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Determinants of Occurrence and Distribution of Fungi in Marine Environments

APPROVED

Wilton R. Tenney

J. C. Strickland

W. S. Woolcott

Warwick R. West Jr.

Jean Dupuis Pierce

Nolan E. Rice

Francis B. Key

APPROVED

B C Holtzclaw

Dean of the Graduate School

Determinants of Occurrence and Distribution of Fungi in Marine Environments

by

Paul W. Kirk, Jr.

A Thesis

Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in the Graduate School of the University of Richmond

August, 1961

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ABSTRACT

Studies were made of the effects of temperature and nutrient level on salinity optima for growth and sporulation of Chaetomium globosum (I) and two marine Pyrenomycetes, Torpedospora radiata (II) and a Halosphaeriopsis sp. (III). Methods of culture and evaluation of sporulation were also investigated.

Ascocarps of (II) developed in artificial sea water containing chelated trace metals, NH_4NO_3, $NH_4H_2PO_4$, biotin, thiamin, cellulose, and tris (hydroxymethyl) amino methane buffer. Tris satisfactorily buffered fungus cultures, but inhibited sporulation, particularly if glucose was the primary carbon source. Precise evaluation of sporulation was accomplished by counting spores in ten fields of each of three 0.03 ml slide preparations derived from suspensions of spores, which were prepared by washing agar from tube cultures with hot water and grinding mycelia with 2 ml of water in a Corning 7725 tissue homogenizer.

Salinities favoring sporulation of (III) decreased as temperature or yeast extract content of media increased. Salinity favoring fructification and growth of (II), and growth of (I), increased with temperature (the "Phoma pattern" of growth) and thiamin-biotin level, indicating the dependence of osmo-regulation upon respiration. Fruiting of (I) decreased as salinity increased, regardless of temperature or vitamin level. Exploratory studies suggested that the oxygen content and specific gravity of sea water, not salinity or pH, may exclude (I) from marine environments. Mineral nutrition studies indicated that the Phoma pattern of (II), more than that of (I), involves salt antagonism and sodium ion.

Discussion focused on ecological implications of data, in particular, on the probable survival value to a marine-fit fungus of a Phoma pattern mechanism involving vegetative and reproductive phases. As the seasons progress, the region where concentrations and proportions of salts are most favorable remains similar to that where vitamin requirements are least and biotic factors are most favorable.

ACKNOWLEDGEMENTS

The author wishes to express his appreciation to the many who have helped make this undertaking possible. In particular he wishes to thank his major professor, Dr. Wilton R. Tenney, for advice on methods of studying the physiology of fungi, for photographs, and for many hours of assistance in the preparation of this manuscript.

For criticizing portions of this manuscript and for bringing to mind certain ecological principles he wishes to thank Dr. William S. Woolcott and Dr. John C. Strickland of the Biology Department of the University of Richmond. For some reagents and equipment he wishes to thank Mr. William A. Dorsey, Director of the Richmond City Public Health Laboratories, Dr. William A. Powell and Dr. J. Stanton Pierce of the University of Richmond Chemistry Department, and Dr. J. Douglas Reid of the Medical College of Virginia. The use of certain facilities of the Virginia Fisheries Laboratories, Gloucester Point, Virginia, is gratefully acknowledged.

For furnishing reprints appreciation is expressed to Dr. Luigi Provasoli of Haskins Laboratories, Dr. Don Ritchie of Barnard College, Dr. Von Willy Höhnk of Bremerhaven, Dr. Helen Vishniac of Yale University, Dr. Irene Wilson of the University College of Wales, Dr. A.B. Cribb of the University of Queensland, Dr. Hugo A. Ferchau of Woffard College, Dr. Nils Fries of Uppsala University, and Dr. I. Mackensie Lamb of the Farlow Herbarium.

The author wishes to thank Dr. Samuel P. Meyers of the University of Miami Marine Laboratories for cultures of _Torpedospora radiata_ Meyers F-187 and _Halosphaeriopsis_ sp. F-115, for many valuable reprints, and for information, advice, and encouragement through correspondence.

Appreciation is expressed to Dr. Terry W. Johnson, Jr. of the Botany

Department of Duke University for the formulae of various media, for many reprints, for loan of continual-flow sea water apparatus, and particularly for allowing portions of his book, Marine Mycology, to be read prior to its publication. The author is also most grateful to Dr. Johnson for taking a personal interest in portions of this project which were sent to him prior to their completion. It was through Dr. Johnson's contributions that the author first became interested in marine mycology.

Finally, great appreciation is expressed to my wife, Evelyn, upon whom fell much of the very difficult job of typing text and tables, and who relinquished many otherwise leisure hours so that her husband's curiosity might be more fully satisfied in the laboratory.

INTRODUCTION

Intensive investigations of the physiology and ecology of marine fungi have originated only within the past six years, although Barghoorn and Linder in 1944 initiated such studies. Among several topics that have received attention are nutritional requirements and determinants of the distribution of estuarine isolates, principally lignicolous Pyrenomycetes and Deuteromycetes.

A relationship between temperature and salinity optima was discovered by Ritchie (1957) in *Phoma herbarum* West. and in a species of Pestalotia, both of which were isolated from Limon Bay, Panama. The fungi were cultured at several temperatures on graded series of sea-water agar containing 0.1% yeast extract and 0.5% glucose, and radial growth rates were determined. Salinity optima were affected by temperature, but conversely, temperature optima were not affected by salinity. Growth at all salinities was best at 25 C. Sub-optimal temperature increases caused salinity requirements to increase slightly, and increases in temperature above 25 C caused salinity requirements to increase markedly. Thus, temperature increases resulted in progressively greater salinity requirements until temperature became limiting. The mechanisms of this phenomenon were not investigated beyond the computation of temperature coefficients, but Ritchie states, "the rise in temperature causes in the system an imbalance which is compensated for by a shift in the reaction of the organism to salinity....Growth at low temperature proceeds fastest when salinity is low, or at high temperature when salinity is high." Ritchie indicated an apparent similarity of this shift to pressure-temperature-volume phenomena in gases and to pressure-temperature-growth relations in bacteria studies by ZoBell and Johnson (1949).

Ritchie (1959) similarly cultured five marine and terrestrial isolates from tropical and temperate climates seeking to indicate whether this temperature-salinity phenomenon, which he termed the "*Phoma* pattern," is a characteristic of most fungi, of marine fungi, or only of isolated cases. Only a species of *Curvularia* isolated from Delaware Bay grew in what Ritchie interpreted as a *Phoma* pattern. Salinity requirements increased with temperature, though not progressively. This pattern of growth was displayed in more highly modified form by a terrestrial fungus and by another marine isolate. Fungi collected from marine habitats varied in their propensities for sea water, but only a terrestrial *Mucor* was inhibited by these salts at all temperatures. Fungi collected from mild climates had temperature optima higher than those of northern isolates. Ritchie suggested that growth patterns so obtained may offer a possible clue to the ecological adaptation, seasonal and geographic distribution of some fungi.

Gold (1959), working independently of Ritchie, counted reproductive structures of fungi occurring on wooden panel traps from salinity gradient stations in the Newport River, North Carolina over a 36 weeks period. Throughout the investigation several species were found exclusively in fresh water portions of the estuary, others apparently were restricted to sea water, and a few were isolated from fresh, brackish, and sea water portions. Five species were distributed in a manner highly suggestive of a *Phoma* pattern, and of these, the pattern of a *Trichocladium* sp. was the most striking. At 4 C this fungus was found only in fresh water. The salinities at which the species could be detected rose with rising temperature, and at 28 C it was found only at salinities approximately 34 o/oo.

As Ritchie (1957, 1959) and Gold (1959) worked with different species, certain assumptions must be made to correlate their findings. Discrepancies

in the laboratory and field data should be mentioned. Barghoorn and Linder (1944) and Ritchie (1957, 1959) have demonstrated that the temperature optima of marine lignicolous fungi are generally 25-30 C. It can readily be assumed that the temperature optima of Gold's five species lie somewhere near or within this range. However, Gold graphically indicated that his possible *Phoma* pattern fungi were detected only in sea-water portions of the estuary when water temperatures were 25-30 C. The range of salinity tolerance of Ritchie's species was greatest at these temperatures. Meyers and Reynolds (1959a) indicated that in nature the same conditions of nutrition may favor both vegetative and reproductive phases of marine Pyrenomycetes, but it has not been demonstrated that both of these phases are favored by the same conditions of temperature and salinity. Neither has it been determined to what extent Gold's fungi responded to factors other than the dual stress of temperature and salinity. There is evidence that this estuarine distribution may have, at least in part, manifested a temperature-affected distribution of nutrients essential for sporulation.

TeStrake (1959) studied the seasonal distribution of saprolegniaceous fungi in the Neuse River, North Carolina, and determined their saline tolerance at several temperatures on nutrient media in the laboratory. The highest salinity at which the water molds occurred in the estuary was 2.8 o/oo, but colonies developed on hypersaline corn meal agar at temperatures 20-30 C. Miss TeStrake concluded that nutrient level, which was higher under laboratory conditions than in the field, is an essential determinant of the estuarine distribution of some fungi. Johnson (1960) proposed from the forementioned evidence and from his own work with the halophilic *Lagenidium chthamalophilum*, that salinity tolerance of some fungi is a partial function of water temperature and nutrient level. Neither TeStrake nor Johnson stated specifically which nutrients may be involved in salinity tolerance mechanisms of fungi.

Barghoorn and Linder (1944) observed that in nature marine fungi commonly become established on materials which, upon decomposition, furnish a supply of organic nitrogen. Gustafsson and Fries (1956) studied nutritional requirements of eight lignicolous Pyrenomycetes in low salinity mineral salts media and reported five of these fungi to be at least partially thiamin heterotrophic. Numerous papers advocate the use of yeast extract, a substance of high organic nitrogen and B-vitamin content, for the propagation of marine fungi. Special attention is called to the work of Meyers and Reynolds (1959a), which shows that all stages of the life cycles of several marine lignicolous Pyrenomycetes are supported by wood and wood products in sea water fortified with yeast extract. These authors also reported that good vegetative growth, but not fructification, was supported by glucose and either yeast extract or a thiamin-biotin-nitrogen mixture in the ASP_2 synthetic sea water of Provasoli et al. (1957). Johnson et al. (1959) surveyed nutritional requirements of 21 marine lignicolous Pyrenomycetes and Deuteromycetes. Peptone was almost invariably the preferred nitrogen source on a vitamin-free synthetic sea-water agar base, though nitrogen contents of substitutions as nitrate and ammonium were in some cases greater, and all fungi appeared to utilize these inorganic sources.

On the whole, these nutritional data suggest that deficiencies for thiamin and biotin may be common among marine lignicolous fungi. As most of the nutritional studies cited were carried out at 20-25 C on high (approximately 34 o/oo) salinity media, one possible reason for some vitamin deficiencies is suggested by the work of Robbins and Kavanaugh (1938). These investigators reported _Pythium butleri_ to be thiamin deficient in a medium containing 16.4 g/liter mineral salts, but thiamin autotrophic when mineral salts were reduced to 1.64 g/liter. Instances in which the demand, in culture, for thiamin and several other vitamins has been affected by conditions of

temperature and salt concentration are cited by Lilly and Barnett (1951), and Cochrane (1958). These texts also point out that some organic nitrogen compounds are vitamin-sparing.

The literature which has been reviewed suggests that saline tolerance of several marine and non-marine fungi is a partial function of temperature and the level of vitamins or their equivalents. One purpose of this thesis is to present experimental evidence which supports that hypothesis. Marine mycology, however, is a young field, and much basic data must be accumulated before effective investigations can be made of the many physiological and ecological problems. Of the problems that have been recognized many are not clearly defined. Because of the nature of marine fungi, and of their environments, new methods of isolation, culture, and examination must be devised. Therefore, the greater intended purpose of this thesis is to meet some of these needs. Experiments have been conducted which shed light on several problems suggested by the literature. All of these can be related to technical and theoretical aspects of the broader problem of determinants of occurrance and distribution of fungi in marine environments. Topics that have been considered are as follows: effects of temperature and nitrogen-vitamin level on salinity optima for vegetative, asexual, and sexual phases of terrestrial and marine Pyrenomycetes; precise technics for evaluation of sexual and asexual sporulation; development of reproduceable synthetic media which support mature ascocarps of marine Pyrenomycetes; natural sea-water media for physiological, ecological, and developmental studies; the utility of tris (hydroxymethyl) amino methane as a buffer in fungus cultures; habitat designations and physiological criteria for dilimiting the marine mycota; mechanisms, prevalence, and functions of the Phoma pattern; possible interrelations of the above as they apply to academic problems of the definition of marine lignicolous fungi.

MATERIALS AND METHODS

A. General Methods

Torpedospora radiata Meyers F-187 and Halosphaeriopsis sp. F-115 were furnished by Dr. Samuel P. Meyers of the University of Miami Marine Laboratories. The former was isolated from yellow pine submerged in Biscayne Bay, Florida, salinity about 30-32 o/oo, temperature 18-25 C. The latter was isolated from basswood submerged at St. Andrews, New Brunswick for 176 days (July 29, 1957 to January 21, 1958). Water temperature during submergence at the Biological Station wharf was 12-2.5 C and salinity 31-32 o/oo (Meyers and Reynolds, 1959c; Meyers, 1961). Chaetomium globosum was obtained from the American Type Culture Collection, number 6205; this is a vitamin autotrophic, lignicolous fungus. Stocks subcultured from the above were maintained in 18/150 mm screw-capped tubes of salinity 34 o/oo media NS-1 and NS-7, to be described.

Inocula for all experiments were of one of four types, which will be described and subsequently referred to by Roman Numeral:

I. Suspensions of conidia and ascospores (F-115) or ascospores (F-187 and 6205) from stock subcultures in 0.05 cc delivery medicine dropper bottles of strength 1/4 artificial sea water AS-1. AS-1 was altered in constitution as necessary. It is to be assumed that liquid inocula contained none of the variables to be tested in nutritional studies, e.g. potassium in potassium studies, or vitamins in vitamin studies. Spores, however, were neither washed nor subcultured free of the variables to be considered. Spores were taken directly from stock subcultures;

II. Similarly prepared and dispensed suspensions of F-187 chlamydospores

derived from colonies on strength 1/4 AS-1 agar which had been incubated at 30 C for two weeks or more. Subculture to AS-1 agar was from original stock subcultures in screw-capped tubes;

III. Small pieces (less than one cubic mm, generally) of colonies on 1/2 strength NS-1 agar incubated ten days or more at 30 C. Inocula for NS-1 agar were derived directly from stock subcultures;

IV. Similarly-sized pieces of colonies on strength 1/2 AS-1 agar incubated ten days or more at 30 C. Inocula for AS-1 were from stock subcultures.

Sea water used in all natural sea water media was obtained (17.5 o/oo) in large quantity from Gloucester Point, Virginia, aged one month, filtered through Pyrex wool, and enough evaporated in enamel pans to make 20 gallons of salinity 68 o/oo, as determined hydrometrically and by AgCl gravimetric methods. This 20 gallons of stock was stored in the dark at about 25 C, in cotton-plugged carboys. Dilution of this stock for the preparation of media was accomplished with graduates and single-distilled water.

For brevity and, in the case of several experiments, much greater clarity, the marine salts content of both natural and artificial sea waters will be designated by the term "strength", rather than by salinity in grams per kilogram. Strengths 2, $1\frac{1}{2}$, 1, 3/4, 1/2, 1/4, 1/16, and 0 refer to the salts content of media, and correspond to salinities approximately 68, 51, 34, 26, 17, 8.5, 2 o/oo, and distilled water media, respectively. A graded series of AS-1, NS-7, or NS-1 agars, strengths 1/4 through 2, refers to salinities approximately from one-quarter to twice those of "normal" (salinity 34.5 o/oo)sea water. These strength designations do not refer to levels of added nutrients, which were held constant for all salinities, or strengths, of sea water base. Dilution series contained a gradient only of those nutrients present in rotted natural sea water. Salinities of natural and artificial sea water media of the same strength did not

correspond beyond the nearest gram per kilogram, but this was considered close enough for the types of comparative studies which were conducted.

The three most commonly used culture media will now be described, and subsequently referred to by letter-number designations.

NS-1 was an aged natural sea water base (strengths 1/4 to 2) containing BBL yeast extract 0.1 %, glucose 0.5 %, and Difco Bacto agar 1.8 %.

NS-7 was as NS-1, but a 6/25 mm (approximately) strip of Whatman No. 1 chromatography paper was substituted for glucose.

Strength 0 NS-1 or NS-7 refers to distilled water-yeast extract-glucose or cellulose media, respectively. Glucose was autoclaved with other ingredients of media. With one exception, all ingredients of all media used in this entire investigation were autoclaved together. Autoclaved chromatography paper was added aseptically to slants of NS-7 sea-water-yeast extract base.

AS-1 was an artificial sea water devised for this investigation largely from information in Sverdrup et al. (1942), Lilly and Barnett (1951), Provasoli et al. (1957), and Johnson et al. (1959). All reagents used in this medium were C.P. grade, with the exceptions of disodium ethylenedinitrilo tetraacetate, to be designated Na_2EDTA, and 2-amino-2-(hydroxymethyl)-1,3-propanediol, to be designated tris. Tris and Na_2EDTA were Eastman Practical grade. The basal medium consisted of salts in the proportions they occur in sea water, of a chelated metals mixture, and of tris buffer. Two ten gallon lots of this base, salinity approximately 68 o/oo, were separately prepared, with double Pyrex-distilled water, and stored in carboys in the dark at 4-10 C. One lot of this base was used for the first run of an experiment, and base for repeats of that experiment was taken from the second lot. AS-1 media were prepared by

single-distilled water dilutions of these salinity 68 o/oo salts-metals-buffer solutions, and the addition of carbon, nitrogen, phosphorus, vitamins, and agar as desired. In some instances the pH gradient resulting from dilution of tris was undesirable, and after dilution of the stock to desired strengths, enough buffer was added to all dilutions to make it constant for all strengths. In Experiments 13, 14, and 16, new AS-1 base was prepared with the appropriate deletions, which will be described in these respective sections.

In Appendixes IV, V, and VI, are data, in tabular form, which were used for manipulation of the constitution of AS-1. While presentation of these data was not considered necessary for adequate description of methods and interpretation of results, they will be of value to any investigator who wishes to use AS-1 or a similar medium for mineral nutrition or other physiological studies, or who wishes to scrutinize aspects of the results which were not thoroughly enough treated by the author.

