically defined media: they incorporate carbon, nitrogen, and sulfur sources of known quantity and constituency, mineral elements, and (often) the salts of trace metals.

Barksdale's (1962a) medium (Table 8) has achieved wide use, sometimes with minor modifications. Lile's (1969) basal medium (Table 10) is similar to that of Barksdale, save primarily for its organic constituents. Because it contains a stock solution of vitamins in addition to the usual ingredients, D. L. McKay's (1967) formulation is more complicated than either of the previous two (Table 11). Another chemically defined but relatively simple medium (Table 9) that readily supports growth of *Aphanomyces* species and some other watermolds is that reported by Yang and Schoulties (1972).

SPECIAL CULTURE METHODS AND MEDIA

Nutrient concoctions or culture manipulations are sometimes necessary to stimulate reproduction in watermolds. A medium devised specifically for the support of sexually reproducing colonies was that of Scott, Powell, and Seymour (1963). All of the eight test species propagated on this substrate (Table 12; compare with Table 5) produced a sexual apparatus, and because of this the investigators proposed that the medium might be substituted for hempseed. Further testing (unpublished) has shown that not all watermolds will reproduce on this medium; thus, while it is not a

universally suitable medium for this purpose, it has wide application as a growth medium.

The production -- on demand, as it were -- of large numbers of planonts of some watermolds usually results when vigorous mycelium is grown in a nutrient solution, then transferred to water. A useful concoction for this purpose is Gleason's (1974) dilute salt solution (amounts in g L^{-1} distilled water): KH_2PO_4 (0.14), Na_2HPO_4 (0.07), $MgSO_4 \cdot 7H_20$ (0.12), and $CaCl_2 \cdot 2H_20$ (0.07). The mycelium is suspended in this solution for 24 hours. Mats of hyphae (of *Aphanomyces* species, for example), may also be propagated in some nutrient-rich medium, then washed and submerged in freshwater (J. E. Mitchell and Yang, 1966; R. T. Sherwood, 1958, among others), or washed repeatedly in a sterile salt solution (Grau, 1975) consisting of $CaCl_2 \cdot 2H_20$, $MgSO_4 \cdot 7H_20$, and KC1. Some degree of sporulation invariably follows. Prior to the foregoing reports, Goldie-Smith (1950) had in effect used the medium exchange technique for stimulating sporulation by first allowing hyphae from an agar plate culture to grow on a sterile hempseed half, then placing the infested seed in a dish of water. The water was changed several times during the first two days of incubation, but then left undisturbed until the fourth day. At that time, the culture water was changed, and in about $^1/_2$ hour sporulation commenced. Unestam (1966b) induced planonts in *Aphanomyces astaci* by agitating mycelial mats at 20 ^oC in redistilled water. He noted (Unestam, 1969e) that lowered oxygen tension enhanced sporulation in some media, while spore discharge was inhibited by minerals in the medium.

Inducing oospore production in watermolds is less certain by means of culture manipulation than is the promotion of asexual spores. Crooks (1937) tried several culture formulations in her attempts to induce the sexual apparatus in some non-fruiting isolates of *Achlya* and *Dictyuchus.* When colonies of some isolates grown in the presence of 1:50,000 auxin in broth cultures were transferred to sterile water they produced oogonia. These cells, however, quickly disintegrated.

The bulk of the work dealing with induced oospore production has been in connection with the phytopathogens in the genus *Aphanomyces.* Perhaps the most novel method of obtaining oospores of *A. euteiches* free of any surrounding mycelium was devised by Bhalla and Mitchell (1970). They simply grew mats of the fungus in broth, fed these to snails (*Helisoma* sp.) and then disintegrated the snail fecal pellets in distilled water to release and harvest the oospores. The zygotes survived passage but the mycelial portions of the mats did not. A less spectacular method for producing oospores that are essentially free of mycelial fragments, spore cysts, and the like, was devised by Rozek and Timberlake (1979). They pelleted a mycelial slurry of *Achlya ambisexualis* (sex cells developed after mating) and suspended the pellets in colloidal silica. This was followed by layering recovered oospores in a gradient series of the silica and then employing additional centrifugation and suspension.

