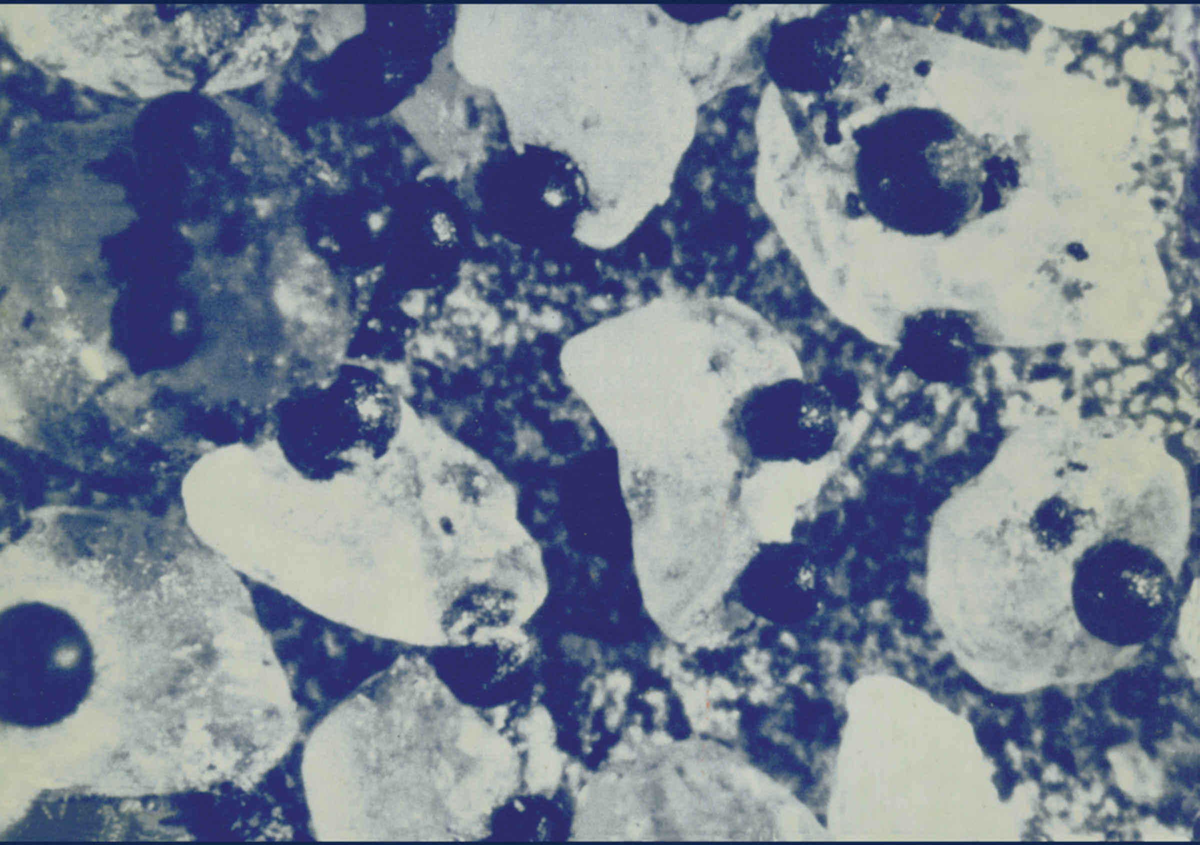
MARINE MYCOLOGY

THE HIGHER FUNGI



Jan Kohlmeyer/Erika Kohlmeyer

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ACADEMIC PRESS New York San Francisco London 1979
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ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1 7DX

Library of Congress Cataloging in Publication Data

Kohlmeyer, Jan.
Marine mycology.

Includes bibliographical references and indexes.
1. Marine fungi. I. Kohlmeyer, Erika, joint author.
II. Title.
QK618.K59 589'.2'09162 79–14703
ISBN 0–12–418350–6

PRINTED IN THE UNITED STATES OF AMERICA

79 80 81 82 9 8 7 6 5 4 3 2 1

To Willy Höhnk
and
to the memory of Frederick K. Sparrow, Jr.
in recognition of their pioneering work
in marine mycology

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Preface

Growing general interest in marine and estuarine habitats in recent years has also led to an increase in studies on marine fungi which were often overlooked as participants in ecological processes. Because of this rapid growth of knowledge in marine mycology, the first comprehensive treatise on marine fungi by Johnson and Sparrow (1961) is partly out-of-date. G. C. Hughes (1975), in a very informative review, has summarized papers published since 1961. However, extensive descriptions and complete keys are not available for all marine fungi. The "Icones Fungorum Maris" and "Synoptic Plates of Higher Marine Fungi" (Kohlmeyer and Kohlmeyer, 1964–1969, 1971b) include diagnoses for only 90 species and keys for the identification of all taxa described up to January 1971, respectively. An updated treatise was clearly needed.

This book deals with the higher marine fungi, i.e., Ascomycotina, Basidiomycotina, and Deuteromycotina. This book combines features of a monograph with those of a text. It includes sections on ecological groups of fungi and other topics, such as phylogeny, ontogeny, physiology, and vertical and geographical distribution, providing information on known facts and open questions. The taxonomic–descriptive part contains complete descriptions of each genus and species, together with substrates, range, etymology of generic and specific names, and literature. There are keys for all species within a given genus, and a general illustrated key leads to the individual species. The taxonomic section is based on our examinations of almost all of the filamentous marine fungi, and unpublished data on new hosts and geographical distributions are included for many species. The filamentous higher marine fungi are represented by 149 Ascomycetes, 4 Basidiomycetes, and 56 Deuteromycetes. The majority, namely 191(91%) of the filamentous fungi, are obligately marine species, whereas the remainder are facultatively marine. One new species and seven new combinations are proposed (see Table I). Excluded

TABLE I
New Species and Combinations Proposed

Ascomycotina
1. *Didymella gloiopeltidis* (Miyabe et Tokida) Kohlm. et Kohlm. (≡*Guignardia gloiopeltidis*)
2. *Haligena amicta* (Kohlmeyer) Kohlm. et Kohlm. (≡*Sphaerulina amicta*)
3. *Halosphaeria galerita* (Tubaki) Schmidt ex Kohlm. et Kohlm. (≡*Remispora galerita*)
4. *Leiophloea pelvetiae* (Sutherland) Kohlm. et Kohlm. (≡*Dothidella pelvetiae*)
5. *Massarina cystophorae* (Cribb et Cribb) Kohlm. et Kohlm. (≡*Otthiella cystophorae*)
6. *Pontogeneia codiicola* (Dawson) Kohlm. et Kohlm. (≡*Sphaerulina codiicola*)
7. *Turgidosculum complicatulum* (Nylander) Kohlm. et Kohlm. (≡*Leptogiopsis complicatula*)

Deuteromycotina
1. *Camarosporium palliatum* Kohlm. et Kohlm. sp. nov.

are 49 *nomina confusa, dubia,* or *nuda.* The yeasts are treated in a separate chapter and comprise 177 species or varieties. Authorities on fungal names and hosts are given in the Organism Index. We have considered all literature except for unpublished reports or theses.