Presented below are the five "systems" of AS-1 ingredients, as conceived by this investigator. Weights of ingredients are based on one liter of strength 1 medium, chlorinity about 19.1 o/oo, salinity 34.5 o/oo. Systems A, B, and C comprise the basal medium. Contents of systems C and D closely approximate the concentrations and proportions of those salts in normal sea water, as presented by Sverdrup et al. (1942). The carbon:nitrogen:phosphorus ratio of the entire medium was 100:7:1, with 2 g carbon, 140 mg nitrogen, and 20 mg phosphorus per liter. Agar was added in 1.8 % concentration as desired. The pH of the medium, after autoclaving, was 8.2 to 8.6.

A. Buffer system: Tris 1 g

B. Metals and chelator system:

Fe^{3+} as chloride 1 mg

Zn^{2+} as chloride 0.3 mg

Mn^{2+} as chloride	0.5	mg
Co^{2+} as chloride	0.01	mg
Cu^{2+} as chloride	0.02	mg
Mo as $Na_2MoO_4 \cdot 2H_2O$	0.01	mg
Na_2EDTA	30	mg

C. Major invariable salts system (abbreviated "major")

NaCl	23.54	g
$MgCl_2 \cdot 6H_2O$	10.63	g
$NaSO_4$	3.92	g
$CaCl_2 2H_2O$	1.47	g
KCl	0.73	g

D. Minor invariable salts system (abbreviated "minor")

$SrCl_2 \cdot 6H_2O$	40	mg
H_3BO_3	26	mg
$Na_2SiO_3 \cdot 9H_2O$	20	mg
NaBr	83	mg
NaF	3	mg

E. C:N:P:Vitamins system

glucose (dextrose)	5	g
NH_4NO_3	0.374	g
$NH_4H_2PO_4$	0.074	g
thiamin HCl	100	ug
biotin (free acid)	5	ug
inositol	5	mg
pyridoxine HCl	40	ug
nicotinic acid	100	ug

Vessels used in this investigation for cultures, and in the preparation of media and reagents, were washed in hot tap water and Alconox detergent with an electric rotary brush set, rinsed in running hot and cold tap water, rinsed once in single-distilled water, inverted until air dried, and finally heated in an oven at 180-250 C from four to six hours. Pipettes were soaked in chromic acid cleaning solution, washed several times in tap water and twice in single-distilled water, and air dried. Sterilization of pipettes and Petri dishes was effected by stacking them in cans in the hot air oven mentioned above.

Liquid media were dispensed into 125 or 200 ml Erlenmeyer flasks in 25 ml amounts with a 25 ml volumetric pipette, the tip of which was filed for rapid delivery. Volumes of liquid media were therefore constant. All flasks were capped with dust covers formed from aluminum foil. No cotton plugs were used in any flasks. Solid media for slants were dispensed hot, after agar had been boiled into solution, with a rapid delivery 10 ml pipette. Thus, slants contained fairly uniform volumes of media. Lips of tubes were in all cases slanted 6 mm above the horizontal. Slants were plugged with absorbent cotton. All media were sterilized in an electric autoclave at 15 lbs (121 C) for 15 minutes. Media for Petri dishes were prepared in 1 liter boiling flasks, in volumes ranging from 200 to 600 ml. These were capped with aluminum foil and autoclaved. Flasks were cooled in 55 C in a water bath, and plates poured. No special effort was made to maintain a constant volume of medium in plates; that is, beyond the accuracy normally expected of a technician experienced in pouring plates. It was estimated that volumes ranged between 15 and 20 ml per plate. The final few ml, which contained heavy precipitates if salinity was greater than 8.5 o/oo, were not used. Due to the large number of replicates and repeats (runs), it is

not likely these differences in plate agar volume constituted much source of error. Colonies of the marine species were grown one to three per plate, as colonies were relatively small and readings never made after 15 days of growth. Plates were stacked three high in incubators, but stacks only contained replicates of the same condition. As will be evidenced later, even this amount of stacking influenced results at higher temperatures.

Failure to maintain constant incubator temperatures was probably the greatest source of error in all experiments. A temperature of 20 C refers to incubation in a darkroom in which temperature fluctuated between 17 and 21 C during the course of this investigation. 25 C refers to the mean temperature in dust-free cabinets in a room where temperature fluctuated between 23.5 and 26.5 C. The 30 C incubator fluctuated between 29 and 31 C. The 35 C incubator, in constant use by other investigators, varied from 33 to 38 C, though the calculated mean was 35 C for any two weeks period. As it was feared experiments would not be reproducible due to these temperature fluctuations, repeats of an original run were set up at one day intervals,, incubated with the original run, and results were gathered at one day intervals after equal incubation times. Experiments which required the use of these incubators did not require extremely constant temperatures. Absolute values were not sought, but only trends at low, high, and intermediate temperatures.

Colonies of *T. radiata* and *Halosphaeriopsis* on agar media were surprisingly circular, so the average of two perpendicular diameters was recorded as the index of growth of these species. Diameter of the inoculum was not subtracted unless there was no growth, but diameters of inocula seldom exceeded 1 mm. Colonies of *C. globosum* on agar media varied from regular to quite irregular, so recorded diameters are the average of from two to six diameters, depending upon the apparent symmetry of the colony. Averages reported for experiments

which were run in triplicate are based upon average diameters of from six to nine colonies; that is, three repeats of three replicates each, less contaminants. Contamination was no problem in tubed media or flask media.

The growth of cultures in liquid media was measured in terms of dry weight of mycelium. For this purpose 28/28 mm aluminum culture tube caps were cut into shallow weighing cups of 3.5+ grams each. These cups were polished with emery cloth, washed, and heated at 100 C until constant weight was obtained. Cultures were poured onto a closely woven circle of cotton fabric in a buchner funnel, both washed with hot tap water and pressed four times, and harvested into weighing cups with probes, policemen, and fingers. When it was estimated that the total amount of harvest from all replicates of a given condition would be less than 2 mg, the contents of all replicates were pooled for a single harvest. Flasks estimated to contain more than this amount of mycelium were harvested individually. Losses through the fabric constituted little source of error except in harvests which averaged less than 2 mg per flask. However, as inocula were neither washed nor subcultured free of the variable ingredients of AS-1 to be tested, little faith was placed in less-than-milligram averages.

Weighing cups containing harvests were heated at 100 C for from four to six hours and weighed immediately upon cooling on a magnetically-dampered chainomatic calibrated to 0.1 mg. Gain in weight due to absorption of atmospheric moisture was not observed to exceed 2 mg per hour per 50 mg of dry harvest. A desiccator was not used. Cups were soaked in distilled water for a few hours, cleaned with a policeman, rinsed in distilled water, and dried. After the first three harvests each cup was reweighed empty, but as weights never varied more than 0.3 mg, only spot checks were taken subsequently.

Determinations of pH were made with a H-20 Hydrion set. Readings obtained using Hydrion papers varied between 0.0 and 0.4 units from pH obtained using a Coleman model 3 D pH electrometer. The greatest discrepancies were in the ranges above pH 6.8. Whenever pH readings were made, these were taken from each of several replicates of the same condition, and a pH range was recorded. In the results section a range was not reported, for the sake of brevity in tables, but it will be understood that plus or minus 0.2 units of the recorded figure would usually include that range.

Microscopic examinations were performed with a Spencer three-objective monocular, diameter of HPF 0.35 mm. A Spencer wide field 10X-40X binocular was used to count ascocarps of the marine species, and a Quebec counter those of *C. globosum*.

B. Experiments Performed and Special Technics

For the sake of organization, experiments have been numbered 1 - 22. Actually, more than 22 discrete experiments were conducted, but different approaches to a common problem, and similar experiments using different organisms, have been placed under the same number. Experiment numbers do not indicate the sequence in which experiments were performed. Experiment 22 was one of the first to be set up, and several experiments were run concurrently.

Experiment 1

The general methods of Ritchie (1957) were followed, using NS-1 in strengths 0 to 2 at 20, 25, 30, and 35 C. Three replicates were run in triplicate for each species. Diameters of *C. globosum* were taken at six days, and the plates re-incubated for ascocarp counts at 12 days. An estimate of all distinguishable perithecia was made without regard for maturity. Colonies of *T. radiata* and *Halosphaeriopsis* were measured at 12 days, and those of the first run also at 15 days. Initial pH readings were made. Inoculation was by method III. Typical colony morphology was observed for comparison with Experiment 2, but no detailed record of these findings was kept.

Experiment 2

This was a repeat of Experiment 1, essentially, but using AS-1, with full nutrients, in strengths 1/16 through 2, at four temperatures. Initial pH readings were taken. Inoculation was by method IV. Typical colony morphology was recorded and a few microscopic examinations made at 15 days growth. As *C. globosum* colonies were irregularly shaped on many dilutions of AS-1, the average for nine replicates was recorded rather than the individual three-replicate averages.

Experiment 3

This was similar to Experiment 2, but vitamins were not added to AS-1. Intended as a screening test, only four replicates of each condition were made, and the experiment not repeated. Various microscopic and macroscopic descriptions were recorded and compared with those of Experiment 2. Readings of *C. globosum* were made at six and 12 days, and those of the marine species at 12 and 15 days. Initial pH readings were taken. Inoculation was by method IV. No special effort was made to keep the size of inocula uniform.

Experiment 4

Four replicates each were made of AS-1 strengths 1/4 and 3/4 containing:

1. complete AS-1 vitamin mixture;
2. no pyridoxine;
3. no nicotinic acid;
4. no inositol;
5. no thiamin;
6. no biotin;
7. no vitamins.

Four sets of plates, each containing four replicates of each condition, were prepared, two sets for each of the marine species. These were incubated for 13 days, one set at 20 C and the other set at 30 C. Media 1 and 7 served as controls, and also as a partial repeat of Experiments 2 and 3, respectively. Inoculation was by method IV. Colony descriptions were recorded.

Experiment 5

Halosphaeriopsis was plated in four replicates on AS-1, strengths 1/4 and 3/4, which contained:

1. thiamin and biotin;
2. thiamin only;

3. complete AS-1 vitamin mixture;

4. no vitamins;

5. biotin only;

6. inositol only;

7. nicotinic acid only;

8. pyridoxine only;

9. AS-1 vitamin mixture less thiamin;

10. AS-1 vitamin mixture less thiamin and biotin.

Torpedospora radiata was plated in four replicates on strengths 1/4 and 3/4 AS-1 containing vitamin mixes corresponding to 1 and 3 above. All plates were incubated 13 days at 30 C. Colony descriptions were recorded. Inoculation was by method IV.

Experiment 6

The periphery of a colony of T. radiata which had developed on vitamin-free strength 1/4 AS-1, at 20 C, was used as a source of inoculum for one plate each of vitamin-free and vitamin-complete strength 1/4 AS-1, and these subcultures were incubated at 20 C. In like manner, inocula from peripheries of colonies which had developed at 25, 30, and 35 C on strength 1/4 AS-1 plates, were subcultured to vitamin-free and vitamin-complete strength 1/4 AS-1 plates, which were then incubated at the temperatures at which inocula had developed. This cross-inoculation was repeated, using as inocula colonies from vitamin-free 3/4 AS-1, vitamin-containing 1/4, and 3/4 AS-1, which had been incubated at 20, 25, 30, and 35 C. Thus, every combination of temperature (20, 25, 30, and 35 C), of salinity (1/4 and 3/4 strength media), and vitamin content (with and without vitamins), was subcultured to one plate each of a vitamin-free and vitamin-complete medium of the strength upon which the inoculum developed, and was incubated at the temperature at which the inoculum had developed.

This was run in three replicates, as three colonies on one plate, and was not repeated. Diameters and colony densities were noted at 13 days. The procedure was repeated identically using Halosphaeriopsis.

Experiment 7

Torpedospora radiata was placed, by inoculation method IV, into 12 200 ml flasks each of 1/4 and 3/4 AS-1 containing:

1. no vitamins;
2. thiamin 100 ug/liter and biotin 5 ug/ liter;
3. biotin 5 ug/liter;
4. thiamin 100 ug/liter;

These were incubated at 30 C, and harvests made at 15, 26, and 42 days. Each harvest resulted in weights for at least three, and most often four, replicates of each condition of salinity and vitamin content. Four replicates of each condition were set up, in anticipation of contamination, which was rare. Due to contamination, however, some averages recorded are for three replicates, and others for four. Initial pH was taken. Cultures were not agitated during the incubation period. When harvests were made, culture, filtrates were collected prior to washing the mycelium, and a cold iodoform test was run, as follows: 3 % iodine in 6 % potassium iodide was mixed one to ten with the filtrate; this was made strongly alkaline with 20 % NaOH; yellow precipitate and iodoform odor were noted. No melting point was taken of the suspected iodoform. This experiment, as described above, was repeated at 20 C.

A similar experiment was run using Halosphaeriopsis in 1/4 and 3/4 AS-1 containing:

1. thiamin;
2. pyridoxine;
3. no vitamins.

This was run at 20 and 30 C, and harvests made at 26, 36, and 44 days.

Experiment 8

In this three part experiment, the tris content of all strengths of AS-1 was held constant at 0.1 %. Initial and final pH of media was determined. Still-cultures were grown in 125 ml flasks, and harvests in all cases were at 20 days. *Torpedospora* was inoculated by method IV, and *Halosphaeriopsis* by method I. There were five replicates of each condition in each of two runs. Cold iodoform tests were run on filtrates, as described in Experiment 7.

a. *Torpedospora* was cultured in strengths 1/4, 1/2, and 1 AS-1, with thiamin constant at 100 ug/liter. Biotin was varied in all salinities as 5, 0.5, 0.05, 0.005, and 0.000, in ug/liter.

b. *Torpedospora* was cultured in these three strengths of AS-1, with biotin constant at 5 ug/liter. Thiamin was varied as 100, 10, 1, 0.1, and 0.0, in ug/liter, the greatest quantity serving as the repeat of the highest biotin level in part a, as these were identical media.

c. *Halosphaeriopsis* was cultured as *T. radiata* above, at three salinities and constant pH, but no biotin was added to the medium. The only vitamin added was thiamin, which varied 100, 10, 1, 0.1, and 0.0, in ug/liter.

Experiment 9

Strength 1/4 AS-1 containing no tris buffer was used in 125 ml flasks for still cultures at 25 C. A measured amount of C.P. $CaCO_3$ was placed into each flask prior to autoclaving. Initial and final pH determinations were made. Six replicates of each condition were set up, and harvests were all at 21 days. The experiment was not repeated. Before harvesting, 10 % of concentrated HCl, reagent grade, was placed into each flask in sufficient quantity to put all calcium into solution. After this was accomplished,

harvesting proceeded in the usual manner. No iodoform tests were run. Inoculation was as in Experiment 8. There were three parts to this experiment:

a. Torpedospora was cultured with thiamin constant at 100 ug/liter, and biotin was varied 5, 0.5, 0.05, 0.005, and 0.000 in ug/liter;

b. Torpedospora was cultured with biotin constant at 5 ug/ liter and thiamin varied 100, 10, 1, 0.1, and 0.0 in ug/liter, the highest concentration serving as a partial repeat of part a of this experiment;

c. Halosphaeriopsis was cultured with thiamin as the only vitamin added. Thiamin varied 100, 10, 1, 0.1, and 0.0 in ug/liter.

Experiment 10

Still cultures in 125 ml flasks were grown at 25 C for 21 days. AS-1 was used in strengths 1/4, 1/2, and 1, but pH was constant for all flasks (tris 0.1 %). Initial and final pH determinations were made. Vitamin level was constant, and high, as biotin 5 ug/liter and thiamin 100 ug/liter. Glucose was constant as 5 g/liter, but added nitrogen and phosphorus, per liter basis, varied as follows:

AS-1, modified	$NaNO_3$	$NH_4H_2PO_4$	$(NH_4)_2HPO_4$	Total N	Total P
1.	4 mg	0.37 mg	none	0.7 mg	0.1 mg
2.	0.054 mg	none	0.004 mg	10 ug	1 ug
3. (control)	none	none	none	none added	none added

These media, therefore, contained inorganic nitrogen and phosphorus in amounts comparable to those in the sea (Sverdrup, 1942); both maximum and minimum levels, plus a control. It will also be noted that, as is characteristic of the sea, the $NH_4:NO_3$ ratio increased as total nitrogen decreased. These fungi are reported to utilize either source (Johnson et al, 1959), and an attempt was made to better simulate natural conditions.

Inoculation was by method IV. Five replicates of each condition were run in duplicate. Mycelia were examined microscopically for conidia.

Experiment 11

AS-1 strength 1 was used with glucose 5 g/liter, thiamin 100 ug/liter, and the AS-1 level of phosphorus was added as K_2HPO_4. Biotin was varied 0.5, 0.05, 0.005, and 0.000, in ug/liter. On the basis of the AS-1 0.140 g/liter of nitrogen, this element was added as follows:

1. all $NaNO_3$;
2. half as $NaNO_3$ and half (by nitrogen content) as aspartic acid;
3. half as $NaNO_3$ and half (by nitrogen content) as casein hydrolysate prepared by boiling Pfanstiehl vitamin free casein in 6N reagen grade HCl for six hours and neutralizing with C.P. NaOH and outside indicator.

No ammonium nitrogen was used. Flasks containing 25 ml of media were inoculated by method II, and then incubated for 19 days at 25 C. Five replicates of each condition were duplicated, including preparation of the hydrolysate.

Experiment 12

AS-1 strength 1 only was used in 18/150 mm screw cap tubes, which were incubated at 25 C in a slanting position. Nitrogen and phosphorus content of all media was standard for AS-1, but 20 different combinations of carbon source and vitamin content were set up. Carbon sources tested for vitamins were as follows:

a. 3/16 by approximately 2 inch balsa stick;
b. approximately 1/8 by 2 inch Puritan pine applicator stick;
c. two 5/25 mm strips of Whatman No. 1 chromatography paper;
d. a small measure of Whatman No. 1 cellulose;
e. C.P. glucose, 0.5 %.

These carbon sources were placed into tubes of AS-1 and autoclaved along

with other ingredients. Each of these five carbon sources contained, in addition, each of four vitamin mixes: biotin 0.5 ug/liter and thiamin 10 ug/liter; biotin 0.5 ug/liter; thiamin 10 ug/liter; no vitamins added. Five replicates of each of these 20 combinations was inoculated by method II with T. radiata, and five replicates each of ten combinations not containing biotin were inoculated by method I with Halosphaeriopsis. Care was taken to prevent liquid from coming into contact with washers of the screw caps, lest these furnish vitamins to the media. Tubes containing glucose served as a control for such chemical contamination. Relative growth was recorded at 20 days, and tubes discarded. When this entire experiment was repeated, however, it was decided to hold tubes longer than 20 days to observe fructification. Photographs were taken of several of these tubes.