Decoctions and homogenates of various natural plant parts are useful (Schneider and Yoder, 1973) for oogonium production by *Aphanomyces cochlioides.* High production of oogonia (and therefore of oospores) occurs in decoctions of oatmeal or sugar beet seedlings, or in homogenates of pearled barley, buckwheat groats, or oatmeal.

Furthermore, oospore production is markedly enhanced in this pathogen if the medium is simply replaced by a fresh supply after a period of incubation.

It is not always necessary to replace media to induce oogonium production by plant pathogenic watermolds; a few media have been devised just for this purpose. One such concoction is the V-8 juice agar formulated by Ayers and Lumsden (1975), and supplemented with cholesterol (1.5% solution in 95% ethanol) at 30 μg L^{-1}. The medium is prepared by centrifuging calcium carbonate (2.5 g) in a mix of 200 ml V-8 juice and 800 ml distilled water.

PRESERVATION OF CULTURES

There are two basic methods for preserving specimens of the Saprolegniaceae, namely, storing viable cultures under some condition that will enhance longevity, or preparing microscope slides of stained material. Each of the various ways of preservation has advantages and disadvantages, and a universally satisfactory method is wanting.

PREPARATION OF MICROSCOPIC MOUNTS

There are both relatively simple and complex methods for preparing microscope slides of specimens. One of the first to detail a procedure for slide preparation was Hartog (1889a). He fixed watermold hyphae in saturated mercuric chloride, stained the material with carmine, and then destained the preparation in acid alcohol followed by a very weak acidulated solution of nigrosin. Prior to mounting the specimens in Canada balsam, Hartog added drop by drop to the specimens in water a mix of equal parts of zinc sulfate and glycerin. He then immersed the specimen in absolute alcohol, and replaced that slowly with a solution of three parts xylol to one part phenol. This method is cumbersome, to be sure, but its most serious defect is that it distorts the oogonia, and characteristics of these are essential for identification purposes.

The first publication advocating use of osmic acid as a fixative for saprolegniaceous fungi seems to be that by Humphrey in 1890. He noted that the fumes alone were sufficiently potent to fix and harden motile cells and very small plants. Humphrey recommended glycerin jelly as a mounting medium, but admitted that it gave poor preservation of members of the family.

A killing-fixing treatment of 70% formalin-acetic-alcohol prior to placing the specimen in a 5% glycerin mounting medium is one method for preserving watermolds on glass slides (T. W. Johnson, 1956b). A stain such as very dilute eosin, basic fuchsin, or cotton blue may be added to the glycerin. A round cover slip is positioned over the specimen, and the whole preparation subsequently ringed with dilute Canada balsam or clear fingernail polish. Amann's medium (20 g phenol, 16 mL lactic acid, 31 mL glycerol, 20 mL distilled water), with or without a stain, is perhaps the best substitute for glycerin as the mounting medium (R. B. Stevens, 1974). Lactophenol has a serious disadvantage: it does not dry throughout the area under the coverslip so that the slides

must be stored flat. Hamid's (1942) method circumvents the disadvantage of Amann's medium, but still induces some distortion in the specimens. Mycelium is killed and fixed in a solution of acetic acid and formalin, and immersed in a 0.5% solution of aluminum sulfate for 2-6 hours. The specimens are washed, mounted in a glycerin solution of erythrosin, and then transferred to glycerin jelly.