Jan Kohlmeyer
Erika Kohlmeyer

Acknowledgments

The investigations have been supported in part by grants GB 27-265, EMS 74-18539, and DEB 74-18539 of the U.S. National Science Foundation and by the Brown–Hazen Fund of the Research Corporation. We are most grateful for this support which made possible the revisions presented in this treatise. We acknowledge gratefully loan of type and other collections by curators of herbaria* ABD, AHFH, B, BM ex K, BPI, C, H, HBG, L, LD, LE, LPS, MA, MPU, PAD, PC, UC, and the University of Sheffield. The following colleagues and friends kindly collected for us or made their own collections of marine fungi from many parts of the world available: J. Acosta, A. A. Aleem, R. A. Barkley, R. D. Brooks, A. B. Cribb, O. Eriksson, G. Feldmann, M. H. Hommersand, R. G. Hooper, D. E. and C. M. Hoss, P. W. Kirk, Jr., L. Malacalza, R. T. Moore, A. Munk, M. J. Parsons, C. Pujals, M. Salmon, I. Schmidt, F. J. Schwartz, R. B. Setzer, G. R. South, A. J. Southward, S. Udagawa, I. M. Wilson, E. M. Wollaston, and H. Yabu. We express our gratitude to these people who gave invaluable information on taxonomic and other matters: J. A. von Arx, R. K. Benjamin, G. H. Boerema, I. M. Brodo, B. Bruce, G. S. Daniels, T. Eckardt, W. E. Fahy, M. L. Farr, J. Gerloff, W. Haupt, M. H. Hommersand, B. Kaussmann, R. P. Korf, I. M. Lamb, N. D. Latham, E. Müller, G. F. Papenfuss, J. Poelt, B. Sahlmann, D. E. Shaw, J. Walker, I. M. Wilson, and J. S. ZoBell. Thanks are due the staffs of the Laboratoire de Biologie Marine, Collège de France at Concarneau (France) and of the Biologische Anstalt Helgoland (Federal Republic of Germany); to I. Gamundi de Amos and staff of the Instituto de Botanica Spegazzini of the Universidad Nacional de la Plata (Argentina); and Instituto Argentino de Oceanografia, Bahia Blanca (Argentina) for kind assistance during our stay at their institutions. Grateful acknowledgment is made to the follow-

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* Abbreviations follow Holmgren and Keuken (1974).

ing colleagues for permission to reproduce illustrations: G. E. Cole (Fig. 34), J. W. Fell (Fig. 129), R. V. Gessner (Fig. 22), and L. Vitellaro Zuccarello (Fig. 31). Stearn's (1966) "Botanical Latin" was invaluable in clarifying the etymologies of many generic and specific names. T. L. Herbert and B. J. Lamore ably assisted us in the laboratory, B. B. Bright and J. W. Lewis provided us with quick library service and administrative assistance, respectively, and J. B. Garner carefully typed the manuscript. We are greatly indebted especially to L. S. Olive, R. V. Gessner, and J. W. Fell for reading parts of the manuscript and making helpful suggestions. Many thanks go also to T. L. Herbert for collecting in Trinidad, for proof-reading and indexing; to B. J. Lamore for help in the preparation of indexes; to P. E. Boyd for typing; and to V. A. Zullo for giving advice on authorities of host species.

1. Introduction

One single publication has probably influenced the development of marine mycology more than any other paper, namely, "Marine Fungi: Their Taxonomy and Biology" by Barghoorn and Linder (1944). These authors demonstrated that there was an indigenous marine mycota, showing growth and reproduction on submerged wood after defined periods of time. The existence of true marine saprobic fungi was often questioned, for instance, by Bauch (1936), who wrote: "Saprobic Ascomycetes which play an important role in forest and soil in the deterioration of organic material, especially of wood, appear to be completely absent in seawater" (translation from the German). Investigations by Barghoorn and Linder and later authors have proven without a doubt that fungi, including Ascomycetes, do contribute to the decomposition of organic substrates in the oceans, although the extent of biodeterioration caused by fungi in the sea is still not fully understood.

The first facultative marine fungus, *Phaeosphaeria typharum,* was described by Desmazières (1849) as *Sphaeria scirpicola* var. *typharum* from *Typha* in freshwater. Durieu and Montagne (1869) discovered the first obligate marine fungi on the rhizomes of the sea grass, *Posidonia oceanica,* and marveled at the most remarkable life-style of *Sphaeria posidoniae* (=*Halotthia posidoniae*), which spends all parts of its cycle at the bottom of the sea. Figure 1 summarizes the numbers of species of higher marine fungi described per decade since 1840. If numbers of new descriptions can be used to indicate the activity dealing with a particular group of organisms at a certain time, this figure shows that up to the period of 1930–1939 there was only sporadic interest in marine fungi. Descriptions of marine species during these first hundred years were supplied mostly by authors working on a wide variety of fungi with no particular interest in the marine habitat. Exceptions may be the Crouan brothers (Kohlmeyer, 1974a), who described five marine fungi in 1867 in

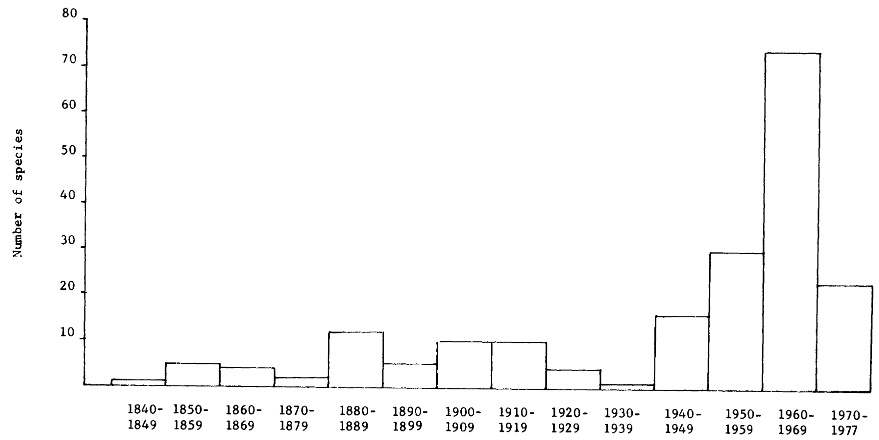

Fig. 1. Numbers of accepted filamentous higher marine fungi described per decade since 1840. Research in marine mycology was stimulated by a paper of Barghoorn and Linder in 1944. The numbers of newly described species show a decreasing trend since 1970.

their *Florule du Finistère* and prepared eleven others for publication (remaining unpublished), and particularly Sutherland (1915a–c, 1916a,b), who published a series of papers dealing exclusively with marine fungi. Almost three-quarters of all recognized higher marine fungi have been described in the last four decades (Fig. 1). This sudden increase in new descriptions after 1940 can be attributed mostly to the publication of Barghoorn and Linder (1944), which stimulated worldwide research in marine mycology. A proliferation of literature on the higher marine fungi occurred, as I. M. Wilson (Great Britain) in 1951, W. Höhnk (Germany) in 1952, S. P. Meyers (United States) in 1953, A. B. Cribb and J. W. Herbert (Australia) in 1954, T. W. Johnson (United States) in 1956, G. Feldmann (France) in 1957, J. Kohlmeyer (Germany) in 1958, and G. Doguet (France) in 1962 and E. B. G. Jones (Great Britain) also in 1962 published their first papers in this field. The first monograph on marine mycology by Johnson and Sparrow appeared in 1961.