Experiment 13

Several variations of the basic AS-1 formula were prepared, but the C:N:P:vitamins system was held constant. To 1/4 strength AS-1, enough metals and chelator, minor, and major systems were added to make the medium strength 1/2 with respect to all systems but the buffer, which remained at 1/4 strength. To more 1/4 AS-1, all systems but the buffer were built up to strength 1. This was also done, keeping in turn metals and chelator, minor, and major at 1/4 strength while all other systems were built up to 1/2 and 1. To determine the exact amounts of each substance present in the eight media which were prepared in this manner, reference can be made to Appendixes V and VI. For the purposes of this survey test, however, it was considered necessary to know only the relative proportions of ingredients present. The proportions of ingredients in each medium are given in Table 13-A.

Fifteen replicates of each of these eight conditions, plus fifteen replicates each of 1/4, 1/2, and 1 AS-1 comparison plates were inoculated by method IV with T. radiata. Five replicates of each of these 11 conditions were incubated at 20 C, five at 30 C, and five at 35 C. This experiment was repeated for Halosphaeriopsis and C. globosum. Diameters of the marine species were taken at 12 days, and those of C. globosum at six days.

Experiment 14

Various ingredients of the major system, and another monovalent cation, lithium, were added to 1/4 AS-1 in the following manner:

Medium No. 1 - Strength 1/4 AS-1 comparison plates;

Medium No. 2 - Strength 1 AS-1 comparison plates;

Medium No. 3 - Enough of the entire major system (Na, K, Mg, Ca, SO_4, and Cl ions) was added to 1/4 AS-1 to build up only the strength of the major system to 1. Buffer, metals, and minor systems remained at 1/4 strength;

Medium No. 4 - All ingredients of AS-1 except NaCl remained at 1/4 strength. Enough NaCl was added to make the medium strength 1 in that salt, and to furnish a sodium-ion molar equivalent of the other major system cations which were not added. Dissociation and ionic strength were not considered;

Medium No. 5 - Strength 1/4 AS-1 was made strength 1 in major system ingredients only, but molar equivalents of LiCl and $LiSO_4$ replaced all NaCl and $NaSO_4$ in the medium but that furnished by the 1/4 strength base;

Medium No. 6 - Strength 1/4 AS-1 was made strength 1 in Ca, Mg, and K only; all were added as chlorides.

In Table 14-A media 1-6 are referred to as additives 1-6. Ten replicates of each of these six media were inoculated by method IV with T. radiata.

Five replicates of each of these six media were incubated at 20 C, and five of each at 35 C. This experiment was repeated for *Halosphaeriopsis* and *C. globosum*. Diameters of the marine species were taken after 12 days of growth, and those of *C. globosum* after six days.

Experiment 15

a. NS-1 agar, strengths 0 through 2, was pipetted into clean deep-well slides, which were placed into Petri dishes and autoclaved. The deep-well contents were allowed to solidify, and then were inoculated with *C. globosum* ascospores. Coverslips were placed aseptically over deep-wells, and observations of the progress of ascospores germination were made at 2, 4, 8, 24, and 48 hours. Incubation was at 25 C, in subdued daylight. Five replicates of each salinity were made.

b. Natural sea water (salinity 26 o/oo) media of three different nutrient constitutions, each containing seven different concentrations of tris, were inoculated by method III with *C. globosum*. Media A-1 through A-7 were NS-1 plus varying amounts of tris. Media B-1 through B-7 were NS-7 plus varying amounts of tris; a circle of sterile filter paper was placed aseptically on the surface of each plate of solidified yeast extract-sea-water agar. Media C-1 through C-7 contained a circle of filter paper, as B media, above, but 0.157 g/liter of NH_4NO_3 and 0.037 g/liter of $NH_4H_2PO_4$ was substituted for yeast extract; N:P content of this medium was one-half that of AS-1. Media A, B, and C, 1-7 contained tris 0.000, 0.07, 0.125, 0.25, 0.50, 1.0 and 2.0 g/liter, respectively. Five replicates of each of these 21 combinations of pH and nutrients were incubated at 25 C, in the dark, for four weeks. Perithecia were then counted, and some from each plate crushed under coverslips and examined for ascospores. The initial pH of all 21 combinations was taken, and the final pH of plates containing 0.2 % tris.

c. The continual-flow sea water apparatus of Johnson and Gold (1958) was used, with slight modifications in the original design. An outlet was inserted between the reservoir and the first culture chamber. An air line was passed through the Sigmamotor pump, through a cotton air filter cartridge, and terminated in the reservoir as a piece of perforated tygon tubing which was plugged with a short glass rod. A good head of air was maintained through this perforated tubing throughout the period of circulation, the Sigmamotor functioning both as air pump and water pump. Pure cultures of Halosphaeriopsis and C. globosum were started on sycamore and balsa, respectively, in salinity 20 o/oo sea water containing 0.01 g/liter yeast extract. Other wooden blocks were autoclaved in their respective culture chambers. The reservoir and circulation tubing was sterilized with 70 % isopropyl alcohol, and the alcohol was washed out of the system with sterile tap water. The four culture chambers were inserted into the system and, in order with the direction of flow, contained:

Chamber 1 - pine, sycamore, yellow poplar;

Chamber 2 - basswood, cork, maple;

Chamber 3 - balsa containing 500+ mature perithecia of C. globosum;

Chamber 4 - sycamore containing vegetative hyphae and ascocarps of Halosphaeriopsis; only about ten ascocarps appeared to be near maturity.

Into the reservoir was placed approximately ten liters of fresh, unsterilized, unfiltered, brackish water, (salinity 18 o/oo), which had been collected by submerging a sterile carboy at Gloucester Point, Virginia. A sterile solution containing 10 g of tris and 0.1 g of yeast extract was added to the fluid, and circulation was begun. Initial pH was taken, and periodic observations were recorded. After one month of constant circulation, 500 ml of this fluid was placed into a flask, 9 g of agar added, and boiled into solution. This sea-water

agar was autoclaved. Several plates were poured, and sterile filter paper was placed on the surface of half of these. Halosphaeriopsis, C. globosum, and the raw circulation fluid were planted, individually, on each of these two media. These plates were examined for growth and ascocarp production. Chaetomium ascospores that developed on the filter paper-sea-water agar medium were examined for ascospores. The final pH of the fluid was taken at the end of the month of circulation. The chambers were drained by clamping off the tubing between the reservoir and the first chamber, opening the outlet which was inserted in that position, and continuing to operate the pump. All fluid in the chambers was returned to the reservoir. The added outlet was plugged with cotton and sterile air was pumped through the chambers for an additional month. The blocks were then examined for ascocarps of the two known species and for other organisms. The balsa block that had originally contained C. globosum was used as inoculum for malt extract agar plates.

Experiment 16

Potassium requirements in media with and without tris were determined, and also potassium requirements at high and low salinity. Strength 1/4 AS-1 liquid was modified to contain 1000, 100, 10, 1, and 0.0 mg/liter potassium, as chloride. The normal amount for 1/4 AS-1 is approximately 100 mg/liter. Calcium carbonate was used as a buffer in one set of these 200 ml flasks of modified AS-1, and both $CaCO_3$ and 0.1 % tris were used in the other set. Strength 1 AS-1 was modified to contain 400, 100, and 40 mg/liter potassium (as chloride), 400 mg/liter being the normal amount. These latter media contained only $CaCO_3$ as a buffer. Initial and final pH was determined for all media. Five replicates of each of these 16 conditions were inoculated by method I with C. globosum, and likewise

with Halosphaeriopsis. Still cultures were incubated at 25 C. Chaetomium was harvested at 15 days, and Halosphaeriopsis at 20 days, $CaCO_3$ being put into solution with HCl prior to harvesting.

Experiment 17

This was a determination of salinity optima for fructification of T. radiata at 20, 25, 30, and 35 C. NS-7 agar in 3.3 ml amounts was used in 12/100 mm cotton-plugged tubes. Whatman paper slips were 6/25 mm, and slants were made by resting the lips of tubes 6 mm above the horizontal. Ten tubes of each of seven strengths of NS-7, 0-2, were made for each of the four temperatures, for a total of 280 tubes. An additional 280 tubes of NS-7 containing 0.1 % tris were made. All tubes were inoculated by method II and incubated in the dark for 30 days. Ascocarp production was then evaluated as follows. All tubes were given a preliminary examination under 20X magnification, and a bank of eight standards was selected. These standards were considered representative of various degrees of fruiting, from none to the most luxuriant.

Standard No.	Description
0	no ascocarps observed
1	trace of immature ascocarps
2	few immature ascocarps
3	several immature, but no mature ascocarps
4	few mature and few immature ascocarps, but mature rather small
5	few mature and many immature ascocarps, but mature rather small
6	many mature and many immature ascocarps, but mature rather small
7	many mature and many immature ascocarps, and mature large
8	luxuriant production of large, mature ascocarps

Each tube was compared to the bank of standards under 20X magnification, and

a whole number or decimal fraction between 0 and 8 was assigned to each tube. The average of these numbers for each set of ten replicates of a given condition was recorded as the relative degree of fruiting for that condition. Therefore, both numbers, and maturity of ascocarps as estimated from their size and color, were used as criteria for estimating optima, but maturity was considered a better criterion than numbers.

At the termination of this evaluation, papers were removed from tubes, perithecia were picked from them and replaced into their respective tubes, and papers were discarded. These tubes were then prepared according to the method described in Experiment 18, and mature ascospores per HPF were determined. Ascospores in 30 HPF were counted for each tube, and the average per HPF was recorded.

Experiment 18

This was designed to determine the optimum salinities for sexual and asexual reproduction of Halosphaeriopsis at 20, 25, 30, and 35 C. NS-1 was used in 3.3 ml amounts in 12/100 mm cotton-plugged tubes. Ten tubes of each of seven strengths, 0 through 2, were prepared for each temperature, for a total of 280 tubes. This entire operation was repeated using NS-1 containing 0.1 % tris, for a grand total of 560 tubes, half with tris and half without. These were autoclaved and slanted, the lip 6 mm above the horizontal. All tubes were inoculated by method I and incubated in the dark for 42 days. Initial and final pH was determined. After the incubation period, contaminated tubes were discarded, and six tubes were selected at random from each set of replicates of a given condition. This was accomplished by scrambling the tubes of a set and selecting six without looking at the pile. The remaining tubes were used for pH determinations, and were held in reserve in case others were broken during centrifugation, or were otherwise lost in the process of evaluating sporulation.

After the sets of six replicates were selected, all tubes were placed in racks in a 100 C water bath, the level of water approximately even with the tops of the slants. Cotton plugs were discarded, and 5 ml of boiling tap water from the bath was added to each tube. Slants were loosened with a probe, and the tubes were heated in the water bath until all agar was in solution. A set of six replicates was removed from the water bath and centrifuged at approximately 2,000 rpm for about three minutes, and the supernatant was then poured out. The entire mycelium remained together in the bottom of the tube. Boiling did not disrupt the perithecia, so ascospores were not lost in the washing process. Microscopic examination of this and subsequent wash waters revealed no conidia or mature ascospores, but only a rare bit of vegetative hypha or an immature ascus. Brownish soluble pigment was present in the first wash water. An additional 5 ml of hot water from the bath was pipetted into each tube of the set, and the six tubes replaced into the bath. All other sets were treated similarly. Each set was washed and centrifuged four times, after which the concentration of agar in each tube was not sufficient to gel upon cooling. After the final centrifugation, 2 ml of hot water was added to each tube with a 5 ml pipette. Mycelium and the 2 ml of water from a single tube at a time were added to a 13/100 mm Pyrex tissue homogenizer, Corning No. 7725, and the mycelium was ground manually until no sizeable particles were observed. Clearance between the ground mortar tube and the pestle is maintained between 0.004 and 0.006 inches, which was sufficient to rupture perithecia and homogenize mycelia, but not sufficient to destroy spores, of the species tested.

After continued useage the clearance became too great for efficient homogenization. In the entire course of this investigation, in which over 500 mycelia were homogenized, only two homogenizers were used, and

only one of these became so worn that it was no longer efficient. This homogeniser may be electrically driven with a slip clutch, but such equipment was not available for trial. From one to three minutes of manual grinding was sufficient to homogenize most mycelia. Suspensions were replaced into their respective culture tubes, and set aside until all tubes had been so treated. The mortar and pestle were washed with tap water between treatments of individual tubes.

A tube of 2 ml of suspension was sealed with the thumb and vigorously shaken. With a 0.5 ml serological pipette, 0.5 ml of the suspension was quickly removed before the contents could settle appreciably, and 0.03 ml was placed on the left end of a one inch by three inches slide. The remaining 0.47 ml was replaced into the tube, and the tube was shaken again. This sampling process was repeated twice, so that three 0.03 ml samples were placed on the slide, one at each end and one in the middle. Glass coverslips (18 mm square), were carefully placed over each 0.03 ml sample, so that there were few air bubbles, and the suspension was distributed beneath the entire area of the coverslip.

All spores observed in ten high power fields per coverslip were counted. Thus,spores in 30 HPF were counted per tube, and six replicates of each condition resulted in averages based upon the spores observed in 180 HPF. Spores were counted in the same general areas of each coverslip, but random selection of fields was accomplished by not looking into the microscope until the mechanical stage was moved into position. General areas counted were: lower left, lower middle, lower right; middle left, center, and middle right; upper left, upper middle, and upper right; approximate center again. In each HPF selected in this random manner, all spores that were observed were counted, even if clumps were present.

Clumps were rare, however, even when counts were high. The only condition that caused a field to be rejected and another in the same general area randomly selected, was the presence of an air bubble.

Several immature ascospores and immature asci were observed, but these were not counted. All refractile bodies appearing as mature ascospores were counted. The word "mature" ascospores has been used, but this is not absolutely descriptive in many cases. Even though internal development of ascospores was not advanced, or appendages not distinct (boiling tended to make appendages poorly defined), a spore was counted if it appeared hyaline, fusiform, and constricted at the midseptum. Ascospores not counted were also hyaline, but were spherical to oblong, apparently aseptate, not constricted, and smaller than "mature" ascospores. Occasionally a group of ascospores was found clinging together, making the general outline of the destroyed ascus wall. In this case, each spore which met the standards of "maturity" previously described was counted.

Defining conidia was a greater problem than defining a mature ascospore, but due to the enormous numbers in which they are produced, it is doubtful that grave errors in counting occurred. Maturity of conidia was not considered. All cells recognized as conidia were counted. When a chain remained intact, each cell was counted individually, and the basal cell was not counted.

Counts of ascospores and conidia were made simultaneously. Conidia were counted with a hand tally while scanning the field, and ascospores were then counted. The total of each type of spore observed was recorded before proceeding to the next field. If a cell was distinguishable as a sexual or asexual spore, it was counted even if more than one-half of it lay out of the field. Focusing on two levels was occasionally necessary to count all spores.

One tube was selected from those several which had not been examined.

This was homogenized to the same degree as the others. Spores were counted in ten HPF of each of three 0.03 ml samples, and the average per HPF of these thirty fields was recorded. This was repeated, using the same suspension, until ten such averages had been recorded. These averages were then compared, for a rough index of the thoroughness of homogenization and precision of the sampling technic.

Experiment 19

Three media similar to NS-1 were prepared. Glucose and agar content were as for NS-1, but yeast extract was varied 100, 10, and 0.0 mg/liter. Ten tubes of each of these three nutrient levels were made in each of four strengths; 1/4, 1/2, 3/4, and 1. Tube size, time of incubation, method of inoculation, and method of spore evaluation was as in Experiment 18. No tubes contained tris. Initial and final pH determinations were made. All tubes were grown at 25 C, in the dark.

Experiment 20

Tubes of strengths 1/4, 1/2, 3/4, and 1 NS-7 were prepared, with and without 0.1 % tris. These were inoculated by method I with Halosphaeriopsis, and grown in ten replicates of each condition at 20 C and 25 C for 40 days. Six tubes of each type were randomly selected from each set of ten tubes. Papers were easily removed, and perithecia on the paper and in the tube were counted. Mature perithecia were picked from papers and replaced into their respective tubes. Papers were discarded. Spores were then evaluated as in Experiment 18.

Attempts were also made to evaluate spores in the entire contents of the remaining tubes, as well as those in some few tubes of T. radiata that were saved from Experiment 17. With papers still in the tubes, agar was removed as in Experiment 18, and Schweitzer's reagent added. The papers

and fungi were allowed to stand in the reagent overnight. Attempts were then made to grind the entire contents of tubes in the tissue homogenizer, and suspensions were examined microscopically as in Experiment 18.

Experiment 21

Torpedospora and Halosphaeriopsis were grown in Petri plates of each of seven strengths of NS-1 containing 0.1 % tris, at 20, 25, and 30 C. Initial pH was taken. Colony diameters were recorded after 12 days.

Experiment 22

Torpedospora radiata and Halosphaeriopsis were each planted, by method I, on twenty replicates of each of several natural sea water (salinity 34 o/oo) media. Ten of each type were incubated in the dark, and ten in natural alternating daylight and darkness, all at about 25 C. Media numbered 1 through 8 were slants containing 1.8 % agar, dispensed in 3.3 ml amounts in 12/100 mm cotton-plugged tubes. Media numbered 9 through 16 were liquids, containing either balsa or pine sticks, dispensed in 7 ml amounts in 15/150 mm screw cap tubes. Odd numbered liquid media (9, 11, 13, 15) were incubated in a slanting position. Even numbered liquid media (10, 12, 14, 16) were incubated in a slanting position for only one week; the liquid contents of these tubes were then poured out, and the wet stick incubated in an upright position until it was examined. Media 2, 4, 6, 8, 11, 12, 15, and 16 contained tris 0.1 %.

Three different nutrient bases were used, but carbon source substitutions, tris content, and conditions of incubation were the same for all media which were similarly numbered. Nutritive bases for NS media, J media, and K media are listed below.

Medium J-1 (after Terry W. Johnson, Jr.; original formula)

Based on one liter of aged sea water

Bacto-peptone	0.1 g
K_2HPO_4	0.05 g

Ferric citrate	0.01 g
agar	18 g
glucose	1 g

Medium K-1

Based on one liter of aged sea water

Bacto-peptone	0.2 g
$Fe(NO_3)_3 9H_2O$	0.0072 g
$NH_4H_2PO_4$	0.0185 g
sodium succinate	0.01 g
agar	18 g
glucose	2 g

Medium NS-1

Based on one liter of aged sea water

BBL yeast extract	1 g
agar	18 g
glucose	5 g

To make NS, J, or K media 2-16, glucose and agar were varied.