Sealing coverslips to slides in the preparation of permanent mounts is troublesome because the slightest break in the seal leads to uneven desiccation of the mounting fluid. The simplest method -- applying balsam or nail polish -- is satisfactory only if it seals out air permanently. Waller's cement is a good substitute, but its preparation and application can be difficult. The cement (Dade and Waller, 1949) is prepared by melting beeswax and gum dammar separately in glass crucibles. The beeswax is then stirred slowly into the gum. Heat must be applied carefully and sparingly during this process, since high temperatures induce color changes in the wax. The wax-gum mix is stored in glass, paper, or cardboard containers. When the sealer is to be used, small bits are placed at several points along the edge of the coverslip, and with a heated bacteriological transfer loop, spread out smoothly to form a seal around the coverslip. Waller's cement is not hardened, dissolved, or discolored by lactophenol mounting medium. A useful modification to retard melting in Waller's cement in warm temperature situations was devised by Thirumalachar and Pavgi (1950). The surface of the cement, after it has been applied to the slide and coverslip, is coated with a dilute solution of Canada balsam. The slide is then placed on a warming table at 60 ^{o}C for $^{1}/_{2}$ hour. The beeswax of Waller's cement melts slightly at this temperature, and in doing so incorporates the balsam into itself. When subsequently cooled, the seal is hardened and translucent.

Although devised initially to make permanent mounts of marine Ascomycetes, the double cover glass method (Fig. 1) of the Kohlmeyers (1972) can be used for preserving specimens of watermolds and some other zoosporic fungi as well. The most satisfactory preparations made by their method are those in which specimens are positioned directly into the mounting medium so that it is unnecessary to blot out water or to change solutions. A 25 mm ^{2} No. 1 coverslip is anchored temporarily (by surface tension) to a clean glass slide by applying a drop of water at two of the edges. A drop of lactophenol (with or without a stain) is put on this coverslip, and the specimen added. An 18 mm^{2} No. 1 coverslip is then dropped into place over the specimen in its mounting fluid. This preparation is allowed to "dry" without disturbance for a few days. During this time, if there is excess lactophenol at the edge of the second coverslip it will evaporate but leave an oily film on the glass surface, and this film must be removed. The best seal is obtained if the quantity of mounting medium is carefully controlled so that no excess drains out around the coverslip. The smaller coverslip is sealed to the larger one with clear fingernail polish (or Waller's cement) and allowed to dry for an additional day. The large coverglass is then pried free of the slide where it has been temporarily held. A drop of Canada balsam or "Caedax" (synthetic balsam; Merck and Co.) is placed on the surface of the small coverslip, and the whole intact preparation of two coverslips is inverted quickly and lowered onto the surface of a

clean microscope slide. The Caedax or balsam spreads out to surround the edge of the small cover glass, thus permanently covering the dried ring of fingernail polish or Waller's cement and coincidentally sealing the two coverslips to the slide. Glass slides for permanent mounts, irrespective of the actual method of preparation, should be of the frosted end type. Label notations can be written directly on the surface in pencil or ink, and sealed, without danger of smudging, with modeler's varnish or lacquer ring of fingernail polish or Waller's cement and coincidentally sealing the two coverslips to the slide.

Even the best mounting and the most permanent sealing method is wasted if the preserved specimens are so badly distorted as to obliterate details. In this connection, it must be admitted that some degree of distortion is inevitable, and no killing-fixing agent or mounting medium is yet known which will preserve in place the lipid deposit in the oospores of the Saprolegniaceae. Reasoning from the fact that osmic acid has long been used as a killing-hardening agent for planonts (Humphrey, 1890; Cotner, 1930a), Armbruster and Langsam (communication) discovered that the initial stages in the standard method for preparing specimens for transmission electron microscopy are useful for preserving watermolds. Whole colonies of watermolds on hempseed are submerged in 1.5% glutaraldehyde in 0.025 M phosphate buffer for five minutes, then transferred to an identical solution and held for 55 minutes. The colony is washed (by submersion) in the phosphate buffer for one hour, then transferred to 1% OsO_4 in 0.025 M phosphate buffer and left for one hour. After this time, the colony is washed, and stored in distilled water in a small snap-cap vial until needed. This technique preserves especially well the antheridial branches and hyphae, although the specimens are blackened by the treatment. Whole-colony wet mounts may be made of the material, or portions of the mycelium can be stored permanently on Kohlmeyer slides.