I. DEFINITION OF MARINE FUNGI

Past definitions of marine fungi have often been based on their ability to grow at certain seawater concentrations (Johnson and Sparrow, 1961;

Tubaki, 1969). It has been shown that marine fungi cannot be defined on a strictly physiological basis (see discussions in Kohlmeyer, 1974a; G. C. Hughes, 1975), and we use a broad, ecological definition, namely: *Obligate marine fungi* are those that grow and sporulate exclusively in a marine or estuarine habitat; *facultative marine fungi* are those from freshwater or terrestrial milieus able to grow (and possibly also to sporulate) in the marine environment (Kohlmeyer, 1974a).

Marine or estuarine habitats are the oceans and ocean-associated smaller bodies of water that contain salt or brackish water, including river mouths, sounds, lagoons, tidal creeks, salinas, étangs, and the like. Fungi from inland salt lakes are not well known, but the mycota of these habitats appears to be identical with that of the oceans (Anastasiou, 1961, 1963b; Davidson, 1974b).

Some authors feel that attempts to define marine fungi are premature (Schaumann, 1975b), but, in our opinion, such efforts are necessary to understand the role of fungi in a particular environment such as the oceans. Fungi are often obtained by culture methods, for example, plating techniques or incubation, from marine substrates. Most investigators using such methods have failed to demonstrate conclusively that the species isolated in the laboratory can actually grow (and, possibly, reproduce) in the marine habitat. The mere isolation of a species from marine samples does not prove that this fungus is active in the marine environment. Such species are possibly dormant in the form of spores or hyphal fractions until the surrounding conditions become favorable for germination and growth. Research by Tyndall and Kirk (1973) and preliminary experiments carried out by Dr. R. V. Gessner and ourselves indicate that a mycostatic factor in fresh seawater inhibits germination of spores of terrestrial fungi but does not affect certain marine fungi. Mycostasis is a well-known phenomenon in terrestrial soils and on leaf surfaces (Lockwood, 1977). The unidentified mycostatic principle in raw seawater is destroyed by heat sterilization. Conidia of terrestrial fungi, for example, *Penicillium* sp., germinate in sterilized or aged seawater or in distilled water, but they do not germinate in the natural habitat. Therefore, fungi isolated from seawater by means of culture methods cannot be considered to be marine species unless their growth in the marine environment is demonstrated. A valid criterion for the definition of a marine fungus might be its ability to germinate and to form a mycelium under natural marine conditions. These conditions may vary, depending on the species. Some fungi might require permanent submergence, whereas others, such as species occurring in beach sands, may need a greater supply of oxygen for germination.

II. NUMBERS OF MARINE FUNGI

About 50,000 fungal species are known from terrestrial habitats (Ainsworth, 1968), but, in contrast, less than 500 species have been described from oceans and estuaries, which cover a much larger area, namely, three-quarters of the world. The higher filamentous marine fungi include 209 species, the marine-occurring yeasts comprise 177 species, and the lower marine fungi comprise probably less than 100 species. The decrease of new descriptions of marine fungi during the period 1970–1977 (Fig. 1) indicates that the most common species have been named and that considerable additions of new species in the future are unlikely. Undescribed fungi can be expected in some less explored habitats, for example, in temperate salt marshes, in mangrove forests, particularly of the Pacific and Indian oceans, and in the deep sea.

The oceans, compared to the land masses, provide stable environments with small changes in temperatures and salinities. Organic substrates, such as algae, marsh plants, and plant litter, are concentrated mostly along the shores, where they provide nutrients for fungi. The open ocean is a fungal desert where only yeasts or lower fungi may be found attached to planktonic organisms or pelagic animals. The existence of such organisms in a free-living state has not been demonstrated convincingly, in our opinion. In contrast to terrestrial habitats, the incubatorlike stable environment of the oceans and the comparable small number of hosts and substrates in the marine environment have probably not exerted enough selective pressure during the course of evolution to induce the formation of a high number of different types of fungi. Ainsworth (1968) estimates the number of named and undescribed terrestrial fungi to be 250,000 or more. We expect the number of marine fungi to be about 1% of the terrestrial species, namely, the same ratio as is found between the presently known marine and terrestrial fungi.

III. SIZES OF MARINE FUNGI

Most of the fungi found in marine habitats are microscopic. The largest ascocarps occur in *Amylocarpus encephaloides,* which do not exceed 3 mm, and the Basidiomycetes *Digitatispora marina* and *Nia vibrissa* have fruiting bodies 4 mm in length and 3 mm in diameter, respectively. Obviously, the marine environment does not permit the development of large, fleshy fruiting bodies, because abrasion by waves and grains of sand impedes formation of such structures. Macromycetes growing in the leaf litter of forests need an extended nutrient-supplying mycelium and an undisturbed habitat. Similarly, soft fruiting bodies of large marine species (*A. encephaloides, Eiona tunicata, D. marina, N. vibrissa,* and *Halocyphina villosa*) develop mostly in sheltered habitats, namely, on firmly anchored wood at or above the high-tide line or protected in cracks of the wood or under bark. The deep sea appears to be another environment where large fruiting bodies could develop because water currents are weak, and, indeed, the Ascomycete *Oceanitis scuticella*, occurring at a depth of about 4000 m, has fleshy ascocarps up to 2 mm in height.

IV. THE MODE OF LIFE AND DISTRIBUTION OF MARINE FUNGI

The higher marine fungi occur as parasites on plants and animals, as symbionts in lichenoid associations with algae and as saprobes on dead organic material of plant or animal origin. Examples of the occurrence of marine fungi in different substrates and habitats are given in the following chapters. These organisms are found in all marine environments from the high-water line down to the deep sea. Their distribution is mainly limited by dissolved oxygen and temperature of the water, less so by salinities. Besides ubiquitous species, there are fungi restricted to temperate waters and others restricted to tropical or subtropical waters.

V. UNSOLVED MAJOR PROBLEMS

Among the topics to be solved in marine mycology, we consider the following three to be of foremost importance.