J, K, or NS No.	Description
1	as presented on preceding page
2	as No. 1, plus tris
3	as No. 1, Whatman No. 1 cellulose substituted for glucose
4	as No. 3, plus tris
5	as No. 3, plus 0.01 % glucose
6	as No. 5, plus tris
7	as No. 1, but slip of Whatman No. 1 chromatography paper substituted for glucose.
8	as No. 7, plus tris
9	liquid medium; no agar or glucose; Puritan pine applicator stick in liquid nutritive base.

10	as No. 9, but liquid poured out after one week incubation
11	as No. 9, plus tris
12	as No. 10, plus tris
13	as No. 9, but 1/16 inch square balsa stick substituted for pine stick
14	as No. 10, but balsa stick substituted for pine stick
15	as No. 11, but balsa stick substituted for pine stick
16	as No. 12, but balsa stick substituted for pine stick

Thus, all media of the same number were similar in physical state, tris content, carbon source, and method of incubation. NS, J, and K media differed in that they contained respectively, a high, low, and intermediate nutrient level base.

After the incubation period, several sticks of the stick-liquid media supported ascocarps. Liquid was poured from the tubes of some of these, and the tubes containing sticks of ascocarps were filled with the following preservative:

glacial acetic acid	30 ml
glycerine	200 ml
formalin	50 ml
artificial sea water (AS-1 base), 34.5 o/oo	720 ml

After four months, sticks were removed and a few observations were made of the morphology of the fungi.

Several sticks of Halosphaeriopsis ascocarps from media NS and K numbers 9, 10, and 13, were placed into a fresh water aquarium, and the feeding activities of fishes were observed.

A semisynthetic medium was also devised and tested.

Medium AS-2E

To one liter of 3/4 strength AS-1 basal medium were added 100 ug of thiamin,

5 ug of biotin, and 1 ml of the following stock nutrient solution.

Sodium succinate	0.01	g
ammonium acetate	0.01	g
ammonium citrate	0.01	g
L-glutamic acid	0.01	g
l-asparagine	0.01	g
L-tyrosine	0.01	g
L-leucine	0.01	g
glycine	0.01	g
inulin	0.05	g
cellobiose	0.02	g
xylose	0.05	g
glucose	0.1	g
KNO_3	0.25	g
$NH_4H_2PO_4$	0.07	g
distilled water	100	ml

To this solution was added 18 g Bacto-agar, and 7 ml slants were made in cotton-plugged tubes. Whatman chromatography paper slips (6/25 mm) were added aseptically to one-half of these slants. Inoculation of these slants with <u>Halosphaeriopsis</u> and <u>T. radiata</u> was by method IV. Tubes were incubated at 25 C in the dark, except for periodic examination, for four months.

RESULTS

Tables and Figures have been placed in a separate section, and in the text of the Results, these will be referred to by number. The number of a Table or Figure corresponds with the Experiment of the same number in the Materials and Methods and Results sections.

Experiment 1

Vegetative Phoma pattern affinities of the three species were sought, and the salinity optima for fructification of C. globosum, using a high nutrient level natural sea water medium.

On NS-1 a salinity approximately one-quarter that of "normal" sea water was optimum for the vegetative growth of C. globosum in the temperature range 20-30 C, (Table 1-A, Figure 1-A). At 35 C the optimum salinity lay between strengths 3/4 and 1, or at approximately 30 o/oo. Fructification decreased as salinity increased, regardless of temperature, and at 35 C no fruiting occurred, (Table 1-C, Figure 1-C). The optimum temperature for fructification was 25 C, and for vegetative growth was 30 C. Colonies on 25 C strength 1½ and 2 were quite regular, puffy, and white, but colonies on NS-1 were generally flat, spreading, yellow to white, and irregular.

The 20 C salinity optimum of T. radiata was approximately 17 o/oo. At 25 C (Table 1-D, Figure 1-D) this optimum did not shift, as determined by the average of 12 day diameters, but the range of salinity tolerance was extended. Fifteen day diameters of one run (Table 1-E, Figure 1-E) indicated that the 25 C salinity optimum is 51 o/oo. The density of all 25 C colonies, as determined visually, was similar for most salinities, but the 51 o/oo colonies were apparently less dense than those from lower

salinity plates. Furthermore, in the lower salinity range more energy may have been devoted to the development of reproductive structures, though the fungus did not fruit in the absence of cellulose. The salinity optimum shifted to strength 3/4 at 30 C, the optimum temperature for vegetative growth. Colonies of _T. radiata_ were dense, white, and disk-shaped, with few to no visible aerial hyphae. Some brown hyphae and orange pigment developed in the centers of the larger colonies.

The optimum salinity for vegetative growth of _Halosphaeriopsis_, in the 20-30 C temperature range, was approximately one-quarter that of normal sea water, and at 35 C approximately one-half. The range of salinity tolerance was greatest at 25 C and 30 C, the latter being the optimum temperature for vegetative growth. Colonies from different salinities and temperatures generally appeared to be of similar densities. Colonies were composed of gray to gray-green aerial hyphae arising from a dense mat of dark green to black hyphae. Many conidia were produced, and were most abundant in the lower salinity ranges.

When replicate plates of _C. globosum_ were stacked three high in the 35 C incubator, growth in the bottom plate of any given stack was almost invariably less than that on the middle and top plates. The cause of this phenomenon was not determined. Data on growth in one such set of stacked plates are recorded in Table 1-B, and calculations derived from those data are presented here. Diameters of middle plate colonies were approximately 70 % of diameters on top plates, with the exception of diameters of strength 2 middle plates, which were 45 % of strength top plates. Diameters of bottom plate colonies were 60-80 % of those on middle plates, and approximately 50 % of those on top plates. Strength 2 bottom plates were 27 % of top-plate diameters. Surface areas of bottom-plate colonies were about 30 % of top plates, except for strength 2, which was only 10 % of the top-plate

colony. Except for strength 2 and possibly some strength 1½ plates an increase in salinity did not intensify the decrease in growth in bottom plates. The salinity optimum at 35 C, as determined by diameters on either the top, middle, or bottom plates, was 51 o/oo. Though decreases in bottom plates were not as marked on vitamin-free AS-1 (Table 3-B), this "stacking" phenomenon was consistent on that medium.

Stacking influenced the growth of T. radiata to a lesser degree, and did not influence the diameters of Halosphaeriopsis colonies. Torpedospora radiata 35 C plates of run number one, Table 1-D, from top to bottom of stacks were: strength 3/4 - 13, 10, 6; strength 1 - 8, 7, 6; strength 1½ - 5, 4, 3.

The reaction of NS-1 was in the slightly acidic to neutral range. The initial pH of strength 0 was 6.4-6.8, and of other strengths was 6.6-7.2.

Experiment 2

The objectives of this were the same as those in Experiment 1, but AS-1 artificial sea water was used instead of NS-1.

The temperature optimum of 30 C, the form of colonies, and the amount of vegetative growth and fructification of C. globosum on AS-1 (Tables 2-A and 2-B, Figure 2-A) was similar to that on NS-1. Salinity optima, however, shifted more in response to temperature increases. The 20 C optimum was between strengths 1/4 and 1/2, the 25 C and 30 C optima between 1/2 and 3/4, and the 35 C optimum between 3/4 and 1.

The amount of growth and form of colonies of the marine species on AS-1 was also similar to that on NS-1. The salinity optima of T. radiata (Tables 2-C & 2-D, Figure 2-C), were strength 1/2 at 20 C, between 1/2 and 3/4 at 25 C, 3/4 at 30 C and between 3/4 and 1 at 35 C.

The greatest diameters of Halosphaeriopsis (Tables 2-E, 2-F, Figure 2-E) in the 20 C to 30 C range were between strengths 1/2 and 3/4. At 35 C diameters of Halosphaeriopsis colonies were greatest between 3/4 and 1; at strength 1/2 there was a marked decrease in growth, and another increase at 1/4. The dip in the 35 C curve was consistent in AS-1, but absent in NS-1 (Figure 1-F).

A pH gradient, as well as a salinity gradient, existed in the AS-1 dilution series. AS-1 strength 1/16 had a pH of 6.2-6.4; 1/4, 6.6-7.0; 1/2, 7.4-7.8; and 8.0-8.4 in other strengths.

Experiment 3

This was a screening test to indicate whether any of the three species is vitamin deficient, and to determine in a general way what effect temperature and salinity have on those deficiencies, and what effect vitamin level has on salinity optima.

Removal of vitamins from AS-1 (Table 3-A, Figure 3-A) depressed growth of C. globosum and caused salinity optima to shift to the left. Though the fungus is not normally vitamin deficient, growth was poor in salinities greater than that in strength 1/4 if temperatures were below 25 C. Growth and salinity optima at 30 C and 35 C were more nearly comparable to those on vitamin complete AS-1, but were still depressed and shifted somewhat to the left. After 12 days the amount of growth was nearly that after six days on vitamin media, but salinity optima were still to the left in the less saline range. Colonies were generally similar in appearance to those of vitamin-complete AS-1, but the 20 C strength $1\frac{1}{2}$ and 2 colonies were quite sparse. Fructification (Table 3-C) was less than that on AS-1 with vitamins, and decreased as salinity increased.

All T. radiata colonies on AS-1 less vitamins were white and growth

was sparse. The only appreciable growth occurred at 20 C on strengths 1/16, 1/4, and 1/2. As growth at other temperatures was so sparse, little faith is placed in those diameters as an index of growth. It did appear, however that the 25 C salinity optimum was at 1/4, and that the 30 C and 35 C optima were at 1½. From these data it was concluded that T. radiata is deficient for one or more vitamins, and that lower concentrations of these vitamins are required at a low salinity, if temperature is also low.

Diameters of Halosphaeriopsis on AS-1 less vitamins (Table 3-E, Figure 3-E) were comparable to those on AS-1 vitamin media, but growth was sparse. Salinity optima were shifted to the left at 20 C-30 C, but not at 35 C. The strength 1/2 dip was present in the 35 C curve.

Colony and microscopic morphology of T. radiata and Halosphaeriopsis on AS-1, with and without vitamins, is recorded in Tables 3-F and 3-G. Drawings of T. radiata are presented in Figure 3-F. AS-1 plus vitamins supported colonies similar in appearance to those on NS-1. In the absence of vitamins colonies were lighter in color and growth was sparse. Production of chlamydospores of T. radiata, and of conidia by Halosphaeriopsis decreased as salinity increased. The initial pH of vitamin-free media was the same as that of vitamin-containing media.

Experiment 4

This was the first of three screening tests (Experiments 4, 5, and 6) run to determine specifically for which vitamins T. radiata and Halosphaeriopsis are deficient, and to indicate the influence of temperature and salinity on these deficiencies.

From Table 4-A and Figure 4-A it appears that T. radiata is totally deficient for biotin and partially deficient for thiamin. Growth in the absence of biotin was as poor as that in the absence of all vitamins. In

the absence of biotin growth was best at a low salinity and temperature. Colony diameters on strength 1/4 and 3/4 less thiamin, at 20, 25, and 30 C were comparable to those on vitamin-complete media of the same strengths, but growth was sparse. The fungus did not appear to be deficient for vitamins other than biotin and thiamin.

In the absence of thiamin, diameters of Halosphaeriopsis on strength 1/4 were comparable to those on vitamin-complete media incubated at the same temperatures. The 1/4 medium, less thiamin, supported diameters smaller than those on 3/4 media with thiamin. Growth was sparse in the absence of thiamin. In the absence of all vitamins, diameters on both 1/4 and 3/4 were less than those on vitamin media of the same strengths, and growth was sparse. It appeared that Halosphaeriopsis is deficient only for thiamin, and that the presence of other vitamins and a low salinity decreases thiamin requirements.

Experiment 5

This was the second screening test to determine the specific vitamin requirements of the marine species under various conditions of salinity and temperature. In particular it was a search for vitamins which increase the efficiency of thiamin for Halosphaeriopsis.

Colonies of T. radiata on AS-1 plus only thiamin and biotin were identical in form and diameter to those on media containing the entire AS-1 mixture of five vitamins. This fungus apparently was not inhibited by the presence of vitamins which it could synthesize.

Halosphaeriopsis colonies which developed on AS-1 plus thiamin only, were similar in appearance to those on media containing the complete vitamin mixture. In the absence of thiamin, growth was increased slightly by the presence of several other vitamins, added singly or in combinations

(Table 5-A). In the absence of thiamin, the addition of biotin resulted in greater increase in diameter than other single additions, but this growth was unusually sparse. Judging from both diameter and density, the addition of pyridoxine appeared to result in the greatest increase of growth in the absence of thiamin.

Experiment 6

This was the third and final vitamin screening test, and essentially was a cross-inoculation test to determine whether diameters recorded in Experiments 3, 4, and 5 were due to residual vitamin content of method IV inoculum.

This test proved that the growth of T. radiata on vitamin-free AS-1 in previous experiments was made possible by the presence of vitamins in the inoculum. Inocula from vitamin-free plates did not grow when subcultured to other vitamin-free plates, but small colonies developed from inocula derived from vitamin-containing plates (Table 6-A).

Halosphaeriopsis inocula derived from vitamin free plates did not grow as well on vitamin-free plates as did inocula from vitamin-containing plates, but some growth did occur (Table 6-B). The reasons for this small amount of growth were not apparent, but there are several possible explanations. Halosphaeriopsis may concentrate thiamin, or thiamin may be present in AS-1 reagents in trace amounts, or this fungus may be only partially deficient under conditions of low salinity and low or optimum temperature.

Experiment 7

This is the first of more precise studies of vitamin deficiencies in the marine species. The experiment was primarily designed to study the ability of T. radiata to synthesize thiamin and biotin, and of Halosphaeriopsis to synthesize thiamin, in a six weeks period under various conditions of

salinity, temperature, and pH.

In the absence of biotin, even though thiamin was present, T. radiata did not grow after six weeks at any temperature or salinity tested (Table 7-A). It was concluded that this fungus is totally deficient for biotin. Growth was not appreciable when biotin was the only vitamin present, but dry weights were sufficiently large to indicate a partial rather than a total deficiency for thiamin. In the absence of thiamin growth was poorest in strength 3/4 at 30 C. Because the concentration of buffer was low in all strength 1/4 media, little could be concluded regarding the influence of temperature and salinity on thiamin demand. Early in the incubation period, pH must have become limiting in strength 1/4 flasks. Filtrates from several flasks gave a slightly positive iodoform test, but this test was grossly positive in flasks containing only biotin.

After seven weeks Halosphaeriopsis had not grown appreciably in the absence of thiamin even though pyridoxine was present (Table 7-B). This suggests a total thiamin deficiency. The data from solid media in previous experiments suggested either a partial thiamin deficiency or traces of thiamin in AS-1 reagents such as agar. In the presence of thiamin, growth was poorest in strength 3/4 at 20 C. Weights from various salinities and temperatures were not comparable, due to the early limiting pH in strength 1/4 flasks. The iodoform test was slightly positive for filtrates of media that contained no thiamin.

Experiment 8

This was designed to determine the quantity of thiamin and biotin necessary for maximum growth of T. radiata, and of thiamin for Halosphaeriopsis, at 25 C and salinities approximately 8.5, 17, and 34 o/oo.

The 25 C salinity optimum of T. radiata was 17 o/oo in AS-1 containing 100 ug/liter of thiamin and either 5 or 0.5 ug/liter of biotin (Table 8-A,

Figure 8-A). Growth was best in strength 1/4 media when the concentration of biotin was reduced to 0.05 or 0.005 ug/liter. The final pH of strength 1/4 cultures containing these lower concentrations of biotin was also slightly lower than that of corresponding strength 1/2 and 1 cultures. The fungus did not grow in the absence of biotin. The iodoform test was most positive on filtrates of cultures containing 0.05 ug of biotin.

When biotin was held constant at 5 ug/liter, and thiamin was reduced to 10 or 1 ug/liter, growth was best in strength 1/4 media (Table 8-B, Figure 8-B). Little growth occurred in media containing 0.1 ug/liter or no thiamin. At these lowest levels, growth was best in strength 1 media, but many chlamydospores were produced in strength 1/4 flasks, and these were not recovered in the harvests. Also the final pH of 1/4 cultures was lower than that of cultures in strength 1. The iodoform test was strongly positive for filtrates of several flasks, but most strongly positive for those containing 1 ug/liter of thiamin.

Contrary to what was previously indicated by colony diameters on AS-1 agar, growth in the strength 1/4 medium containing 100 ug/liter of thiamin (Table 8-C, Figure 8-C) was much greater than that in strength 1/2 and 1. As the concentration of thiamin was reduced to 100 or 1 ug/liter, growth at all three salinities was comparable. No appreciable growth occurred in the absence of thiamin, or at a 0.1 ug/liter level. The iodoform test appeared to be most positive for filtrates of cultures containing 1 ug/liter of thiamin. Conidia developed in these media, and some harvest was lost as a result. Though it does appear that the data on solid media give a false impression of the effect of salinity on thiamin demand, the results on solid and liquid media so drastically contrast that further investigation seems warranted. This experiment should be redesigned, perhaps using inoculation method IV, a lower initial pH, and other nitrogen sources.

Experiment 9

As tris has not often been used as a buffer in nutritional studies of fungi (see Provasoli et al., 1957), this experiment was set up to determine whether results of Experiment 8 would be much different had a more conventional buffer been used.

Growth of the marine species in high vitamin level AS-1 with $CaCO_3$ buffer (Table 9-A) was comparable to growth in similar media with tris buffer (Tables 8-A, 8-B, & 8-C). As vitamin level was reduced, however, growth in $CaCO_3$ media was generally greater than that in tris media, and the partial thiamin deficiency of T. radiata was more obvious. Judging from final pH readings, one cause of this discrepancy may have been an early limiting pH in tris media.

Experiment 10

This was to determine what effects inorganic nitrogen and phosphorus levels have on salinity optima of T. radiata.

The fungus grew better in strength 1/2 AS-1 than in strengths 1/4 or 1 when nitrogen-phosphorus levels were 0.7:0.1 mg/liter or 10:1 ug/liter. In media to which no nitrogen or phosphorus had been added, growth was best in strength 1. This was probably due to nitrogen and phosphorus furnished by the reagents of AS-1 base. Growth in strength 1 was in all cases better than that in strength 1/4. In AS-1 containing 140 ug/liter of nitrogen as ammonium nitrate, growth in the strength 1/4 medium was greater than that in 1(Experiment 8).

Mycelia which developed in these predominantly nitrate-nitrogen media were darker than those from ammonium-nitrate media, but microscopic examination revealed no conidia.