CULTURE STORAGE

In his culture manipulation work with saprolegniaceous fungi Coker (1923) experimented briefly with methods to maintain viable cultures. For example, he put colonies in aquarium jars with algae or grew cultures on insect larvae in vials, then tested the specimens months or years later for viability. Coker also kept mycelium on cornmeal agar; such cultures seemed generally to have survived up to several months. These methods of maintaining viable specimens are chancy at best, and more effectual techniques have been devised.

Among the first to adapt a modification of the bacteriologist's liquid paraffin technique to fungal culture storage was Sherf (1943). He successfully maintained several phytopathogens for long periods as did Norris (1944). Buell and Weston (1947) applied a modified Sherf's methodology to their fungus cultures. Their technique consists of filling a sterile separatory funnel with autoclaved or hot air-sterilized (150 ^{o}C, 1 $^{1}/_{2}$ hours), high-purity mineral oil. This oil is then decanted over a slant culture to a depth about one cm beyond the uppermost level of the medium, and the culture stored upright. Although transferring mycelium directly from mineral oil

culture to water (and hempseed) is possible, the residual oil sometimes interferes with microscopy. This problem can be avoided by seeding a plug of agar (with hyphae) from the mineral oil slant onto a plated medium. After new hyphae appear (two days or so), plugs are cut from the edge of the colony, transferred to water, and the culture baited with hempseed. Mineral oil cultures are effectively mite-proof, and this important factor may well outweigh any disadvantages of the method.

The most extensive study of mineral oil storage of members of the Saprolegniaceae was that by Reischer (1949a). She stored 71 isolates (seven genera) under oil at 4-10 °C. Some fungi were not viable after 6-11 months, but she nevertheless thought the method could be expected to sustain viability over a period of 10-30, months. Lyophilized culture storage employed so successfully by K. B. Raper and Alexander (1945), Fennell (1960), and Fennell, *et al.* (1950), Reischer found, was not suitable for members of the Saprolegniaceae. Encysted spores of *Achlya* spp. disintegrated under lyophilization (Reischer, 1949a), and oospores did not preserve well (of 12 isolates of *Achlya* with abundant oospores, none was viable three months after being lyophilized). Fennell and her associates (1950) had earlier stated that strictly mycelial cultures or those with few spores or conidia were best maintained under mineral oil. Indeed, this seems to be the method of choice where it has been applied to storage of watermolds and other zoosporic fungi and phytopathogens (Goldie-Smith, 1956; Wernham, 1946; Krizková and Balan, 1975). Over a long period of time, however, even mineral oil preservation may fail (Iyengar, *et al.*, 1959) to maintain viable specimens.

Water also has been used as a storage medium for cultures of the Saprolegniaceae. Axenic colonies on hempseed have been kept viable for eight months or longer (for some species) in one-quart Mason jars approximately two-thirds full of sterile, distilled water, and stored at 8 °C (T. W. Johnson, 1956b). An improvement on this method is to use Emerson's (1958) mix of pond and distilled water. Dick (1965) maintained viable cultures in water in Erlenmeyer flasks. He included in each storage flask a single, intact hempseed (autoclaved in water), and inoculated the container with hyphae growing on agar blocks cut from plate cultures. The flasks were cotton-stoppered and the closure covered with paper. Dick reported the isolates were viable in water storage up to 15 months at 20 °C, and up to 24 months at 5 °C, but recommended that specimens of *Geolegnia* be transferred often.