A. Quantification

So far we have no means to measure the biomass, numbers or activity of marine fungi in a particular habitat. Microscopic observation of decaying marsh plants or marine wood indicates that fungi play a major role in the decomposition of these plants and wood, but fungal hyphae and substrate cannot be separated.

B. Definition of Marine Fungi

To obtain a better understanding of the role of fungi in the marine environment, we need to separate the indigenous marine species (obligate and facultative) from the "contaminants." The latter are terrestrial or freshwater species [sometimes called "transients" (Park, 1972b)] that are dormant in marine habitats. A way of separating indigenous from nonindigenous species appears to be a test of their germination ability in the natural environment, as discussed above.

C. Fossil Records

In the absence of fossil higher marine fungi, discussions on the phylogeny of this group are more or less speculative. A search for parasitic fungi on fossil marine algae, for instance, on calcified Rhodophyta, might supply data on the origin of marine fungi.

2. Methods

Techniques used to collect, preserve, and culture higher marine fungi do not differ greatly from those applied in research on fungi from other habitats, and recent handbooks (Norris *et al.*, 1971; Hawksworth, 1974; Stevens, 1974) should be consulted for general information. We explain only those methods that are particularly relevant to research on the filamentous higher marine fungi. Detailed descriptions of methods used in marine mycology have been published by E. B. G. Jones (1971b) and Höhnk (1972), whereas G. C. Hughes (1975) reviewed the literature appearing between 1961 and 1974.

I. COLLECTING TECHNIQUES

As discussed in Chapter 1, collecting techniques in marine mycology are crucial to determine if a fungus can be considered marine, that is, an active inhabitant of marine habitats. Therefore, the collecting methods based on direct observations are always preferred over the incubation (or "indirect") techniques.

A. "Direct" Examination Methods

Material collected according to the methods described in the following paragraphs is examined under the dissecting microscope for the presence of ascocarps, basidiocarps, pycnidia, or conidia. Fruiting bodies are transferred with a needle to a microscope slide, torn apart in a drop of water to expose the spores, and carefully squeezed under a cover glass. For the identification of an unknown species, the observation of ascospores, basidiospores, or conidia usually suffices. In some cases it is necessary to search for asci and sterile elements of the ascocarps, such as paraphyses and pseudoparaphyses. Additional information leading to the identification of higher marine fungi is given in the introduction to the key (Chapter 23).

1. Wood and Other Cellulosic Matter

Fungi found growing on cellulosic materials in marine habitats belong to the Ascomycetes, Basidiomycetes, and Deuteromycetes. Substrates include breakwaters, pilings, and other shoreline fortifications, driftwood, ropes, and mangrove roots, all of which are submerged in, or wetted by, seawater for some part of the day. Specimens are collected by cutting or sawing off pieces of the substrate and placing them in sterile plastic bags.

In the laboratory, the material is either examined at once under a dissecting microscope or stored in a refrigerator at about 5°C if the investigation can be made during the following days. If a longer storage period is necessary, the specimens should be frozen at about −18°C.

a. Driftwood. Some pieces of driftwood, ropes, or other matter washed ashore may not yet contain any marine fungi because they have been in seawater for only a short period. Terrestrial fungi growing on such material may then be mistakenly considered as marine. Therefore, care should be taken to collect only specimens that have been in the marine habitat for several weeks, as indicated by the attack of marine fouling or boring organisms, such as barnacles, algae, or shipworms. Wood wedged between jetties or natural rocks is often inhabited by marine fungi. G. C. Hughes (1968) distinguishes between "driftwood" *sensu stricto,* namely, wood that is actually drifting or capable of drifting, and "intertidal wood," that is, wood that has become wedged between rocks or partially buried in sand for long periods of time. Intertidal wood also includes submerged parts of permanently fixed wooden structures such as pilings and bulkheads.

b. Test Panels and Poles. Marine mycologists often use wooden panels to trap fungi. In this method, first described by Meyers (1953) and later modified by T. W. Johnson *et al.* (1959) and by other researchers, panels are placed on a string or chain and submerged for several weeks until recovered and examined. Wood pieces, about 15 × 13 × 2 cm, are arranged in a sandwich fashion on a nylon or polyethylene cord 6 to 10 mm in diameter, separated from each other by brass washers. Smaller wood specimens (e.g., 3 × 2 × 0.5 cm) separated by pieces of plastic tubes have also been used successfully (Kohlmeyer, 1963c). The optimal length of exposure depends on location and the species of wood. It is to be expected that in tropical or subtropical areas untreated wood is deteriorated much faster by boring animals than it is in colder regions. After the wood is removed from the water, it must be examined as soon as possible to disclose fungal structures present at the time of recovery. If desired, the wood can be incubated for a certain time (Meyers and Reynolds, 1958; see p. 15 on "indirect" methods) after the initial examination has been performed. Some investigators (e.g., Johnson and Sparrow, 1961) recommend scraping the test panel with a putty knife to remove the fouling organisms. This procedure usually damages or even removes fungal fruiting structures present in or on the softened outer layers of the wood. If growth of bryozoa, algae, or tunicates impedes handling of the panel under the dissecting microscope, it is preferable to cut these organisms carefully off with a sharp knife without damaging the wood surface. Complete cleaning of the wood by scraping should be done only if the specimen must be incubated in order to obtain a secondary growth of fungi (p. 15).

Our experience in two decades of collecting marine fungi has shown that submerged wood panels yield a smaller number of species than do wooden substrates found in the natural habitat, such as pilings, driftwood, lobster pots, eroded trees, or roots of mangroves. The cause for the greater variety of fungal species on the latter substrates is probably related to the different age, origin of growth, and diversity of tree species that produced these materials. Panels used in submergence experiments are restricted to a few species of wood that usually come from one source of supply, may even be derived from one tree, and are decorticated. Bark that is often found on driftwood branches is host to a variety of higher marine fungi (Table XVI), some of which may not be found if only dressed wood panels are used in ecological surveys. G. C. Hughes (1975) also concluded that studies of intertidal wood give a better estimate of species diversity and distribution of lignicolous fungi in a certain area than do trapping experiments with wood panels. On the other hand, an advantage of the panel method over the collecting of driftwood and other substrates is that the species of wood and the time of submergence of the panels are known, whereas these data are usually missing for material washed up on the beach or for shoreline fortifications.