Experiment 11

The biotin-sparing effects of three nitrogen sources were investigated. None of the three nitrogen sources tested replaced biotin completely (Table 11-A). Aspartic acid did replace this vitamin to a slight extent, and casein hydrolysate replaced it to a considerable extent. Because inoculation was by method II, a comparison of growth on these nitrogen sources to that on ammonium nitrate (Experiment 8) cannot be made. Method II probably results in much greater growth than method IV. Mycelia were darker on these nitrogen sources than on ammonium nitrate, but microscopic examination revealed no conidia.

Experiment 12

This was to determine whether thiamin and biotin are supplied by purified cellulose, filter paper, balsa wood, and pine wood in sufficient quantity to support marine fungi which are deficient for these vitamins. Data are presented in Table 12-A and Figures 12-A, B, C, D, E, and F.

The marine species grew poorly or not at all in AS-1 with glucose, filter paper, or cellulose, unless essential vitamins were also added to these media. Torpedospora radiata produced sparse white mycelia on these carbon sources if only biotin was added, but papers were highly degraded by the fungus. Vegetative growth was excellent in glucose media with added vitamins. Absence of growth in controls indicated that screw caps did not furnish biotin or thiamin to the media.

Growth on balsa was poorer than that on pine, but growth on these woods without added vitamins was as good and often better, than that on wood media with added vitamins. The woods contained substances which satisfied the vitamin requirements of the fungi tested, but it cannot be said from this evidence that these substances were actually thiamin and biotin. Essential vitamers and other organic compounds could produce similar results.

No asexual spores of _T. radiata_ were observed in these media, but after one month mature ascocarps were supported by the following, in order from the most abundant: filter paper plus biotin and thiamin; pine plus biotin; cellulose plus thiamin and biotin; balsa plus biotin; pine with no added vitamins; pine plus thiamin and biotin; balsa plus thiamin. Ascocarp counts were made from five replicate tubes of each condition, and mature ascospores were observed. As this experiment was terminated after 40 days of growth, the abilities of other media used, to support ascocarps of _T. radiata_, were not fairly tested. This fungus apparently does not fruit if glucose is the sole carbon source, but does fruit on cellulose of high purity.

Halosphaeriopsis produced conidia in all media in which it grew, but sparsely in the glucose-thiamin medium. Numerous mature ascocarps (ascospores observed) were produced on pine without thiamin, and fructification was nearly as good on pine with thiamin. A few ascocarps were produced on balsa plus thiamin. Ascocarps containing immature asci had begun to develop on paper and cellulose media containing thiamin, but mature ascospores had not developed in these at the time this experiment was terminated (40 days). Ascospores of _Halosphaeriopsis_ which had been introduced into media as inoculum were observed to have undergone bipolar germination and growth into mycelia. Only ascospores that had undergone unipolar germination were observed to remain for considerable periods of time in the solid media of Experiments 18, 19, 20, and 22, though no special effort was made to study the methods of ascospore germination of this species.

Experiment 13

From Experiment 2, it was obvious that requirements of the three species for certain constituents of AS-1 are temperature-affected.

Experiment 13 was a survey mineral nutrition study of the requirements of these fungi for constituents of AS-1 when temperatures are supra-optimal, optimum, and sub-optimal. Results of this experiment are presented in Table 13-A, and in the nine parts of Figure 13-A.

At 20 C strength 1/2 media containing 1/4 major salts supported colonies of C. globosum which averaged 10 mm less in diameter than those on 1/2 AS-1 controls (Part 1). Strength 1 with 1/4 major salts grew colonies 9 mm greater in diameter than those on strength 1 controls. It appeared that this fungus, at 20 C, was inhibited by a high concentration of major salts, but stimulated by a lower concentration of these. At 30 C diameters on strength 1/2 with 1/4 major salts were comparable to those on strength 1/2 controls, but diameters on strength 1 with 1/4 major salts averaged 8 mm less than those on strength 1 AS-1 controls (Part 2). Holding the buffer at 1/4 strength while other systems were raised to 1/2 or 1 resulted in diameters 10 mm less than those of 1/2 and 1 AS-1 controls. Holding the minor salts constant at 1/4 while other systems were raised to 1/2 or 1 did not greatly effect colony diameters. At 30 C it appeared that a high concentration of major salts favored growth if the concentration of other AS-1 systems was high, and that a high pH was favorable. At 35 C a high concentration of major salts was essential to growth when the concentration of other AS-1 ingredients was high, and a high pH favored growth (Part 3).

As colony diameters of C. globosum probably offer an inaccurate index of the growth of that species, only generalizations can be made. Holding AS-1 ingredients other than major salts constant at strength 1/4 did not result in curves which could be superimposed over control curves, but all curves were nearly parallel. Lines which connected the diameters

on strength 1/2 and 1 containing constant major salts, and incubated at 20 C and 35 C, bisected these other lines. At the optimum 30 C temperature, as would be expected, it appeared that the organism's tolerance of any variations in the medium was greatest.

It was obvious that concentration of the buffer noticeably affected growth, but this and other ingredients of AS-1, did not influence the diameter of C. globosum as drastically as did those of the major system. The requirement for major salts, however, appeared to be a function both of temperature and of the concentration of other AS-1 ingredients. If an increase in the concentration of systems other than the major to greater than 1/4 strength produced little effect on growth, diameters on all media containing a constant 1/4 major system would be expected to approximate those of strength 1/4 controls more closely than was the case. When the concentration of the major system was 1/4 and that of other systems 1, at 20 C and 30 C diameters were similar to those of the 1/4 controls, but at 35 C diameters on this medium were much less than those on the 1/4 AS-1 control plates.

The opposite was true of strength 1/2 media containing 1/4 major salts. At 35 C, diameters on this medium approximated those of the 1/4 control plates, but diameters at 20 C averaged approximately 10 mm less, and at 30 C 10 mm more, than those on 1/4 AS-1 35 C control plates.

Thus, C. globosum responded favorably to an increased salt concentration as temperature was increased, but the presence of a high concentration of major salts was vital at 35 C only if the concentration of other AS-1 ingredients was strength 1.

Torpedospora radiata grew too slowly in the 20 C incubator to make conclusions possible (Part 4). The temperature of the darkroom averaged

approximately 18 C during the 12-day growth period of this portion of Experiment 13. At 30 C diameters of colonies on strength 1/2 and 1 media containing 1/4 major salts were 4 mm less than those of the strength 1/2 and 1 AS-1 controls (Part 5). The buffer concentration had some influence on growth, but the concentration of this and other AS-1 systems had less effect on growth than did the concentration of major salts. At 35 C only the buffer and major salts lines deviated from the control lines (Part 6). Apparently, a high pH was favorable at a high salinity, and a lower pH at a lower salinity. The constant 1/4 major salts line deviated drastically from the control line, indicating the requirement for a high concentration of major salts at this temperature. As temperature was increased, T. radiata responded favorably to a high major salt concentration.

At 35 C diameters of T. radiata colonies on strength 1 containing 1/4 major salts were the same as those on strength 1/4 controls. Diameters on strength 1/2 with 1/4 major salts were somewhat greater than those on 1/4 controls. Therefore, concentrations above 1/4 of systems other than the major had some effect on growth, but less effect than the major system concentration.

Altering the concentration of systems other than the major had no effect on Halosphaeriopsis diameters at 20 C (Part 7). Diameters on strength 1/4 major salts media were slightly greater than those on controls, which indicated that a high concentration of salts was unfavorable at this temperature. At 30 C the concentration of metals and minor salts had little effect on colony diameters (Part 8). A high pH appeared to be favorable in strength 1/2 media. Diameters of colonies on strength 1 media containing 1/4 major salts were comparable to those on strength 1 AS-1 control plates, but the former colonies were much darker, with

fewer aerial hyphae than those on controls. At 35 C reduction of the concentration of buffer, metals, or major salts to 1/4 in strength 1/2 media removed the characteristic strength 1/2 dip from the curve (Part 9). The dip persisted in strength 1/2 media containing 1/4 minor salts. Apparently a lower pH and a lower concentration of metals, but a fairly high concentration of major salts, were favorable at 35 C.

Experiment 14

It having been discovered from Experiment 13 that the major system, more than other systems of AS-1, is required in greater concentration as temperature increases, this experiment was designed to determine whether NaCl alone would satisfy this requirement.

All data for Experiment 14 are in Table 14-A. At 20 C the diameters of C. globosum colonies on strength 1/4 and 1 controls were comparable, but strength 1 colonies appeared to be the thinner. At 35 C growth on control plates was better at the highest salt concentration. Increasing the major salts or the NaCl content of 1/4 AS-1 decreased diameters at 20 C, but increased them at 35 C. Lithium was obviously toxic. Increasing the Mg, Ca, and K salts concentration of 1/4 AS-1 (additive number 6) affected diameters differently from additives 3 and 4. At 20 C, diameters on plates of additive number 6 media were greater than those on either the strength 1/4 or 1 controls. At 35 C, diameters on this medium were greater than those on the 1/4 controls, but less than those on the strength 1 controls. At either 20 C or 35 C a greater-than-strength-1/4 concentration of Mg, Ca, or K, or some combination of those, was favorable. A high concentration of NaCl was favorable only if accompanied by a high temperature. At 20 C the advantage of a greater concentration of Mg, Ca, and K salts was apparently nullified if accompanied by a high concentration of NaCl, but at 35 C a

high concentration of NaCl increased colony diameter nearly as much as a high concentration of the entire major system.

Some of these remarks on C. globosum are partially true for T. radiata. Lithium was less toxic at 35 C than at 20 C. This monovalent cation appeared to replace sodium partially at the higher temperature. The addition of Mg, Ca, and K salts to 1/4 AS-1 resulted, as for C. globosum, in a slight increase in diameter over that of the 1/4 control at 20 C, but at 35 C T. radiata diameters on this medium were equivalent to those on the 1/4 controls. Addition of these salts, therefore, did not decrease diameter at 35 C, but neither did this addition increase growth, as it did for C. globosum. This was the most notable difference in the response of these two fungi to salts. The diameters of T. radiata colonies, as those of C. globosum, were increased by a high concentration of either NaCl or the major system at 35 C, but decreased by a high concentration of these at 20 C. The advantage of increased Mg, Ca, and K at 20 C was nullified if accompanied by an increased NaCl concentration, and at 35 C a high concentration of NaCl increased diameter nearly as much as a high concentration of the entire major system.

Diameters of Halosphaeriopsis colonies on all media at 20 C were so similar that differences were not discernible. It did appear that a high concentration of major salts decreased growth, and a high concentration of Mg, Ca, and K salts increased growth at this temperature. Lithium was less toxic for Halosphaeriopsis than for the other species tested, and a high concentration of this cation was more favorable at a high than at a low temperature. At 35 C, diameters on the lithium medium averaged 3 mm greater than those of the 1/4 control, and 3 mm less than the strength 1 control. Addition of Mg, Ca, and K salts to 1/4 AS-1 increased diameter

4 mm, and addition of the major system or NaCl increased diameter 5 mm at 35 C. It appeared from colony diameters that temperature affected the salt requirements of Halosphaeriopsis in a manner similar to that of the other species tested, but to a lesser degree. It is believed that dry weights would have furnished more reliable data on all three species.

Experiment 15

This consisted of a series of tests and observations designed to determine what factors of marine environments might exclude C. globosum. The influence of salinity on ascospore germination, the influence of high pH on fructification and ascospore development, and the ability to disseminate and compete in a simulated marine environment were considered.

a. Ascospores of C. globosum had begun to germinate after two hours, and on all strengths of NS-1 used were observed eventually to develop into young mycelia. It was estimated that germination and development were most rapid on strengths 1/2 and 3/4.

b. Ascocarps from all media listed in Table 15-A were examined microscopically, and all except young peripheral perithecia contained typical lemon-shaped ascospores. The final pH of media A, B, and C number 7 was 7.9, 8.4, and 8.4 respectively.

c. After three days of circulation the liquid became cloudy, apparently due to bacterial growth, and abundant vegetative growth of C. globosum was observed to have developed on the balsa block. After six days the liquid was again clear, but with a yellow tint that was not present originally, and the vegetative growth of C. globosum was much reduced. Apparently this fungus did not continue to fruit in the circulation fluid, and perithecia that had been introduced into the system became

darkly colored during the first week of circulation. An additional 0.1 g of yeast extract was introduced after six days, but failed to restore the initial cloudiness of the fluid, or to rejuvenate the vegetative growth of C. globosum.

Chaetomium globosum from pure culture (inoculation method III) grew sparsely on plain sea-water agar prepared from the month-old fluid, but growth of the fungus was better where filter paper was added. Ascocarps developed slowly, but contained ascospores. Halosphaeriopsis from pure culture grew sparsely on the sea-water agar. Better growth was obtained on the filter paper medium, but after two months on this, Halosphaeriopsis produced no ascocarps. No organisms other than bacteria were isolated from the month-old fluid.

Examination of the wooden blocks and cork revealed only remnants of C. globosum perithecia on the balsa upon which this species was introduced into the system, and no trace of the fungus on other blocks. Subcultures to malt extract agar from the surface and from deep within the balsa failed to isolate C. globosum. The balsa and other blocks were overgrown with an unidentified phycomycete and a free-living nematode. These organisms were numerous on the balsa block, and the end of the maple block in contact with the cork had been converted into a paste containing nematodes and their eggs. Halosphaeriopsis fruited luxuriantly on the sycamore block upon which it was introduced into the system, and on the sycamore in the first culture chamber. The phycomycete and nematode were also growing on these sycamore blocks. The growth of all species was poorest on the pine block in the first culture chamber. Final pH of the circulation fluid was 8.1.

Experiment 16

Chaetomium globosum will fruit on NS-1 plus 0.1 % tris (Table 15-A), but Halosphaeriopsis will not (Table 18-B). Before it was discovered that the marine species will fruit on cellulose with tris (Table 20-A), this experiment was set up to explore the possibility that failure to fruit on glucose (NS-1) media was due to potassium antagonism. Should a marine species be one that requires, for fructification, a delicately balanced K/:X ratio, it was supposed, tris might be a valuable tool for delimiting the marine mycota.

The data in Table 16-A do not suggest that potassium is antagonized by tris. As results were quite the opposite of what would be expected were tris antagonistic, this experiment should be redesigned. A comparison series containing only tris buffer would have yielded more information concerning the effects on growth of soluble calcium and the relatively inert $CaCO_3$ substratum. The potassium requirements of C. globosum were between 40 and 100 mg/liter in AS-1 strength 1/4 and 1. Fructification of this species was best in media containing 100 mg of potassium per liter, and did not occur in flasks containing 1 mg/liter or no potassium added. it was again noted that the growth of Halosphaeriopsis in strength 1/4 media was better by far than that in strength 1, further evidence that colony diameters of this species might be misleading. The final pH of $CaCO_3$ media was 6.4-6.6, and that of tris - $CaCO_3$ media 8.0-8.4.

Experiment 17

As T. radiata did not fruit after several months in NS-1 stock cultures, NS-7, which supports luxuriant fructification of this species, was used to determine salinity optima for development of ascocarps. Data for this experiment are in Table 17-A and Figure 17-A.

Salinity optima for fructification on the cellulose medium were less than optima for vegetative growth on the glucose medium (Figure 1-D). However, optima for both types growth increased with temperature. Considering desiccation of medium NS-7, the salinity optima for sexual reproduction are probably closer to those for vegetative growth than data indicate. Desiccation proceeded more rapidly at high than at low temperatures. Because salinity increased with desiccation, all 35 C tubes were unsatisfactory. It is probable that the reported 20 C optimum is nearly correct, the 25 C optimum slightly higher, and the 30 C optimum at least 8.5 o/oo higher than that reported.

The numbers in Table 17-A represent averages of the relative degree of fructification in eight to ten tubes. At 20 C, strength 1/4 tubes were given minimum values of 0 and maximum values of 6, and strength 1/2 tubes values of 3 and 6, but the average of 1/4 tubes was greater than that of the 1/2 set. The 25 C strength 1/4 and 1/2 sets each had minimum and maximum values of 4 and 7, and the 30 C 1/4 and 1/2 sets 4.5-8 and 5-8, respectively. It is interesting that T. radiata produced mature ascocarps (ascospores observed) in yeast extract-paper media without sea water at 25 C and 30 C.

Spores per HPF corresponded to the standards selected, as is evidenced below.

Standard No.	Mature ascospores per HPF
0, 1, 2, and 3	0.0
4 and 5	0.5
6	0.7
7	1.9
8	3.6

Thus, production of large carbonaceous ascocarps of *T. radiata* was a good index of its ascospore production. However, counts of ascocarps, without regard for maturity, were not a good index. The total number of ascocarps in some tubes of NS-7 decreased as these ascocarps became more mature.

On high pH media, mature ascospores developed only at 25 C in strength 3/4 media (maximum value 5). The addition of 0.1 % tris to NS-7, therefore, decreased fructification in nearly every case, but most drastically in the lower salinity range. Salinity optima were to the right of those recorded from standard NS-7 media, but this was obviously due to greater inhibitory effects of the high pH at low salinities rather than a favorable response to the higher pH at high salinities.

Experiments 18, 19, and 20

Halosphaeriopsis demonstrates some of the reasons for the general lack of precise methods for evaluation of sporulation in the mycological literature. The mycelium of this fungus is carbonaceous, and perithecia, imbedded in the agar, are difficult to detect and enumerate. Sclerotial structures morphologically similar to perithecia are produced by several of the marine Pyrenomycetes. Sterile ascocarps are produced along with fertile ones, and fertile ascocarps vary in size and degree of maturity. As was indicated by Experiment 17, the number and maturity of ascocarps may not always be positively correlated. Evaluation of conidia by inspection is certainly not a precise practice.

A method of evaluating the production of conidia and mature ascospores was developed in this laboratory and applied to problems involving *Halosphaeriopsis*. Experiment 18 was to determine the effects of temperature

and of tris buffer on salinity optima for asexual and sexual reproduction. Experiment 19 dealt with the effects of organic nitrogen-vitamin level, as yeast extract, on these optima. Experiment 20 was an abbreviation of Experiment 18, using cellulose as a carbon source rather than glucose. As all of these experiments were designed to furnish data on the sporulation of Halosphaeriopsis, and all employed the new technic for evaluation of sporulation described in Experiment 18 of the Materials and Methods, the results of all three will be considered together. Data pertaining to the present discussion are located in Tables 18-A, 18-B, 19-A, 20-A, and 20-B, and Figures 18-A, 18-B, and 18-C.

a. Factors Affecting Sporulation

The final pH of NS-1 media containing 0.1 % or 0.01 % yeast extract and no tris was 6.8-7.2. The final pH of NS-1 with 0.001 % yeast extract was 6.0-6.6. Tris NS-1 media had a final pH of 7.9-8.3. NS-7 with tris had a final reaction of 8.0-8.4, and NS-7 without tris 6.8-7.4. In all cases the lowest final pH readings were taken from strength 0 tubes.