The water culture viability study of longest duration was that by G. Clark and Dick (1974): a test of 355 cultures of Oomycetes that had been stored 1-8 years. At the time viability was tested, 251 fungi were alive (110 were 1-2 years old; 127 were 5-6 years old). Krizková and Balan (1975) did not find water storage of saprolegniaceous fungi to be a suitable method, even when isolates were kept at 4° or 10 °C. Their hempseed cultures were maintained in vials, and thus the volume of storage water was not comparable to that used successfully by Dick (1965), or T. W. Johnson (1956b). Although she noted that oak leaf decoction was harmful to some extent to watermolds, Goldie-Smith (1956) recommended cool temperature storage of specimens in water with a very weak leaf extract. Boesewinkel (1976) did not use watermolds in his study on

long term storage, but did report that some other Oomycetes were viable up to seven years submerged (growing on agar blocks) in tightly capped bottles half full of sterile water and held at room temperature. His method can be adapted readily to the watermolds.

Low temperature storage of slant cultures is a standard practice for maintaining watermolds, but, of course, such cultures must be transferred regularly and rather frequently. Kramer and Mix (1957) stored agar cultures in a deep-freeze box. Of the two watermolds they used, *Saprolegnia* sp. survived for five years at -18 ^{o} C, but *Aphanomyces* sp. was dead when tested for viability at 12 months storage. Using several species and strains of Saprolegniaceae as test organisms, Hwang (1966) suspended spores or fragmented mycelium in a 10% (v/v) glycerol-water mix, sealed them in ampoules, and cooled the specimens to -165 to -196 ^{o}C in liquid nitrogen. *Achlya bisexualis* (both mating strains), one strain of *A. ambisexualis, A. americana, A. flagellata, Aphanomyces euteiches, Aphanomyces raphani,* and *Thraustotheca clavata* were viable when tested at 37-38 months. Belyakova *et al.* (1979), on the contrary, found that the three species of watermolds they tested did not remain viable in liquid nitrogen (without lyophilization). Nevertheless, ultra-low temperature storage may have promise as a method for maintaining living watermolds; pertinent papers by Hwang (1960, 1968) and Hwang and Howells (1968) should be consulted.

Other culture storage techniques have been tried with nonsaprolegniaceous fungi; whether they can also be applied to stock cultures of watermolds remains to be tested. Two methods show promise: storage in soil (Bakerspigel, 1953, 1954; but *see* Remy, 1950, and Apinis, 1964), and over silica gel (R. F. Elliott, 1975). Excellent reviews of the spectrum of fungal culture storage are to be found in the papers by Fennell (1960), and Onions (1971).

SPECIAL PURPOSE TECHNIQUES

One of the first techniques applied to watermolds for a very particular purpose was that of osmic acid fixation of spores. With this method, Humphrey (1891) detected flagella on the emerging primary spores of *Achlya (polyandra?).* Since Humphrey's time, investigators have devised useful -- and sometimes unique -- methods by which to manipulate watermolds and thus accomplish specific purposes.

Work with the Saprolegniaceae ultimately brings the mycologist into contact with parasitic species, and with specimens that are themselves parasitized. Whiffen (1938) demonstrated the ease with which the alga-inhabiting *Aphanomyces phycophilus* can be propagated in artificial culture. She seeded invaded filaments of *Spirogyra* sp. directly onto 27% water agar. When hyphae had grown out onto the agar some were transferred to nutrient media. Mullins and Barksdale (1965) successfully separated mixtures of spores of *Achlya* species from those of the parasite *Dictymorpha dioica* Mullins by taking advantage of differences in spore sizes. They filtered spore suspensions through sintered glass with a pore diameter that would pass the spores of *D. dioica,* but retain those of the host species.

Rather than separate two-member cultures, it is equally possible to propagate them together in bacterial-free form as Slifkin (1964) demonstrated. She washed a colony of a watermold parasitized by *Olpidiopsis incrassata* Cornu, and submerged it in a dish of sterile lake water. A 24-hour-old hempseed colony of the uninfected host was added, and the culture incubated for 1-2 days. At this time, another 24-hour-old axenic hempseed culture of the host was put in sterile lake water, and the now parasitized colony from the first dish of lake water placed in with it. Following an incubation of about four hours, this exposed hempseed colony (now invaded by the *Olpidiopsis*) was blotted on sterile agar, to remove excess water, and planted on 2% water agar. After 24 hours, infected hyphae had grown out onto the agar, away from bacterial contamination, and the two-member mycelium could be transferred to hempseed in water. The infected colonies could be "passed through" water agar repeatedly in the event they were not bacteria-free from the first transfer.