Untreated wooden poles of at least 5-cm diameter are valuable substrates for determining the vertical distribution of marine fungi. Schaumann (1968) described the zonation of fungi along a partly corticated oak pole from Bremerhaven, Germany. Experiments made by us in North Carolina with fir and pine poles (5 × 5 cm in diameter, 1.5 m long) placed in salt marshes showed the suitability of such poles for experimental studies. Optimal length of exposure varies with the locality and depends largely on the degree of attack by wood-boring animals. The poles must be recovered shortly before destruction by borers causes fracture of the wood at the intertidal zone. Zonation of fungal species below, at, and above the high-tide line can be determined.

c. Roots and Submerged Branches of Mangroves and Other Trees. Partly submerged trees along eroding shorelines and roots of mangroves are promising substrates for wood- and bark-inhabiting marine fungi (Cribb and Cribb, 1955, 1956; Kohlmeyer and Kohlmeyer, 1965). Fungi grown on permanently or intermittently submersed roots and branches are doubtlessly aquatic. Salinity determinations in the vicinity of the trees must establish whether the habitat is marine, brackish, or freshwater. Mangrove trees (*Avicennia, Rhizophora* spp., and others) are able to grow in full salinity seawater as well as in freshwater, even developing best in the latter. Overlapping marine, freshwater, and terrestrial mycotas in the mangrove habitat offer fascinating opportunities for ecological studies.

Submersed roots of mangroves or other trees are cut immediately above the sediment with a saw or knife. Parts of roots embedded in the soil are generally useless for mycological investigations because the surrounding substrate is low in oxygen and full of anaerobic bacteria, as indicated by the black color and smell of H_2S. Only those parts of roots and branches that are submerged for some part of the day in water are suitable for collecting aquatic fungi. Exposed parts above the water line are inhabited by terrestrial fungi. Thus, the distribution of terrestrial and aquatic fungi overlaps on mangrove roots and branches at the high-water mark (Kohlmeyer, 1969d). Injured, partly decorticated roots should be collected because fruiting bodies and conidia of higher marine fungi develop more readily on such wounded areas. Roots are placed in sterile plastic bags and further treated as explained above (Section I, A, 1, a and b).

Large root systems of mangrove trees can also be collected for the study of vertical distribution of fungi along the roots. Pieces of horizontal *Avicennia* roots up to 1 m long, with their attached vertical pneumatophores, can be cut, folded, and placed in a plastic bag. In the laboratory the roots are unfolded and examined under a dissecting microscope. Young damaged *Rhizophora* seedlings with prop roots, up to about 1.5 m in length, can also be collected *in toto* for further examination.

2. Algae

Representatives of all fungal groups except Basidiomycetes have been found on marine algae. Living algae are generally collected at low tide from the shore by dredging or by skin diving. Algae washed ashore and lying on the beach for a certain time are often contaminated by terrestrial molds, and critical examination has to prove which of the fungi are indigenous marine species. In general, collecting algicolous marine fungi is not easy because it requires time-consuming examinations of great numbers of algae in order to detect a few fungal parasites, symbionts, or saprobes. Fungal infestations occur particularly on Rhodophyta and Phaeophyta, rarely on Chlorophyta. Discolorations of the plants indicate attack by fungi, but similar spots may be caused by parasitic algae or bacteria.

a. Ascomycetes. Among the 39 Ascomycetes occurring on marine algae, 31 have been collected on living plants (see Table V, p. 56). The majority of algicolous fungi can be found on algae washed up on the beach. *Haloguignardia irritans* is a distinct parasitic, gall-forming species that can be easily collected along California beaches on *Cystoseira* and *Halidrys*. Other gall-causing Ascomycetes occur on a small number of plants of *Cystophora* and *Sargassum*. Black, stalked ascocarps of *Thalassoascus tregoubovii* (see Fig. 100) develop on the stalk near the holdfasts of *Aglaozonia* and *Cystoseira* in the Mediterranean and on the Canary Islands. This fungus is also uncommon and grows predominantly in habitats with stagnant or contaminated water. *Phycomelaina laminariae,* forming black patches on stalks of *Laminaria* spp., is easily gathered with drift or attached algae, especially in New England, but in northern European countries as well. At low tide, this fungus can be seen *in situ* on *Laminaria* stalks and holdfasts. An endemic Ascomycete in brown algae, *Mycosphaerella ascophylli*, forms ascocarps in receptacles of *Ascophyllum nodosum* and *Pelvetia canaliculata.* A common Ascomycete in *Chondrus crispus,* namely, *Didymosphaeria danica* (see Fig. 88d), is collected either *in situ* or with plants washed ashore. This fungus is readily detected by the black spots formed in the tips of the thalli, in the region of the cystocarps. Perthophytic *Didymella fucicola* develops in damaged lower side branches of *Fucus* spp. with ascocarps embedded in the central midrib. Many specimens of *Corallina, Halimeda,* and *Jania* must be examined under the dissecting microscope before one of the small epiphytic ascocarps of *Mycophycophila corollinarum* will be found.

Collected specimens are placed in plastic bags on ice in a cooling box during transportation. Yeasts, bacteria, and terrestrial molds develop rapidly on the surface of algae and tend to grow over the marine fungi, unless the material is kept cool. Therefore, the algae should be examined immediately or stored in a freezer at about −18°C.

b. Deuteromycetes. Direct observation of algicolous Deuteromycetes is more or less restricted to Coelomycetes, whereas conidia of Hyphomycetes appear mainly after incubation of the substrate and, in most cases, cannot be considered to be true marine fungi. Ascocarps of several Ascomycetes are associated in nature with pycnidia or spermogonia (Table

XXI), for instance, in *Didymella fucicola* (on *Fucus*), *Didymosphaeria danica* (on *Chondrus*), *Mycosphaerella ascophylli* (on *Pelvetia* and *Ascophyllum*), *Phycomelaina laminariae* (on *Laminaria*), and *Thalassoascus tregoubovii* (on *Cystoseira*).

3. Marine Phanerogams

Collecting techniques for fungi occurring in marine phanerogams are similar to those described above for algicolous and lignicolous species.

a. Permanently Submerged Plants. Host plants of marine fungi include sea grasses, such as *Posidonia oceanica, Ruppia maritima, Thalassia testudinum,* and *Zostera marina.* Rhizomes and leaves of these plants are often washed ashore and are collected along the beach or gathered by dredging or skin diving. The large ascocarps of *Halotthia posidoniae* on *P. oceanica* are easy to detect on bare parts of the rhizomes near old leaf bases. The foliicolous Ascomycete on *Thalassia, Lindra thalassiae,* occurs on leaves washed up on the beach. Brown and whitish spots on the tips of the leaves often indicate attack by the fungus. Fruiting bodies can be found *in situ,* or they develop after incubation of damaged leaves.

b. Partly or Intermittently Submerged Plants. Higher marine fungi are easily collected in salt marshes on *Juncus, Salicornia, Spartina,* and other hosts. Old rhizomes, culms, or leaves of these plants lying on the sediment are deteriorated by a number of fungi, predominantly Ascomycetes (Table XI). Dead standing upper parts that have never been submerged may be inhabited by terrestrial fungi. Ascocarps and pycnidia of marine fungi are often embedded in the substrate without being visible from the outside. The cuticle or outer cell layers of stems or leaves are cut off in a tangential section with a razor blade under the dissecting microscope, or the specimens are split lengthwise, in order to make visible the fruiting bodies, which are often arranged in rows parallel to the long axis of the culm. Black spots on stems and leaf sheaths of *Spartina* spp. indicate the location of hidden ascocarps. Salt-marsh plants offer a wide field for new discoveries of marine fungi; however, descriptions of new species should be made only after a careful literature search because some of the marsh fungi may have been described before from other hosts of freshwater or terrestrial habitats.