On NS-1 with tris Halosphaeriopsis did not fruit, and produced few conidia (Table 18-B). On NS-7 fructification was reduced by the addition of 0.1 % tris, but fruiting did occur, as well as asexual reproduction (Table 20-A). Salinity optima for sporulation were greater on media with tris than on lower pH media. As with T. radiata in Experiment 17, this was obviously due to inhibition by tris at lower salinities rather than a favorable response to tris at high salinities. This fact prompted Experiment 16, through which it was hoped to indicate whether this inhibition was due to the high pH of tris media, or to potassium antagonism.

On NS-1 without tris the salinity optima for fructification of Halosphaeriopsis shifted slightly to the right as temperature or nutrient

level decreased (Tables 18-A, 19-A, Figure 18-A). If this was not due to more rapid desiccation at higher temperatures, then Halosphaeriopsis responds to salinity in a manner quite the opposite of what would be expected of a Phoma pattern fungus. However, due to more rapid desiccation of media at high than at low temperatures, salinity optima for ascospore production may lie progressively farther to the right than data have indicated. Many immature ascospores were observed in suspensions from tubes of strengths 0 to 1 in Experiments 18, 19, and 20.

Production of conidia on NS-1 without tris (Tables 18-A and 19-A, and 18-C) decreased as salinity increased at 20 C and 30 C. Except for a slight increase in the number of conidia per HPF observed from 25 C strength 1/2 tubes, at this temperature asexual reproduction also diminished as salinity increased. A dip in the 25 C asexual reproduction curve occurred at 1/4, the optimum strength for sexual reproduction. As the level of yeast extract was decreased to 100 mg/liter, asexual reproduction was rather constant through the strength 1/4 to 1 range. In media containing 10 mg/liter of yeast extract, asexual reproduction was not great, and was rather constant on strengths 1/4, 1/2, and 3/4 at 25 C. Production of conidia increased markedly, however, in strength 1 tubes, and was greater than that on strength 1 media containing 1 g/liter yeast extract.

Low nutrient-level, glucose-sea-water agar tubes, served mainly as a control to indicate the extent to which sexual and asexual reproduction in Experiments 18, 19, and 20 were affected by the dilution gradient of natural sea-water organics. The data in Table 19-A indicate that the organic nutrient level of all strengths of natural sea water media used can be considered constant, providing at least 10 mg/liter of yeast extract is added to all strengths. On glucose-sea-water agar, no sexual and little

asexual reproduction occurred. Conidia from this medium were long, thin, slightly granular, and hyaline to light brown.

Depending upon stage of development and conditions of growth, conidia from higher nutrient level media were characteristically cylindrical, sausage-shaped, ellipsoidal, or spherical. Color and internal development were dependent upon maturity. Luxuriant production of conidia was generally accompanied by darker, more clearly defined, and internally differentiated conidia. These were deep brown with one or more central, greenish, slightly refractile bodies, though immature conidia near the basal cell sometimes were similar in appearance to those from the glucose-sea-water agar. (Figure 18-C)

No correlation between salinity optima for the production of conidia and the length of conidial chains was recognized. The maximum chain length observed (22 cells) was most common in strengths 1/4 and 1/2 NS-1 containing 100 mg/liter yeast extract. Maximum chain length observed in NS-1 containing the standard 1 g/liter yeast extract was 15 cells. In lower salinities other asexual cells were also produced. Some of these were chlamydospores and others appeared as thin-walled, collapsing, darkly-colored, oil-containing cells. A few of these could have been mistaken for conidia, but the distinction was usually sufficient for differentiation.

From Table 20-A it is evident that counts of Halosphaeriopsis carbonous perithecia on NS-7 and ascospores per HPF from media incubated at 25 C are positively correlated. After a sufficient incubation period, it seems likely that either ascospore or perithecia counts would offer an accurate index of fructification. The mycelia of Halosphaeriopsis on NS-7 were less abundant and less carbonaceous than those on NS-1, and perithecia could be counted easily. On NS-7 with tris, Whatman papers were most moist, and perhaps more degraded than those on NS-7 without tris. On the tris

medium all perithecia developed on the agar beneath the paper, while on the lower pH medium perithecia developed on and beneath the paper.

Though production of sexual and asexual spores on NS-7 was less than production on NS-1, it was noted that the trend of sporulation in response to salinity was similar on both media. The decrease in asexual reproduction with increase in salinity, and the similar salinity optima for fructification on both media prompted computation of the ratios in Table 20-B. The number of conidia or ascospores per HPF or the number of carbonaceous perithecia per tube from strength 1/4 of a given medium was divided by numbers of these from strength 1/2 of the same medium. Strength 1/2 values were divided by those from 3/4, and finally 3/4 by strength 1 values. Ratios from NS-1 media containing 1 g/liter yeast extract were compared with those from NS-7 media of the same yeast extract content.

The ratios of conidia per HPF of strength 1/4 to 1/2 for NS-1 at 20 C and NS-7 at 20 C and 25 C were 1.4, 1.3, and 1.5, respectively. Strength 1/2: 3/4 conidia per HPF ratios for these same media and temperatures were all 1.1. Strength 1/4: 1/2 ascospores per HPF ratios for NS-1 and NS-7 at 25 C were 1.1 and 1.2, respectively, and the NS-7 1/4: 1/2 25 C carbonaceous perithecia ratio was 1.2. From either curves or ratios it is obvious that asexual reproduction decreased as salinity increased in 0.1 % yeast extract media, whether glucose or cellulose was used as a carbon source. These ratios demonstrate that the homogenization and counting technics used in this series of experiments could find application in investigations requiring a precise method of analysis. However, data have also demonstrated the need for refinement of the basic technic.

b. Technic for Evaluation of Sporulation

When counts of ascospores in adjacent strengths of the graded series were low, results were not comparable due to sampling error. A tube in

which one ascospore was found per thirty HPF was re-sampled, and three ascospores were found per thiry HPF. Percentage-wise, this is a great amount of error. However, resampling of low count tubes never produced high counts. The function of the technic in these experiments was to determine optima, at which ascospore production was markedly greater than in adjacent strengths. The magnitude of error in sampling was much less at optima, where counts were high. This was demonstrated by repeating ten times the sampling of a suspension and counting of conidia in 30 HPF. The similarity of 30 HPF counts was striking:

Trial No.	Conidia per 30 HPF
1	412
2	437
3	436
4	424
5	439
6	416
7	431
8	445
9	424
10	431

No other tube was so thoroughly examined, but assuming homogenization of other tubes was as complete, these data are reason enough to assume that a count of conidia in 30 HPF was representative of the number of conidia in any 30 HPF of the same suspension.

Clumping of ascospores was more serious than clumping of conidia in a field, as counts of the former were comparatively low. A drop of Tergitol No. 7 was added to one tube of a suspension in which clumps of ascospores

appeared to be common, and samples were withdrawn and examined microscopically to determine whether this made homogenization more complete. Apparently it did, but only a few observations were made. This use of detergents and wetting agents should be more thoroughly investigated.

If cellulose is used as a carbon source, as it must be for some marine Pyrenomycetes until more is known of their nutritional requirements (see Experiment 17), some growth will be lost due to the necessity of removing papers from growth to be homogenized. Neither ascospores of T. radiata, nor ascospores and conidia of Halosphaeriopsis were distorted beyond recognition by Schweitzer's reagent, if at all, but neither were papers completely degraded, so this technic was not investigated further.

Though 0.03 ml samples were withdrawn from spore suspensions, it is believed that a lesser volume, perhaps 0.02 ml, may be preferable. Using 0.03 ml there was some run-out from the edges of many coverslips, and spores tended to concentrate somewhat in such areas. If sufficient spores for high counts were not present in a 0.02 ml sample, the volume of water used in the preparation of spore suspensions could be decreased, thus increasing the concentration of spores. A few attempts were made to count spores in a haemocytometer, but the size of spores and accompanying hyphal debris made this impractical. However, this technic was designed to permit reproduceability, not to determine the absolute number of spores supported per unit volume of medium. Data indicated that neither the counting technic nor the method of preparing spore suspensions were great deterents to precision.

One purpose of Experiments 18, 19, and 20 was to explore this new technic. Not until certain of the suggested refinements have been considered, it is believed, would statistical analysis of data be worthwhile.

For that reason, and for the sake of brevity in Tables pertaining to more theoretical problems, the HPF tallys and replicate averages upon which Table averages were based have been placed in Appendixes I, II, and III. Data in these Appendixes need not be scrutinized for it to be obvious that the greatest source of error in this technic was the method of culture. While samples from the same suspension yielded similar results, counts of spores in replicate suspensions of the same condition varied considerably. The minimum tube value for six replicates of a given strength often overlaps the maximum tube value for six replicates of the next lower strength. This variation within replicates was attributed to several factors: slight differences in tube diameter, volume of medium, or angle of slant; stimulating or antagonizing substances not homogeneously dispersed in natural sea water; differences in exposure to light; slight temperature differences; most significant of all, different rates of desiccation. In this exploratory initial consideration of this technic, no more than ordinary precautions were taken to keep these factors constant. Certainly these sources of error would be reduced to a minimum by using a greater volume of medium in screw cap tubes, but such tubes were not available in sufficient numbers for this investigation. The use of media other than NS-1 and NS-7 agars should also be considered (see Experiment 22).

Experiment 21

This was to determine the influence of constant high pH on salinity optima of the marine species at different temperatures.

The salinity optima for vegetative growth of *T. radiata* on NS-1 plus tris, as determined by colony diameters, were the same as salinity optima for fructification reported from NS-7 plus tris (Table 21-A). The salinity optima for vegetative growth of *Halosphaeriopsis*, as determined by colony

diameters, were the same as salinity optima for fructification reported from NS-7 plus tris (Table 21-A). The salinity optima for vegetative growth of Halosphaeriopsis, as determined by colony diameters were strength 3/8 at 20 C and 3/4 at 25 C and 30 C (Table 21-A). Salinity optima for asexual reproduction on NS-1 plus tris were strength 3/4 and 20 C and strength 1 at 25 C and 30 C, but no sexual reproduction took place (Table 18-B). On NS-7 plus tris the optimum for sexual reproduction was strength 1/2 at 20 C and 25 C (Table 20-A). Tris inhibits several activities of both of these fungi, but less at high salinities than at low.

Experiment 22

This was to consider the utility of various culture media for physiological, developmental, and other studies of marine fungi.

Neither T. radiata nor Halosphaeriopsis grew appreciably on AS-2E without Whatman paper. On AS-2E with paper T. radiata produced small, mature perithecia (ascospores observed) in the agar around the periphery of the paper. Halosphaeriopsis grew well, but did not fruit on this medium.

Fructification of the marine species on natural sea water agars was generally better in the dark than in alternating light and darkness, but fructification on liquid media with sticks was better in alternating light and darkness. Solid media in the light desiccated more rapidly than tubes of the same media incubated in darkness, and desiccation, rather than radiation, caused the light-darkness tubes to support fewer perithecia.

Both the initial and final pH readings on solid media without tris were in the range 6.6-7.2, and 7.8-8.4 for those with tris. The initial pH of liquid media with sticks was as follows:

Number	NS	J	K
9-10	6.2	6.2	6.0
11-12	7.6	8.4	8.4
13-14	6.4	6.4	6.4
15-16	8.4	8.4	8.4

Final pH readings taken on sticks and liquids were similar to initial readings as a rule, though slightly lower in some tubes without tris. The lowest pH reading made was 5.5, at which no ascocarps were produced. Fructification was abundant in some tubes with a final pH of 6.2.

Of all liquid media containing sticks, only K-16 from alternating light and darkness supported mature ascocarps of _T. radiata_ after two months, at which time this experiment was terminated. Ascocarps of this species on K-16 were superficial, carbonaceous, with a short neck, and were growing in clusters on the sticks, within 15 mm of the slurry in the bottoms of tubes. The greatest number of perithecia counted on any stick was 27, and a few of the K-16 sticks supported no ascocarps.

Fructification of _T. radiata_ on solid media was much better than that on sticks, but still less than that on NS-7 strength 1/4 or 1/2 from Experiment 17. After one month fruiting bodies were produced, (in order from the most abundant), on the following media: NS-7 dark; NS-8 dark; NS-7 and NS-8 dark, and alternating light and dark; K and J numbers 7 and 8 dark, and from light and dark. A very few perithecia were produced on J-6, K-6, NS-5, and NS-6. No fruiting occurred on media without cellulose.

Fructification of _Halosphaeriopsis_ at the end of one month was better on most media than that of _T. radiata_ on NS-7, strength 1, at the end of two months. Of the solid media, NS were superior to K, and K superior to J of the same pH and carbon source. No sexual, and little asexual

reproduction of Halosphaeriopsis took place on NS-2, K-2, or J-2, which contained tris and glucose. Of the agar media, NS-7, K-7, and NS-1 supported the greatest numbers of Halosphaeriopsis conidia and ascocarps. Sporulation was also fair on K-5, and perithecia could easily be counted due to the light background and lack of dark vegetative hyphae. The final pH of K-5 was 7.2.

Before examining Halosphaeriopsis on stick-liquid media it was necessary to remove sticks from tubes and allow them to dry for a few minutes on paper toweling. Otherwise an abundance of vegetative growth on NS medium sticks masked perithecia and made counting difficult. This was less of a problem on K media, and no problem on J media. Perithecia on all stick-liquid media were superficial, carbonaceous when mature, and neck length varied with maturity and the medium.

All numbers of J media supported fewer perithecia and less vegetative growth of Halosphaeriopsis than did corresponding numbers of NS and K media. Of the J media, number 9 appeared to be the best, and then 10, 11, and 12, in that order. Larger, more carbonaceous and mature perithecia, and greater numbers of ascocarps were produced on NS than on K media as a rule, and large numbers of uniformly mature ascocarps were produced more rapidly on NS 14, 16, and 10 than on any other media upon which the species was grown. However, fructification on K-9 was comparable to that on the best NS media, and provided the organism developed typically, K-9 may be superior to NS media for developmental studies. Ascocarps were less masked by vegetative growth on this medium, and more stages of perithecial development were observed than on NS media. Media K-14 and K-16 supported fewer perithecia than did K-9, and on most of these sticks structures resembling small

rhizomorphs were observed (Figure 22-A). Many sticks in other media were sheathed with vegetative growth, but K-14 and K-16 supported well defined, more highly organized structures, which branched and often contained perithecia. It was not determined whether these structures were true rhizomorphs, but repetition of this experiment with microscopic examinations may prove interesting.

All structures of the fungi that were placed in the preservative described in Experiment 22 of the Materials and Methods were well preserved by that solution, and remained attached to sticks. A similar technic may be useful in taxonomic and developmental studies, in which it is desirable to preserve material.

Fresh sticks of Halosphaeriopsis were placed in a marine aquarium primarily to consider their utility in ecological studies, in particular, studies of the role of animals in the (fecal) dissemination of marine fungus ascospores. Of the four fishes in the aquarium, a juvenile Rhinichthys atratulus was most voracious, and stripped pine sticks of ascocarps in preference to vegetative hyphae. Semotilis atromaculatus juveniles were also very active, but ate both ascocarps and vegetative hyphae from a balsa stick at the surface of the water. Hybopsis leptocephalus juveniles fed more on vegetative hyphae than on ascocarps. Young Moxostoma rhotheaca were not observed to feed.

These fishes were quite hungry, being fed only occasionally on dog food, which they would eat in preference to fungi when both were present. However, it is significant that after three weeks of feeding on the fungus none of these fishes showed any outward signs of poisoning or disease, and continued to eat ravenously whenever the fungus was supplied.

Disadvantages of this technic are the residual peptone and the

superficial location of ascocarps on sticks which might make marine fungi more available to fishes than they are in nature.

DISCUSSION

While aspects of the Phoma pattern have been the central theme of this investigation, a greater understanding of that phenomenon has not been its sole objective. The experiments conducted have resulted in an accumulation of basic technical and physiological data much needed in several areas of marine mycological research. While some investigations were thorough, many were exploratory. Data are in some respects intentionally diversified. However, integration of these data offers support to discussions of tris buffer, nutrition and culture of marine fungi, their morphology, determinants of their distribution, and means for distinguishing which marine isolates are indigenous. Consequently the following discussion is under these topic headings.

Tris and pH

This was added in a 0.1 % concentration to one-half of the units of media in several experiments to consider its utility as a buffer in fungal cultures, and to study the effects of pH on activities of Pyrenomycetes.

When provided the proper nutrients and salinity, all three species completed their life cycles in tris-buffered media. Dry weights of mycelia, a generally reliable index of growth, gave no indication that tris inhibits vegetative fungal growth through its antagonistic effects on potassium. Fruiting took place on tris-buffered distilled water-0.1 % yeast extract-cellulose media in which potassium concentration is low, showing that reproductive activities are not seriously affected by antagonistic action of the buffer. Growth of the marine species on distilled water media also disproved a hypothesis that marine fungi

might be delimited by the requirement for fructification of a delicately balanced K/:metal ratio.

The failure of Halosphaeriopsis to fruit and produce abundant conidia on tris-glucose-yeast extract-sea-water may be due to pH. But why pH 8.4 is tolerated on cellulose media and not on glucose was not apparent. The requirement by T. radiata and Halosphaeriopsis for cellulose suggests that cellobiose may be utilized during fructification.

It is perhaps fortunate that the marine species did not fruit in artificial sea water-glucose-tris media. Nutritional and biochemical data gathered from sporulating cultures are often different from those obtained from vegetative mycelia, and are less reproducible. If several marine fungi will grow only vegetatively under these conditions, such media will be useful in physiological and biochemical studies.

Many advantages of tris buffer may be cited: it does not form precipitates when autoclaved; it does not provide a substratum, as do insoluble buffers, for the accumulation of substances which influence growth; it does not require special treatment prior to the harvest of mycelia, as does $CaCO_3$; apparently it is not utilized as a nutrient, and may, therefore, be considered an inert component of media; it does not interfere with vitamin synthesis, except through its effect on pH; it can be obtained in high purity. As a buffer for fungus cultures in liquid media, 0.1 % tris was comparable to an excess of $CaCO_3$, provided harvests were made after two to three weeks of growth. After longer periods of incubation, pH in many tris-buffered cultures became limiting. This was especially true of low vitamin level cultures in which various products accumulated due to the blockage of metabolic pathways.