Oospores have proven to be important sources of inoculum (Chapters 27, 28) for plant pathogenic species and have other uses as well. A method to increase the production of these cells thus is essential. Schneider and Yoder (1973) found that certain natural product media favored oogonium and oospore development in specimens of *Aphanomyces,* and that the level of production was increased simply by replacing the medium in which colonies had been growing with fresh substratum of the same constituency. Some of the conditions for synchronous oospore production in *Achlya ambisexualis* have been established by Rozek and Timberlake (1979). To obtain matings in this species they mixed encysted primary spores of the male (E87) and female (734) strains in various proportions and the resulting mycelium then was examined for the products of sexual reproduction. The most rapid production of presumed normal oogonia (with oospores) by the female strain (734) of *A. ambisexualis* takes place at incubation temperatures of 18-22 ^{o}C. Moreover, the number of oogonia developed is directly related to the proportion of encysted spores -- from the female thallus -- used in the initial mixed inoculum. The ratio of seven spores from the female plant to three from the antheridial (male) thallus is evidently optimal for the formation of oogonia with oospores in this species.

For investigating the effects of medium-incorporated chemicals on mycelial growth, D. A. Smith's (1976) half-plate method is useful. A nutrient medium containing the test chemical is poured into a small, sterile Petri plate. When the agar has solidified, one half of it (a semicircular segment) is removed. Fresh medium without the test chemical is then poured into the vacant semicircle, and when the substratum is solidified the plane of the agar surface is reestablished. The plate is inoculated with mycelium on a plug of agar positioned over the line of juncture (test chemical on one side, control medium on the other). Interpretation of the results from use of this half-plate method must take into account diffusion rates of the chemical.

Ordinarily, glass rings or depression slides are adequate for microscopy and the viewing of living material over periods of time. Pavgi and Singh (1971), working with chytrids, simply raised a coverslip off the slide surface with a few small beads of molten wax, and viewed the specimens in water in the intervening space. In the absence of

depression slides or glass rings, their method is a suitable substitute for hanging drop preparations.

In situations where maintaining fresh water around specimens in a slide preparation is desirable, the continuous flow system devised by Hoch and Mitchell (1972c) has not been improved upon. A coverslip is raised off the slide surface by means of glass rods, and with a capillary pipette fresh liquid medium fed into one side of the chamber. Using a vacuum line attached to an opposing capillary, the medium is simultaneously withdrawn. Thus, the Hoch and Mitchell device achieves a method of washing the mycelium and increasing its longevity. A mammalian tissue culture chamber has been adapted (Salkin and Robertson, 1970) for use in medium perfusion studies. Although this simple, continuous culture system has not been employed for propagation of watermolds specifically, it seems suitable for this purpose.

Three techniques illustrate a diversity of methodology applicable to studies of spores of watermolds. Schoulties and Yang (1971) obtained reliable spore counts of *Aphanomyces euteiches* by using a Coulter electronic particle counter. A suspension of spores was placed in an electrolyte solution, and passed through a minute aperture conducting a current between two platinum electrodes. The momentary increase in resistance in the electrolyte as the spores passed the aperture was recorded. While it is usually not difficult to induce sporulation in watermolds, the planonts or encysted spores from a single colony are not necessarily always of the same age. Temperature shock, Holloway and Heath found (1977a), induced synchrony into populations of spores of *Saprolegnia* species. The method is as follows. A vegetative colony is transferred to a dilute salts medium (Holloway and Heath, 1974). Discharged primary spores and cysts are collected from the mycelium on this medium, and incubated at 3 ^{o}C for eight hours in a small amount of the solution of salts. These spores and cysts are then exposed suddenly to 24 ^{o}C, and within 1 $^{3}/_{4}$ hours most of the spores become motile. Neish (1976), working with saprolegniaceous fish pathogens determined levels of planont abundance in experimental fish tanks. He submerged Petri plates of YpSs agar horizontally for 6 hours in the tanks. By simply examining the agar surface (microscopically) at the end of this time, he could locate and count settled spores.