c. Litter-Bag Experiments. Most research on marine phanerogams has been carried out with plants collected *in situ,* except for some litter-bag experiments. In such tests, sterilized and weighed plant material is submerged in estuarine or marine habitats in noncorroding plastic containers, usually made of nylon mesh. Anastasiou and Churchland (1969) submerged leaves of terrestrial plants (*Arbutus menziesii* and *Prunus laurocerasus*), whereas Fell and Master (1973) used mangrove leaves

(*Rhizophora mangle*). In both of these investigations leaves were recovered at regular intervals and fungi were isolated after incubation. Gessner and Goos (1973a) employed the litter-bag method to investigate the mycota of *Spartina alterniflora* in a Rhode Island tidal marsh. These authors examined the grass microscopically after recovery, determined the loss in dry weight, and prepared dilution plates with the decomposing plants. The pour-plate technique proved to be selective for terrestrial molds, a fact that points to the importance of direct microscopic examination after collection. Finally, Newell (1976) utilized untreated and injured seedlings of *Rhizophora mangle* submerged in nylon mesh bags. The extensive sampling program of Newell included direct observation at the time of collection and after incubation, as well as plating, baiting, and use of the wash water.

Most litter-bag studies did not consider the influence of tides, since the plant material was submerged at all times. Certain fungi of the salt marshes and mangroves may require an alternation between immersion and drying out, and future investigations should take this possibility into account.

4. Sea Foam Containing Fungal Spores

The environment between sand grains in marine beaches, the endopsammon, harbors a rich microfauna and microflora (see Chapter 8). Higher marine fungi occur in the interstitium, and ascospores and conidia of marine fungi are released from fruiting bodies and conidiophores, respectively, and carried away with the waves. Ascospore appendages or tetraradiate shapes of spores and conidia favor their entrapment in foam between air bubbles. Foam is easily collected along the shore with a spoon-type skimmer. This slightly convex spoon, about 11 cm in diameter, with about 100 holes, each 2 mm in diameter, is used to scoop up foam, which is poured into a widemouthed bottle. Care should be taken not to collect any sand or water with the foam. Bottled samples are refrigerated at about 5°C for several hours to allow foam to "dissolve" and spores to settle. Storage at low temperatures is necessary to keep spores from germinating. Finally, a droplet of the sediment is taken out with a pipette, placed on a slide, and covered with a large cover glass (25 × 25 mm). Fungal spores are sought out by scanning the slide under a 40× objective, possibly in dark field or phase contrast, where ascospores, basidiospores, or conidia stand out by their refraction between nonrefractive amorphous particles (see Fig. 12). If samples cannot be examined the same day, formalin (45%) is added to the dissolved foam to yield a 5% solution.

On certain days, when foam formation on the shore is heavy, receding water leaves thick patches of foam along the upper edge of the high-tide line. Eventually, this foam collapses and dries, forming greyish-green spots on the sand and covering plant and animal debris. The dry foam can be carefully scraped off the sand or debris and shaken in water, and the sediment examined for fungi. Fungal spores found in foam give a good indication of the mycota present in the sand of a particular beach (Kohlmeyer, 1966a).

5. Marine Animals

The Ascomycete *Pharcidia balani* has a worldwide distribution on shells of living balanids, as well as limpets and other mollusks growing in the intertidal zone. Ascocarps and pycnidia of this fungus appear as black dots on the surface of the shells.

Other substrates of animal origin attacked by marine fungi are the cellulose mantle of tunicates and calcareous linings of empty shipworm tubes in rotten wood. Ascocarps of *Halosphaeria quadricornuta* occur in both materials. Imperfect fungi may also be found in the tube linings, forming conidia inside the substrate and breaking through the surface. Perithecia and hyphal aggregations appear as dark spots in the white calcareous tubes. These $CaCO_3$-inhabiting fungi are more frequent in tropical and subtropical waters than in temperate zones.

B. Indirect Collecting Methods (Incubation)

1. Sediment and Water Samples

Sediment or soil samples are collected on beaches, or in mangrove or salt-marsh communities, placed in sterile plastic bags (e.g., Nasco Whirl-Pak), and refrigerated at about 4°C until used. Soil from offshore locations is gathered with a bottom grab or coring tube lowered from shipboard. Several types of sampling devices have been used successfully by mycologists. Höhnk (1972) describes the use of the van Veen bottom grab, which was applied in the Indian Ocean to depths of 3450 m. Another device used by Höhnk is the 2.5-m-long coring tube devised by Pratje. Parts of the sediment can be taken for examination from any depth of the core. This type of tube works satisfactorily in every location except when it hits bare rock.

Water specimens from below the surface can be collected with the Cobet–Weyland sampler (Höhnk, 1972) or other devices, for example, Nansen bottles. Sediment or water specimens are used in dilution plates to obtain fungal cultures after incubation.

2. Natural Substrates

At this point, one must be cautioned again that the incubation method does not exclude isolation of fungi of nonmarine origin. Nevertheless, some widely used techniques are as follows: Pieces of wood, living or dead algae, or marine phanerogams collected in marine habitats are incubated in moist chambers, for example, petri dishes or larger containers lined with wet filter paper. Mycelium or fruiting bodies developing on the surface of the substrates can be used for the isolation of fungi. Incubation of algae or other plants in sterilized seawater from the collecting site is another method employed.

Marine animals also serve as a source of fungi. The contents of the intestines of fishes, sea cucumbers, sea urchins, and starfishes or the fluid of sponge colonies are used for dilution plate isolations. Yeasts can be obtained from surface smears of fishes, algae, or other organisms.

3. Baits

Depending on the fungal species, different substrates should be used for trapping. Wood panels as a source of higher fungi have been mentioned in Section I, A. Some authors (e.g., Johnson and Sparrow, 1961) advise submerging the panels for certain periods of time, followed by removal of the fouling organisms after recovery and incubation of the wood in moist chambers. Another method uses cores cut with a borer from pilings or other wooden substrates and subsequent incubation of the specimens. Again, it is emphasized that nonmarine fungi may develop in addition to marine species after incubation in the laboratory.

II. PRESERVATION

In view of the large number of doubtful and rejected marine species (Chapter 27), we recommend strongly not only that type material be deposited in recognized herbaria, but also that other specimens which are the bases for check lists or other research should be deposited there. Such thoroughly labeled material will permit later investigators to confirm or correct earlier identifications. As Dennis (1968) so aptly stated: "Lists of records that cannot be verified are mere waste paper."