The high initial pH of 0.1 % tris media may be a disadvantage in

studies of some species, but *C. globosum*, which is usually cultured in neutral to slightly acid media, grew well and completed its life cycle in liquid and solid media containing this concentration of the buffer. Some mature ascocarps developed even in a 0.2 % concentration. Initial pH was lowered by using 0.075 % tris, and approximately 100 mg of dry *T. radiata* mycelium was obtained from some flasks containing this concentration before pH and other factors became limiting. The high initial pH of 8.4 and the early limiting pH of some tris-buffered liquid media could be alleviated by using a 0.075 % concentration initially, and aseptically adding more buffer after a period of incubation. Highly concentrated solutions of tris can be prepared, but whether these can be autoclaved without appreciable chemical change was not investigated. In solid media a less-than-0.1 % concentration of tris can be used successfully, as acids diffuse through agar.

Because of its apparent inertness, with respect to fungus nutrition, tris buffer may help resolve some of the problems of salts of organic acids in ammonium nitrogen cultures, (see Lilly & Barnett, 1951). For salinity tolerance studies, it may in some cases be advantageous to use a substance which possesses the other merits of tris, but which buffers in a slightly lower pH range, perhaps 7.4 to 7.8.

A pH gradient exists between some rivers and the sea, and species would be expected to vary in their response to such a gradient. Even from this preliminary study, it is evident that the optimum pH for growth of some fungi is not the same under varying conditions of salinity and temperature. The extreme low pH of river water is 6.6, and the extreme high pH of most sea water is 8.6. An intermediate pH of 7.6 may not be optimum in either high or low salinity water, but may be more nearly

optimum for a great range of salinities and temperatures than would either of the extremes cited. Using colony diameters as an index of growth on artificial sea water-glucose-nitrate-vitamin media, a survey should be conducted to determine the hydrogen-ion requirements of several species at three or more salinities and temperatures. Data on the individual species would be pertinent to several problems, but the discovery of trends, and the determination of a "general purpose" pH for studies of marine lignicolous Pyrenomycetes and Deuteromycetes, would be of greater theoretical and technical value. A similar survey of trace metals requirements would also be a contribution toward the development of a general purpose synthetic sea water medium for these organisms. However, until such surveys are completed, it is believed that 0.1 % tris and the strength 1 AS-1 chelated metals mixture are adequate for studies of some marine lignicolous species.

Fortunately, the growth response of T. radiata and C. globosum to salt concentration was measurably greater than response to pH and trace metals concentration, but because the latter do affect growth, they should be held constant in salinity tolerance studies. If the optimum pH at 35 C and a salinity between 26 o/oo and 34 o/oo is higher than the pH optimum at lower temperatures and salinities, response to a pH gradient might be falsely interpreted as a Phoma pattern response to a salt gradient. Also, a pH gradient might so modify growth response to salt concentration that a Phoma pattern would either go undetected, or be greatly accentuated. The concentration of trace metals available to fungi, through water and food materials, may be nearly the same in fresh, brackish, and sea water. In the future, the basal synthetic sea water medium, for Phoma pattern detection and other purposes, should contain only major and minor salts. This base should be diluted to the desired strengths, and the most generally

favorable concentrations of buffer and chelated trace metals added to each dilution of the base, rather than diluting all four systems proportionally.

The high pH afforded by 0.1 % tris was not required by the fungi cultured in stick-liquid and other natural sea-water media. However, a pH higher than that of autoclaved, fortified natural sea water may favor sexual reproduction of some marine fungi, and autoclaving certain woods and wood products in liquid media lowers pH considerably (Meyers & Reynolds, 1959a). In this study, pH was lowered more by pine than by balsa. Experiment 22 demonstrated that 0.1 % tris may be a suitable buffer for natural sea water cultures of marine fungi, when a buffer is needed.

The use of tris in culture media is discussed by Provasoli et al. (1957). Fungi were cultured in the ASP_2 medium of Provasoli by Ritchie (1957) and by Meyers and Reynolds (1959a). From these, and the present investigation, it appears that the only marked affects of 0.1 % tris on the growth and reproduction of several lignicolous Pyrenomycetes and Deuteromycetes results from the compound's buffering action.

Nutritional Requirements and Synthetic Media

This investigation demonstrated that *T. radiata* is less fastidious than may be inferred from the work of Meyers and Reynolds (1959a) or Moore and Meyers (1959). Substitution of cellulose for glucose in ASP_2 may well support ascocarps of the fungus. Perithecia of either *T. radiata* or *Halosphaeriopsis* should develop on pine in ASP_2 with added nitrate, but *Halosphaeriopsis* is probably the more fastidious of the two. Longer incubation or addition of more buffer to AS-1 plus cellulose may have enabled ascospores of this fungus to mature, but it is very doubtful that perithecia would have been as typical on the synthetic medium as

were those of *T. radiata*.

While the full nutritional requirements of *Halosphaeriopsis* were not determined, data do conclusively demonstrate that neither of the marine species studied require marine salts in sea-water proportions or exotic marine growth factors for fructification. Thus, a graded series of natural sea water, supplemented with 10 mg to 1 g per liter of common laboratory digests or extracts, provides satisfactory media for studying the salinity optima for fructification of euryhaline lignicolous species.

Though euryhaline, *T. radiata* and *Halosphaeripsis* are obviously halophilic, true marine species. Therefore, strength 1 AS-1 may be an adequate base for studies of the nutritional requirements for sexual reproduction of several euryhaline and stenohaline Pyrenomycetes. Judging from this investigation, and those of Johnson et al. (1959) and Meyers and Reynolds (1959a), the full nutritional requirements of many marine lignicolous species including saprophytic *Lulworthia* species, should be satisfied by thiamin, biotin, phosphate, nitrate or ammonium nitrogen, and cellulose, in a synthetic sea water base containing tris buffer and a chelated metals mixture. The development of perithecia free from wood, in the slurry of some stick-liquid tubes of Experiment 22, suggests that provision of a substratum by cellulose is not a vital function of the polysaccharide, and that it may be replaced by glucose, xylose, or cellobiose, or mixtures of these and other sugars.

Fructification of some species may be favored by, or dependent upon, organic nitrogen and non-cellulosic polymers of plant materials. It is doubted, however, that morphologically adapted, and particularly stenohaline lignicolous Pyrenomycetes require lignin of phanerogamic origin for completion of the life cycle. White-rotters are most commonly Basidiomycetes and though

some Ascomycetes utilize this substance efficiently after degradation is advanced, they do not commonly require it. Also, though wood is commonly used to detect and isolate fungi from marine environments, this substance of terrestrial origin is not present in the oceans in sufficient quantities to support the great populations of so-termed lignicolous Pyrenomycetes present. Marine saprophytic fungi undoubtedly perform tasks similar to those of terrestrial wood rotters but the "litter" of sea-water is largely of animals, Monocotyledons, and Cryptogams.

Before proceeding further with the development of a general-purpose synthetic medium which will support perfect stages of marine Pyrenomycetes, the inhibition of sporulation by 0.1 % tris should be more thoroughly investigated. If sexual and asexual reproduction of Halosphaeriopsis were also inhibited on inorganically-buffered glucose media of pH 8.0-8.4, it could be assumed that tris functions as a buffer, and little else, in fungus cultures. The AS-1 vitamin mixture should suffice for any of the marine lignicolous Pyrenomycetes and Deuteromycetes. It is likely that these fungi are deficient only for biotin, thiamin, and pyridoxine, but few species should be inhibited by the presence in media of vitamins for which they are autotrophic. The growth and sporulation of most species studied in culture has been accomplished on 0.1 % yeast extract. For sexual reproduction, a reduced level of vitamins may be favorable in some cases.

If AS-1 proved adequate for the task, a comparative study of the nutritional requirements for fructification of euryhaline and stenohaline, free-living and parasitic species, would provide information of general value to marine microbiologists and ecologists. For the initial mineral-

nutrition studies of marine lignicolous fungi it is also believed that a medium such as AS-1 would be preferable to one of reduced salinity and altered proportions of major marine salts, as it has been demonstrated (Experiment 14) that requirements for some ions may be dependent upon the concentrations of other ions. Mineral nutrition studies so conducted would reveal ion antagonisms, and would greatly contribute to the development of a medium better suited for biochemical studies than is AS-1.

ASP_2 is a medium of low salinity, with a Ca/Mg ratio different from that of natural sea water. Because of a low concentration of salts which form insoluble phosphates and carbonates, ASP_2 has a high degree of reproducibility. AS-1 of strength 1/2 or greater forms abundant precipitates upon autoclaving. The phosphate concentration of this medium is higher than is necessary, but even if this were reduced, or glycerophosphate used, as in ASP_2, the concentrations of Ca, Mg, and Sr would still be a source of precipitates. Experiments indicated that for euryhaline species, the concentrations of Ca, Mg, and probably K ions, may be held to between 1/4 and 1/2 the concentration of these in sea water, and salinity built up with NaCl. Sodium partially replaces potassium for some species (Lilly and Barnett, 1951). The Mg/Ca ratio is also alterable, as indicated by growth on strength 0 NS-1 and strength 0 NS-7. A less-than-strength 1 concentration of minor salts may be adequate for fructification, if required at all. Cobalt may be unnecessary, but the addition of other trace metals, should be investigated.

Species which require a high salinity probably evolved from euryhaline fungi, Therefore, Experiments 13 and 14 suggest that AS-1, so modified, might also support ascocarps of stenohaline sea-water Pyrenomycetes, providing NaCl concentration of the medium is high enough. Properly

fortified, the ASP_2 or ASP_6 of Provasoli (1957) might also be sufficient for several Lulworthia spp.

In any event, the development of synthetic media which support the perfect stages of marine Pyrenomycetes will probably be less of a problem than that of obtaining morphologically typical ascocarps under other than natural conditions.

Morphology

One purpose of Experiment 22, and indirectly Experiments 17 through 20, was to consider what types of media might be suitable for morphological and taxonomic studies. Several stick-liquid media of Experiment 22 favored sexual reproduction of Halosphaeriopsis more than did agar media. Examination and preservation of variously matured ascocarps on sticks is convenient, and in many respects well-adapted to developmental studies. This study is not complete, however, until the morphology of species grown on sticks is compared with that of fungi isolated from natural conditions. It is not known whether ascocarps which developed superficially on sticks or free in slurry are as suitable for morphological studies as those imbedded in wood. The morphology of ascocarps which developed on the various media used in this investigation was not carefully considered. The neck length, color, position on substrata, number of necks, and fertility of ascocarps were noted. Examinations were thorough enough to strongly support Meyers (1957) evidence that the morphology of ascospores is consistent, and is a better taxonomic character than the structure of the perithecium. When Halosphaeriopsis was grown under different conditions of pH, light intensity, nutrient constitution, and physical state of the medium, ascospores were morphologically indistinguishable from one condition to the next. Perithecia in which these ascospores occurred varied greatly

as to size, neck length, and number of necks on the venter. The dark color of mature perithecia was their most consistent characteristic. It is reasonable that these variations would occur in nature. Perithecia of T. radiata which developed on HS-7 were quite different in appearance from those on AS-2E, the latter being minute, brownish rather than carbonaceous, and without a prominent neck in all cases. Ascospores from these two media, however, were identical in appearance. Variations in temperature and salinity in media of the same nutrient constitution did not produce as great changes in the structure of perithecia as did variations in nutrients at a constant salinity. Boiling perithecia caused ascospore appendages to be indistinct, but these were present on the mature ascospores from all salinities and temperatures in which these occurred. Ascospores from the various combinations of temperature and salinity were morphologically indistinguishible. One conclusion drawn from these observations is that ascospore appendages might better be termed adaptations to an aquatic, rather than to a marine environment. There is no evidence that these processes developed only in marine environments, nor that their function in fresh water is not the same as that in sea water. Evidences indicate that stick-liquid media may be suitable for taxonomic and morphological studies which place more emphasis on spore morphology than on perithecium structure. Both marine species, as they appeared on sticks, were easily keyed to genus by the key of Meyers (1957).

Conidia of Halosphaeriopsis generally arose basipetally, but the possibility of other methods was not excluded by this study. In low salinities this species produced conidia, chlamydospores, and oil-containing cells, as well as filamentous hyphae.

Torpedospora radiata produced many chlamydospores in low-salinity artificial sea water. A great diversity of undetermined forms was also produced on this medium. Under conditions of low salinity and nearly optimum temperature, the mycelium apparently fragmented, or failed to produce filamentous hyphae. A mass of chlamydospores, and budding cells suggestive of yeasts, were present. Conidia have not been reported, for this species, and some effort was made to detect these asexual spores in several natural and artificial sea water media. Only a few structures suggestive of microconidia were observed (Figure 3-F). No asexual spores of any kind were observed in the T. radiata cultures of Experiment 22.

Determinants of Distribution

Of the three Pyrenomycetes studied, only T. radiata possessed a Phoma pattern involving vegetative and reproductive phases. From this evidence, one would expect that the fungus has a Phoma pattern distributed in an estuary. Gold (1959), however, found ascocarps of this species in fresh, brackish, and sea water portions of the Newport River in warm and in cold seasons.

Sexual reproduction curves of T. radiata did not peak at 25 C and 30 C (Figure 17-A). At these temperatures, the range of salinities at which fructification occurred was greatest. It is not understood, therefore, why perithecia of supposed Phoma pattern fungi collected by Gold did not occur over a great range of salinities when water temperature was 25-30 C. Certainly nutritional factors, as well as temperature, affected the salinity optima of these species, but these factors should also have affected the distribution of biotin-thiamin heterotrophic T. radiata. There is no evidence that the species studied by Gold are so fastidious that they must follow peaks of the production of specific organics. More likely,

the *Phoma* pattern mechanism in these fungi is more highly developed than that in *T. radiata*, if the North Carolina *T. radiata* possesses such a mechanism at all.

It is possible that the North Carolina *T. radiata* is physiologically distinct from the Florida isolate used in this investigation. While Johnson (1958) did report some morphological differences, nutritional requirements of these were shown to be similar (Johnson et al. ,1959). A comparative study of the growth pattern in culture and the seasonal distribution of *T. radiata* in Florida and North Carolina waters may reveal a seasonal shift in population density, though not necessarily a *Phoma* pattern like that of *Trichocladium* sp. (Gold, 1959). Such a pattern, if it does exist, should result from the combined action of water temperature and the level of biotin and thiamin. Other factors should also be considered as influences of distribution.

A comparison of salinity optima on NS-1 and AS-1 gives some clue to the nature of the other determinants of distribution. The NaCl concentrations of these two media were the same. No assay of the BBL yeast extract used in NS-1 was available, but it is reasonable that the thiamin, biotin, nitrogen, and phosphorus levels of these media were adequate, Yet, salinity optima were higher, and the degree of *Phoma* pattern expression was greater, on AS-1 than on NS-1. Experiment 13 indicated that this discrepancy is only in part due to the pH and trace metals gradient in a series of AS-1 agars. It was demonstrated in Experiment 10 that nitrogen-phosphorus level has no effect upon the salinity optimum of *T. radiata*, but Experiment 11 and others suggest that the forms in which nitrogen is available might have a great effect. By partial replacement of biotin, organic nitrogen increases the salinity

tolerance of *T. radiata* in biotin-free media, but the effects of nitrogen sources on salinity optima in high-vitamin level media should also be investigated. Dry weights of mycelia from three salinities and three temperatures, in the presence of NH_4, NO_3 or organic nitrogen, may reveal a heretofore unsuspected determinant of estuarine distribution.

Concentrations of inorganic substances other than NaCl also affect salinity optima. Requirements for trace metals, calcium, and sulfur were probably satisfied by 0.1 % yeast extract, but not by strength 1/16, and perhaps not by strength 1/4 AS-1. Regardless of temperature, maximum growth of *T. radiata*, *C. globosum*, and *Halosphaeriopsis* could not be attained on AS-1 until the salinity was increased to strength 1/2, and with it the concentrations of Ca, Mg, SO_4, and trace metals. These nutritional requirements caused salinity optima on AS-1 to lie farther to the right than those on NS-1. At 20 and 35 C, *C. globosum* and *Halosphaeriopsis* responded favorably to a greater-than strength 1/4 concentration of a mixture of Ca, Mg, and K chlorides in sea water proportions. This indicates a nutritional or an osmotic function of these salts. *Torpedospora radiata* responded favorably to an increased concentration of Ca, Mg, and K salts at 20 C, but unfavorably at 35 C. Thus, response to this mixture of salts was the opposite of response to NaCl concentration. Though the biochemical mechanisms of the *Phoma* pattern are not understood, Experiment 14 suggests that in *T. radiata* it is principally an ionic phenomenon.

Data on *T. radiata* suggest salt antagonism. The effects of sodium and potassium ions on the permeability of cells are opposed by the effects of calcium and magnesium ions. In this preliminary survey, Ca, Mg, and K concentrations of media were increased or decreased simultaneously,

though, above a certain concentration, the monovalent and the bivalent cations may affect growth differently. Despite this, some inferences can be drawn from Experiment 14. Precisely, not only the higher calcium level, but also the greater Ca/Na ratio of 0.1 % yeast extract media may have caused salinity optima of T. radiata to lie farther to the left on NS-1 than on AS-1 at sub-optimal temperatures. Dilution of AS-1 lowered calcium level, but the Ca/Na ratio remained the same for all strengths of the medium. In an estuary, increases in the Ca/Na ratio, brought about by calcium-rich river water, might favor the growth of Phoma pattern fungi in low salinity regions during winter months.

Additional mineral nutrition studies, using dry weights of mycelia as an index of growth, are needed to determine the best approach to the Phoma pattern mechanism, but flame photometry, respirometry, and other tools of biochemistry will probably be necessary to resolve this problem.

The present nutritional studies have indicated that the osmoregulatory devices of T. radiata and C. globosum are dependent upon energy furnished by aerobic respiration. Sodium-ion tolerance increased with temperature and thiamin-biotin level, and pyruvate probably accumulated in absence of sufficient amounts of these vitamins. This suggests active transport, but does not rule out a diffusion mechanism. The presence of thiamin pyrophosphate provides rapid entry into the krebs cycle. As temperature increases so does respiration. It is possible that the greater water of respiration produced at high temperatures must be accompanied either by a more rapid entry of salts or a more rapid loss of water. The concentration gradient and Ca/Na ratio of sea-water would enhance diffusion, and the maintenance of the proper tonicity and viscosity of the protoplasm.

For a Phoma pattern mechanism to be functional in an estuary, those conditions of temperature and salinity which favor vegetative growth must also favor sporulation and the development of adequate means of dissemination. Fungus populations will not become established anywhere in an estuary that spore production, dispersal, and attachment are ineffective. It is, therefore, conceivable that a Phoma pattern could be expressed by T. radiata, and be of definite survival value. In winter months, when organic production is low in sea water, a low salinity and high Ca/ Na ratio favor growth and reproduction. When temperature is low, vitamin requirements are less and nutrient level greater at low than at high salinity. When temperature is high, high salinity favors growth and reproduction. The Phoma pattern fungus is established in a region where organic nutrients are abundant, and the appendaged ascospore has a decided advantage. Seasonally adventitious non-marine fungi therefore are less successful than those having such aquatic adaptations.