Watermolds have, of course, been found in saline waters (Padgett, 1978a; TeStrake, 1958, 1959, for example), but refined ecological study of these fungi has been hindered because of inadequate means of experimentation and observation. To some extent, this problem has been resolved by the reservoir-pumping system developed by Padgett and Lundeen (1977). With reservoirs of water of different salinities, and flow controlled by electro-hosecocks and interval timers, these investigators have simulated estuarine tidal cycles in a culture chamber. The mechanism is adaptable to cyclic input of two kinds of liquid media as well, and thus the apparatus has more general application than that of simply providing salinity cycles.

Various techniques have been devised for photographing the colonies of watermolds (and other fungi) without disturbing their morphology. Barnes (1933) cut an elliptical hole in a Petri dish cover, fastened a coverslip to border strips cemented on the inner surface of the cover, and then substituted this “window” cover for an ordinary

one when colonies on plates were to be observed. While the method devised by Cole *et al.* (1969) was applied to Hyphomycetes, it has a very restricted application to observation of watermolds. A channel is cut out of a plate of nutrient agar supporting colony growth, and a coverslip laid over the channel. Hyphae growing out into the space can then be examined or photographed undisturbed.

The most elaborate "window" system for examining submerged cultures (including Saprolegniaceae) is that reported by J. Fletcher, in 1976. A rectangular hole 35 x 20 mm is cut in the bottom of a plastic culture dish (Fig. 2), and the edge raised with a hot knife. A 40 x 22 mm No. 0 coverslip is then cemented (with a solution of polystyrene in chloroform) over this window. The dish bottom with its window is then covered with the plastic lid, and the intact plate is exposed to formalin vapor in a closed desiccator for 24 hours. The plate is then removed and the formalin allowed to dissipate. After this modified Petri dish has been vapor-sterilized, a thin layer of agar is poured into the dish bottom, and, when cool, inoculated with a mycelial plug at one of the short sides of the rectangular window (Fig. 2). The seeded dish is then incubated, and when growth appears is inverted on the microscope stage and the fungus examined or photographed. Fletcher found no contamination of plates even after the cultures had been used in observation for 5 days. The advantage of Fletcher's method over that of Cole and his associates in reducing desiccation is at once apparent.

For certain experimental approaches to the study of watermolds, very particular techniques are indispensable. Two examples follow. In 1975, E. A. Ellis and Mullins published details of a method that very satisfactorily prepared watermold hyphae for freeze-etching. In a drop-wise fashion, over an 8-10 hour time span, they gradually infiltrated (augmented by shaking) the hyphae of the specimen with the cryoprotectant prior to freezing the preparation with Freon 22. This slow infiltration process prevented plasmolysis when the filaments are immersed directly into the cryoprotectant. Taylor and Cameron's (1973) review of preparative techniques for the quantitative analysis of fungal cell wall constituents is particularly useful as a guide for studies of this nature.

Viable cultures are ordinarily shipped with the least chance of loss as colonies on slants in screwcap tubes. A cheap, less bulky substitute was suggested by Desai and his associates (1971). They used hospital-tissue, distilled water ampoules (70 x 14 mm) as culture "tubes", putting in 2.5 mL of a nutrient agar, and sealing the open end with cotton before sterilizing. Small screw cap or snap cap vials are also quite suitable as agar culture vessels for shipping. Living specimens, on hempseed, need only be kept moist during transit. A colony is placed on a small piece of sterile, wet filter paper lying in a film of water in a Petri plate. The paper is folded to form a small packet, and this container enclosing the colony is then wrapped lightly in wax paper, parafilm, aluminum foil, or plastic bag, and placed in a vial or other container for shipment.