Collected specimens can be preserved for future examination in the following ways: alive in a pure culture, frozen, dried, in liquids, or on a microscope slide. There is no rule for the most suitable preservation technique of marine fungi. Every species may have its own requirements, but freezing, freeze-drying, and microscope mounts are probably the best preservative methods.

A. Freezing

Algae- and wood-inhabiting marine fungi are well preserved if stored at temperatures around −18°C (Kohlmeyer, 1968e, 1969c). The morphology of Ascomycetes and Deuteromycetes remains unaltered after repeated freezing and thawing. Conidia of *Robillarda rhizophorae* survived this treatment and germinated at room temperature in distilled water. To prevent drying of the specimens in the freezer, the substrate is stored in seawater in a plastic bag. Fungi kept in frozen seawater will probably remain morphologically unchanged indefinitely.

B. Drying

Drying techniques depend on the substrate on which the fungus has developed. Bulky, hard material such as wood, bark, rhizomes, or calcareous algae can be air-dried (not in the sun!). Soft algae containing fungi are handled like other algal collections, that is, dried in a plant press between pieces of cloth (Dawson, 1956). Leaf-inhabiting fungi are dried with the substrate between drying papers in the plant press. Pure cultures are made into dried reference specimens according to the method described by Pollack (1967). The partially dried agar culture is removed from the petri dish, 15 ml of hot 2.5% glycerol agar is poured into the lid of the plate, and the culture is floated on the hot agar until the lower layer is solidified. The culture is left uncovered and is then peeled off the plate after it is completely dry. The pliable agar can be mounted in a cardboard folder for filing.

Freeze-drying of marine fungal specimens is recommended if a freeze-dryer is available. This technique guarantees the best possible preservation of the material and prevents, for instance, collapsing of fruiting bodies, which often occurs during slow air-drying.

C. Liquid Storage

Preservation of marine fungi in the most widely used preservatives, namely, ethyl alcohol (about 70%) or 5% formaldehyde in seawater, is not always satisfying. Delicate structures, such as asci, interthecial tissues, or spore appendages, may shrink and become useless for future work. Identification of species preserved in alcohol or formalin is often difficult or even impossible if spore appendages have lost their natural shape. The use of alcohol or formaldehyde as fixative is suitable if the material is to be sectioned after dehydration and embedding in paraffin wax. However, sectioning the living material in a cryostat is preferable.

Liquids containing fungal spores, such as seawater resulting from "liquified" foam, should be examined as soon as possible after they have been collected. These specimens can be preserved by adding formalin to yield a 5% solution.

D. Permanent Microscope Slides

Squash mounts or cryostat sections of living specimens are examined in water and then made into permanent slides according to the double-cover-glass method (Kohlmeyer and Kohlmeyer, 1972c). For the initial examination of a fungus, a square cover glass (25 × 25 mm, No. 1) is placed on a slide (76 × 26 mm) and tiny droplets of distilled water are placed with a dissecting needle at two sides of the cover glass to make it adhere to the slide (Fig. 2a). A larger droplet of distilled water is dropped in the center of the cover glass, and the specimen is placed in the water and covered with a small cover glass (18 × 18 mm, No. 1; Figs. 2b and 2c). Now the living material is ready for microscopic investigation, including under oil immersion if required. After the necessary observations are made and drawings or photographs are prepared or measurements are taken, a drop-

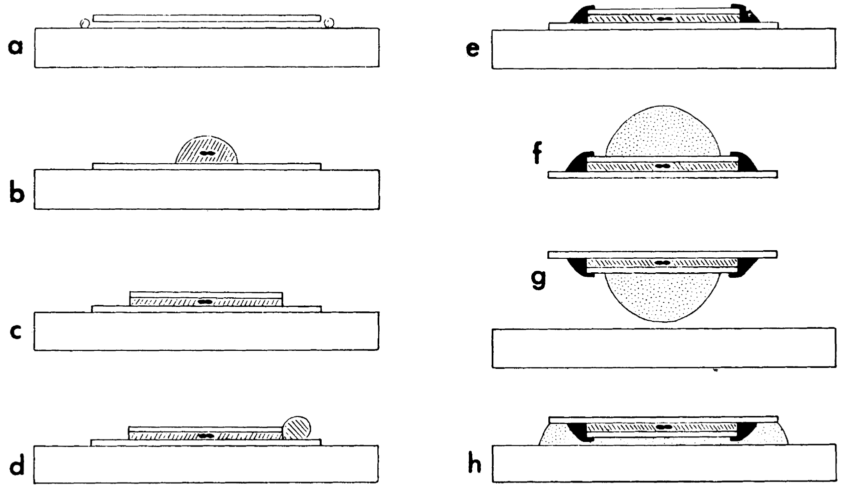

Fig. 2. Double-cover-glass method for permanent microscopic mounts. Preparation in vertical section; drawings not to scale. See the text for explanation. From Kohlmeyer and Kohlmeyer, *Mycologia* **64,** 667 (1972), Figs. 1–8. Reprinted with permission.

let of concentrated glycerin is added to the water from the side (Fig. 2d). The slide is stored horizontally in a dust-proof container for several days to permit the water to evaporate. After cleaning the edges, the mount is sealed twice with a ring of clear fingernail polish (Fig. 2e). The large cover glass is detached from the slide and a drop of mounting medium (e.g., synthetic Canada balsam "Caedax," E. Merck) is placed on the small cover glass (Fig. 2f). The preparation is turned over with the drop of mounting medium hanging down and then placed on the slide (Fig. 2g). The drop of balsam flattens out and surrounds the edges of the small cover glass, thereby permanently covering the dried ring of nail polish (Fig. 2h). A slowly hardening mounting medium is more suited for permanent preparations than fast-drying media, which tend to become brittle and crack over the years.

A reference collection of permanent slides is probably more valuable than other kinds of preserved specimens, because such slides keep indefinitely and are immediately available for comparison.

III. SECTIONING

The preparation of sections is essential for the investigation of fruiting bodies of the higher marine fungi. Squash mounts are often sufficient for the identification of known species, but sections must be made for thorough examinations of critical or new fungi. Hard substrates, such as wood or stems of marsh plants, can be hand-sectioned with a razor blade. Small specimens or fruiting bodies embedded in algae are sectioned on a microtome, preferably in a cryostat, where fixation of the fungi is not required. We have had excellent results in sectioning frozen algicolous fungi enclosed in the host tissue and have been able to obtain sections as thin as 2–5 μm in some species by using an International Cryostat (Model CTI) of the International Equipment Company. Delicate internal structures of ascocarps and pycnidia remain unaltered during the freezing, sectioning, and thawing process. The sections are transferred into a drop of distilled water on a slide, covered with a cover glass, and examined under the microscope. Stains can be added from one side of the preparation by drawing water out from the other side with blotting paper.

IV. MICROSCOPIC EXAMINATION

Many higher marine fungi are characterized by morphological adaptations to the aquatic habitat, in particular, the ascospores (Chapter 3).

Gelatinous or mucilaginous sheaths or appendages of the spores are often invisible in the bright-field microscope; therefore, phase-contrast optics, differential interference contrast, or stains must be used to detect their presence. The following stains are useful for this purpose: acid fuchsin–lactophenol, aqueous nigrosin, Delafield's hematoxylin, picric acid–anilin blue, or violamin. India ink added to an ascospore or conidial suspension will make those sheaths visible that do not take up stains, for example, in *Haligena amicta* and *Leptosphaeria pelagica*. If the chemical composition of ascospores and their appendages is to be examined, we recommend consulting Kirk's (1966) extensive treatise on the cytochemistry of marine Ascomycetes, where tests and procedures are described in detail.

Studies with the transmission electron microscope have been carried out on only a few higher marine fungi (Lutley and Wilson, 1972a,b). The scanning electron microscope (SEM) has been employed by Brooks *et al.* (1972) and Gessner *et al.* (1972) to investigate surfaces of wood and *Spartina,* respectively, and Moss and Jones (1977) have examined the ascosporogenesis of *Halosphaeria mediosetigera*. Basidiocarps of *Halocyphina villosa* have been illustrated in SEM pictures by Kohlmeyer and Kohlmeyer (1977). The application of transmission as well as scanning electron microscopy in marine mycology will probably increase in the near future, because both instruments have proven to be valuable in detecting previously unknown features in marine fungi.

V. ISOLATION AND CULTURE

Substrates collected as suggested in the preceding sections usually contain several species of marine fungi. In addition, the surface of the material recovered from a marine habitat may be contaminated by bacteria and dormant propagules of terrestrial fungi. Therefore, care must be taken not to isolate such contaminants in place of the usually slower growing marine fungi. Wood, leaves, or other substrates are blotted with sterile filter paper and externally dried at room temperature. Superficial fruiting bodies or conidia are located under a dissecting microscope. Immersed ascocarps or pycnidia are found by making thin sections with a sterile razor blade parallel to the surface. Some ascospores or conidia are picked out with a sterile glass or metal needle and transferred onto a slide for identification, others from the same fruiting body are used to prepare a spore suspension in 2 ml of sterile distilled water for dilution plates, or the spores are spread directly on the surface of agar. The superficial drying of the natural substrate compresses the fruiting bodies and often presses the spores out, causing them to accumulate at the orifice, where they can be taken up with a needle. Conidia of Hyphomycetes developing on the surface are transferred onto a slide, directly onto a solidified agar medium, or into water to be poured into dilution plates. After inoculation, agar plates are examined regularly under a dissecting microscope, if possible in dark field, to observe germination. Germinating single spores are transferred into test-tube cultures for permanent maintenance.

Media used for isolation should contain a bacterial inhibitor, such as 0.03% streptomycin sulfate (Kirk, 1969), potassium tellurite (20–50 mg/liter), or tetracycline (500 mg/liter) (G. C. Hughes, 1975). Streptomycin sulfate (100 mg/liter) plus penicillin-G (100 mg/liter) is used routinely with good success by R. V. Gessner (personal communication). Glucose–yeast extract agar (Johnson and Sparrow, 1961) is generally employed for isolating marine fungi.* Natural seawater must be aged, that is, stored for at least 1 week before use, or artificial seawater can be used, for instance, made up with Rila Marine Salt Mixture (Rila Products, Teaneck, New Jersey). For general maintenance of cultures, we have also used Emerson's YpSs agar,† available from Difco Laboratories (Detroit, Michigan). The addition of a cellulose source [e.g., birch applicator sticks—*Betula papyrifera* (Kirk, 1966, 1969)] to the agar enhances the fruiting of cellulose-decomposing fungi. We have also had good results in growing cellulolytic species on 10-cm-long strips of filter paper or cellophane or on birch sticks in test tubes half filled with seawater plus yeast extract (0.1%). Such cultures can be maintained over long periods without drying out.

A liquid medium particularly recommended for experimental use is that employed by Sguros *et al.* (1962), later modified by Meyers and Simms (1965) and Meyers (1966).** These authors developed a procedure to supply uniform inocula and to measure gravimetrically the growth of marine fungi. This standardized method yields data with a high degree of reproducibility, and it is easily modified. As G. C. Hughes (1975) pointed out, the reproducibility is highly significant statistically ($P < 0.001$) with only three replicates.

A closed system used in nutritional studies by most investigators has the disadvantage that nutrients become exhausted, pH and osmotic changes occur, and toxic metabolites accumulate. Therefore, a continuous culture system, such as that developed by Churchland and McClaren (1976), appears to be advantageous over the closed (or batch culture) system. The growth of three marine Hyphomycetes tested by these authors was significantly greater in continuous culture than in batch culture. Churchland and McClaren (1976) conclude that "the continuous system can possibly be regarded as a closer approximation to the marine environment than the batch culture system." Another continuous culture chamber was devised by Padgett and Lundeen (1977) in which the salinity can be varied in a cyclic pattern, simulating the conditions in estuaries. The continuous flow systems require a large apparatus with 6-liter reservoirs, which limit the use of this excellent method when extensive experiments are required.

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* Glucose, 1 g; Bacto-yeast extract, 0.1 g; agar, 18 g; aged seawater, 1 liter.

† Bacto-yeast extract, 4 g; soluble starch, 15 g; dipotassium sulfate, 1 g; magnesium sulfate, 0.5 g; Bacto-agar, 20 g; seawater, 1 liter.

** Glucose, 5 g; $MgSO_4 \cdot 7H_2O$, 2.4 g; NH_4NO_3, 2.4 g; yeast extract (Difco), 1 g; Tris buffer, 1.21 g (pH 7.5); natural seawater, 1 liter.

Temperatures for isolating and maintaining pure cultures of marine fungi depend on the source of the species. The majority of ubiquitous fungi can be grown at temperatures between 15° and 25°C [e.g., *Nia vibrissa* (Doguet, 1968)]. Some tropical species, for example, *Halosphaeria quadricornuta,* require temperatures around 28°C for germination (e.g., Kohlmeyer, 1968c), whereas some fungi from temperate waters, such as the Ascomycete *Herpotrichiella ciliomaris,* germinate only at about 16°C (Kohlmeyer, 1960), and growth of the Basidiomycete *Digitatispora marina* ceases above 23°–25°C (Doguet, 1964).

Small laboratories are usually unable to maintain pure cultures of microorganisms in perpetuity, and it is suggested that cultures of marine fungi be made generally available by depositing subcultures of them in large collections, for instance, in the American Type Culture Collection (Rockville, Maryland) or in the Centraalbureau voor Schimmelcultures (Baarn, The Netherlands).