During warm seasons the level of dissolved vitamins need not be high for T. radiata to invade the oceans, as the level of organic nitrogen is high. Nitrate, ammonium, and organic nitrogen are utilized. The latter also increases salinity tolerance by partially replacing biotin. Cellulase is actively produced, even in absence of thiamin. Thus, entrance could be gained into wood cells and other plant materials, and biotin-thiamin requirements more fully satisfied.

It is difficult to evaluate results of the experiments to determine the effects of temperature and nitrogen-vitamin level on salinity optima for vegetative and reproductive activities of Halosphaeriopsis. This is due to discrepencies in data obtained from solid and liquid media, to

desiccation of tube media, and to high C/N ratios of some tube media of Experiment 19. Thus, only generalizations can be made. Reproductive activities followed a pattern somewhat the opposite of that which might be expected of a *Phoma* pattern fungus. Reduction of temperature or vitamin level caused salinity optima for sporulation to increase, rather than decrease, as was the case with *T. radiata*.

These data are of ecological significance in that they indicate determinants of the distribution of this fungus. However, it is difficult to surmise, from these data, how *Halosphaeriopsis* is distributed in various marine habitats throughout the seasons. This is partly due to lack of knowledge of the level of nutrients that are available to this fungus. Data generally indicate that a mesohaline range of salinities would favor most activities of this species regardless of season or climate. Sporulation may be favored by salinities less than 17 o/oo in tropical estuaries, or in south temperate estuaries during summer months. In north temperate estuaries or in winter months in south temperate estuaries a salinity greater than 17 o/oo may favor sporulation. Though the temperature optima for activities of this fungus lie between 25 C and 30 C, this species is a common representative of the northern marine mycota (Meyers and Reynolds, 1959c).

Distribution can be determined with certainty only by distributional studies. The function of laboratory studies should not be so much to predict, as to explain the distribution of fungi. More conclusive results would have been obtained had fungi been used which displayed the *Phoma* pattern under natural conditions. Much more distributional data is needed, and in the light of present data, field studies of *T. radiata* and *Halosphaeriopsis* may be of value.

For prediction or explanation media more closely simulating natural conditions might be prepared, and field and laboratory findings more closely correlated. Some of the stick-liquid media of Experiment 22 may be useful in this respect. Also, on these media perithecia could be counted easily even if vegetative hyphae were carbonaceous. It has been demonstrated with _T. radiata_ and _Halosphaeriopsis_ that a count of carbonaceous perithecia is an accurate index of ascospore production. The mycelium, which grows predominantly on the surface of stacks and in the liquid, could be harvested with little loss, and either dry weights of mycelia or conidia per HPF, could be determined.

The ecological significance of a _Phoma_ pattern in _C. globosum_ is not apparent. It is very doubtful that _C. globosum_ invades even oligohaline sectors of estuaries, except temporarily on floating debris or structures above water level. Data indicated that oxygen content and specific gravity of sea water, not salinity and pH, exclude _C. globosum_ from marine environments (Experiment 15 and Table 1-B). However, the effects of vitamin level and temperature on salinity tolerance of this organism are undoubtedly exemplary of several other non-marine fungi which are more marine-fit than this Pyrenomycete. This is evidenced by the invasion of estuaries, especially during summer months, of a hoard of variously designated fungi.

Delimiting the Marine Mycota

The number of presumably non-marine species that can survive in marine environments is so great that some have reasonably questioned the use of habitat designations such as "terrestrial" and "fresh water" fungus as opposed to "marine" fungus (Ritchie, 1954; TeStrake, 1959; Siepmann and Johnson, 1960; Wolf, 1960). It is conceded that "lignicolous" does not allude to the actual and potential nutrient sources of many fungi

as inclusively as would "hyllytic", (Gr. hylos - wood, matter; lysos - to dissolve). Also, criteria for delimiting marine species may be particularly difficult to recognize, if there is any distinction at all, in the case of some yeasts (Fell et al. 1960) and Deuteromycetes (Meyers and Reynolds, 1959b; Meyers and Moore, 1960). However, despite forms that apparently are pre-adapted or adaped to the extent that ubiquity is possible, in the majority of cades it seems unnecessary to revert to calling a fungus a fungus.

The definition of a marine fungus is a problem in biology and semantics. The biological problem is that of determining whether a particular marine isolate is: (1) actually marine, (2) an opportunist which could legitimately be designated "terrestrial" or "fresh-water," (3) a transitional form which requires both marine and non-marine habitats, or (4) a generalized fungus whose genetically identical representatives are adapted to both marine and non-marine environments, though not dependent upon both for completion of the life cycle. Some features of marine Pyrenomycetes were set forth by Meyers, (1957) "(1) the continuous and regular occurrence of these fungi in oceanic areas; (2) their association with marine algae and higher plants, sometimes as parasites; (3) morphological adaptation and various growth responses in culture." To this list one may add, their absence from environments other than oceanic and estuarine. The following definition is now proposed in an effort to restrict the meaning of the term "marine fungus", ("Thalassiomycete?" see Moore and Meyers, 1959) and thereby make that designation more meaningful.

A marine fungus is a morphologically and physiologically distinct species, variety, or forma of fungus to which the most favorable natural conditions for perpetuation are provided by brackish and sea-water habitats,

to which it is restricted except under conditions favorable for facultative invasion of adjacent fresh-water and shore habitats.

Opportunists may be distinguished by poor or periodic representation in marine environments, greater representation in low than in high salinity regions, greater representation in non-marine environments, and by lack of morphological and physiological features of potential marine survival value. These general features, of course, are difficult to evaluate. Some characteristics are undoubtedly of greater survival value than others, but no one of these would be held in common by all fungi which are indigenous to the sea, yet absent from all other fungi. The oceans and estuaries provide a number of environmental situations, all of which must be considered marine, but none of which are optimum in all respects for any particular fungus species. Several species must be either marine-fit or unfit to comparable degrees, but for different reasons. Some non-marine fungi may be fit for survival in certain marine situations from which they are geographically isolated.

This study has indicated that neither the demonstration of saline-tolerant vegetative mycelia nor of vegetative growth in a _Phoma_ pattern in culture are necessarily indicative of marine fitness. It is predicted that future exploration will fail to isolate _C. globosum_ from natural marine situations, and that pure cultures, started on sterile wood panel traps and submerged in estuaries, would either perish or fail to become established on other panels placed in the immediate vicinity of the initially pure culture panels. Some degree of _in vitro_ vegetative _Phoma_ pattern expression is apparently a characteristic of several fungi from different habitats. Affinities to this pattern have been demonstrated in three of five marine isolates, and in one of two terrestrial isolates

by Ritchie (1957, 1959), and in two marine and one terrestrial species used in this investigation. All of these fungi were selected at random. Terminology might be revised, however, to indicate the distinction between the vegetative *Phoma* patterns of undetermined function in fungi such as *C. globosum*, and the *Phoma* patterns of marine-fit fungi, which involve both vegetative and reproductive phases. The present study indicates that salinity optima for fructification may be a reliable index of marine-fitness. For the purpose of assigning descriptive habitat designations to fungi, saline tolerance data are most useful if obtained through field studies. Cultures in graded series of nutrient sea water media determine barriers created solely by salinity, and are measures of salinity tolerance under a particular, (usually favorable), set of conditions. Field studies reveal actual boundaries which are merely defined in terms of salinity. Survival and dissemination tests, using continual-flow, or using panel trap cultures in estuaries, might provide a practical index of marine-fitness.

Transitional forms, if they exist at all, may be of two types. One may be a species extremely sensitive to temperature change, which can be compensated for only by a shift in the organism's reaction to salinity. This would be a *Phoma* pattern fungus which, in temperate climates can only exist in fresh-water during winter months, and can only exist in sea water during the summer months. The only evidence that such a fungus exists was found by Gold (1959), "the absence of reproductive structures under certain conditions does not indicate that specific fungi were absent but rather that they may have failed to reach reproductive maturity..." The other type of transitional form may require that physical conditions, chemical constituents, or hosts from sea water for one stage of its life

cycle, and from fresh water for the other stages. However, the extensive works of Höhnk (1939, 1952a and b, 1953a and b, 1955, 1956) have not indicated that such a condition exists in the Phycomycetes, in which it would be most likely to occur.

The term, "terraquatic", might be applied to those fungi which are permanent inhabitants of marine and non-marine habitats, though are not transitional forms. Some fungi have been found to occur in several habitats, and the marine and non-marine isolates appear morphologically to be the same species (Fell et al. 1960; Meyers and Reynolds, 1959b; Siepmann, 1959a and b; Siepmann and Johnson, 1960). In some cases, legitimate species or lower taxa may have been distinguishable by features other than the morphology of mature reproductive structures. Developmental morphology will likely be a profitable approach to this problem in some groups of fungi. However, in this investigation, efforts have been directed more toward the discovery of physiological criteria for delimiting the marine mycota. To distinguish between two different, but morphologically similar fungi, one a marine isolate and the other not, and to indicate whether a marine isolate might be indigenous to that environment, the following experimental approaches are suggested: (1) determinations of the specific gravities, and the amounts and types of oil in conidia; (2) salinity optima for asexual reproduction on low-thiamin level media; (3) study of nutrition on a reproducible artificial sea water; (4) determinations of cellulolytic activity, as suggested by Meyers and Reynolds (1959b); (5) evaluation of the abilities of species to disseminate in nature or in simulated natural conditions; (6) respirometric studies. On consideration of the last approach the following observations were made. Growth of Halosphaeriopsis, but not

C. globosum, in the continual flow device of Experiment 15, the "stacking" phenomenon of C. globosum, and the shift to the right of salinity optima for some activities of Halosphaeriopsis on low-thiamin level media, all suggest that the fermentative abilities and oxygen requirements of marine fungi are different from those of fungi which cannot be indigenous to marine environments. The aerobic, and particularly anaerobic respiration of filamentous fungi has never been thoroughly investigated, but respirometry may well become a valuable tool in several areas of marine mycology.

SUMMARY

Terrestrial Chaetomium globosum and two marine Pyrenomycetes, Torpedospora radiata and a species of Halosphaeriopsis, were subjects of extensive laboratory investigations. Topics considered were: nutrition and culture of fruiting marine Pyrenomycetes; technics for precise evaluation of sporulation; utility of tris (hydroxymethyl) amino methane as a buffer for fungus cultures; effects of temperature and levels of nitrogen and vitamins on salinity optima for growth and sporulation; temperature-affected mineral salts requirements which result in a Phoma pattern (Ritchie, 1957 and 1959); characteristics of Chaetomium globosum which make it unfit for marine environments.

1. When cultured on graded series of sea-water agars at four temperatures, Phoma pattern affinities were displayed by Torpedospora and Chaetomium, and were weakly indicated in Halosphaeriopsis.
2. At 35 C Torpedospora required Na, and was inhibited by a high concentration of a mixture of Ca, Mg, and K. At 35 C the other species responded favorably to Na or to the mixture. At 20 C all species were inhibited by a high concentration of Na, and stimulated by the mixture. This suggests involvement of salt antagonism in the Phoma pattern of Torpedospora.
3. Chaetomium grew poorly on vitamin free media if salinity was high and temperature low. Torpedospora was deficient for thiamin and biotin. Casein hydrolysate partially replaced biotin. Vitamin requirements were least when temperature and salinity were low. Inorganic nitrogen: phosphorus level did not affect salinity optima. Halosphaeriopsis was thiamin deficient. High thiamin level favored growth in low

salinity, as determined by dry weights of mycelia.

4. Chaetomium fruited on sea-water agar, but not in a marine aquarium. This, and buoyancy of mycelia in sea water, indicated that oxygen content and specific gravity of sea water, not salinity and alkalinity, exclude Chaetomium from marine environments. This demonstrated that a vegetative Phoma pattern is not a reliable index of marine-fitness.
5. Growth and fructification of Torpedospora were favored by similar conditions of temperature, salinity, and nutrient level. This fungus fruited in artificial sea water containing chelated trace metals, ammonium nitrate and phosphate, biotin and thiamin, cellulose, and tris buffer. In this artificial sea water Halosphaeriopsis required wood for sexual reproduction.
6. Tris satisfactorily buffered fungus cultures, but inhibited sporulation if glucose was the sole carbon source. Torpedospora required cellulose whether the buffer was present or not.
7. A potentially precise technic for evaluation of sporulation was devised. Agar was removed from tube cultures with boiling wash waters and centrifugation. Mycelia were ground in 2 ml of water in a Corning 7725 tissue homogenizer. Three 0.03 ml samples were taken from each spore suspension so prepared, and three coverslip preparations were made. Counts of spores in ten HPF of each of three samples were averaged, and "spores per HPF" were reported. Reproducible spore counts proved the effectiveness of the method.
8. Asexual reproduction of Halosphaeriopsis decreased as salinity increased on 1 g/liter yeast extract, but increased with salinity on 10 mg/liter. In 100 mg/liter asexual reproducation was similar in all salinities 8.5 to 34 o/oo. Salinity optima for fructification apparently increased

slightly as temperature and nutrient level decreased. As salinity tolerance of the other two species was a function of respiration rate, in turn dependent upon temperature and thiamin pyrophosphate, it was suggested that Halosphaeriopsis may have greater fermentative powers.

9. Operating in an estuary, a Phoma pattern would function to establish a fungus in regions where vitamin efficiency and organic production were greatest; that is, in low salinity in winter, and high salinity in summer.

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Table 1-A

Colony diameters (mm) of C. *globosum* after six days on seven strengths of NS-1 at four temperatures; averages for each of three runs, and average of three runs.

Strength NS-1	Temperature (C) 20				25				30				35			
				(avg)				(avg)				(avg)				(avg)
0	29	42	26	32	50	54	48	50	50	56	48	51	3	0	7	3
1/4	49	50	48	49	70	70	67	69	78	76	80	78	5	4	8	6
1/2	47	50	46	47	60	70	58	62	70	70	65	69	11	12	11	11
3/4	44	46	43	44	56	68	51	58	64	68	58	63	22	30	12	21
1	38	42	36	38	51	53	46	50	51	53	48	51	17	35	12	21
1½	28	30	25	28	37	42	38	39	42	42	38	41	12	24	14	17
2	18	18	19	18	31	32	29	31	32	32	30	31	9	10	6	8

Table 1-B

Colony diameters (mm) of C. *globosum*; individual replicates of 35 C run number three of Table 1-A. Plates were stacked three high.

Strength NS-1	Top Plate	Middle Plate	Bottom Plate
0	9	6	5
1/4	11	7	6
1/2	14	11	8
3/4	16	11	9
1	15	11	8
1½	19	14	9
2	11	5	3

Table 1-C

Relative abundance of C. globosum perithecia after 12 days on seven strengths of NS-1 at four temperatures; average of three runs.

Strength NS-1	Temperature (C) 20	25	30	35
0	4	6	3	0
1/4	3	5	2	0
1/2	2	5	2	0
3/4	1	2	1	0
1	?	1	0	0
1½	0	0	0	0
2	0	0	0	0

Table 1-D

Colony diameters of T. radiata after 12 days on seven strengths of NS-1 at four temperatures; averages for each of three runs, and average of three runs.

Strength NS-1	Temperature (C) 20				25				30				35			
				(avg)				(avg)				(avg)				(avg)
0	4	3	3	3	8	6	6	7	8	8	8	8	0	0	0	0
1/4	5	3	4	4	10	8	8	9	10	12	12	11	0	1	2	1
1/2	6	5	5	5	10	13	14	12	19	17	16	17	5	4	5	5
3/4	4	5	4	4	10	12	14	12	20	18	18	19	10	9	9	9
1	3	4	4	4	11	13	12	12	20	18	19	19	7	7	6	7
1½	3	2	2	2	11	9	9	10	16	13	14	14	4	3	3	3
2	1	2	1	1	4	1	2	3	6	5	5	5	0	1	1	1

Table 1-E

Torpedospora radiata colony diameters (mm); first run of Table 1-D after 14 days growth.

	Temperature (C)			
Strength NS-1	20	25	30	35
0	5	11	10	0
1/4	8	12	12	2
1/2	10	13	25	8
3/4	6	13	26	12
1	5	13	24	9
1½	3	14	20	4
2	2	5	9	1

Table 1-F

Halosphaeriopsis colony diameters (mm) after 12 days on seven strengths of NS-1 at four temperatures; averages for each of three runs, and average of three runs.

	Temperature (C)															
Strength NS-1	20				25				30				35			
				(avg)				(avg)				(avg)				(avg)
0	7	7	8	7	13	15	10	13	14	13	11	13	9	3	6	6
1/4	10	10	10	11	15	18	14	16	22	24	17	21	9	4	6	6
1/2	9	10	7	9	15	18	15	16	20	22	20	21	12	10	9	10
3/4	10	10	8	9	14	18	15	16	18	20	19	19	8	6	4	6
1	6	9	5	7	11	13	13	12	14	13	17	15	7	2	3	4
1½	4	7	4	5	7	8	9	8	7	6	10	8	2	2	1	2
2	2	2	4	3	4	2	5	4	3	3	5	4	1	1	0	1

Table 2-A

Colony diameters (mm) of C. *globosum* after six days on seven strengths of AS-1 at four temperatures; average of three runs.

	Temperature (C)			
Strength AS-1	20	25	30	35
1/16	30	40	42	10
1/4	47	57	60	23
1/2	48	60	70	30
3/4	45	60	70	48
1	38	51	65	49
1½	22	38	43	35
2	17	30	31	21

Table 2-B

Relative abundance of C. *globosum* perithecia after 12 days on seven strengths of AS-1 at four temperatures; average of three runs.

	Temperature (C)			
Strength AS-1	20	25	30	35
1/16	3	6	3	0
1/4	2	5	2	0
1/2	1	3	1	0
3/4	0	2	1	0
1	0	1	0	0
1½	0	0	0	0
2	0	0	0	0

Table 2-C

Colony diameters (mm) of _T. radiata_ after 12 days on seven strengths of AS-1 at four temperatures; averages for each of three runs, and average of three runs.

	Temperature (C)															
Strength AS-1 Base				20				25				30				35
				(avg)				(avg)				(avg)				(avg)
1/16	2	3	3	3	4	4	3	4	4	5	4	4	2	3	0	2
1/4	6	8	9	8	11	12	12	12	14	11	11	12	7	4	2	4
1/2	10	11	12	11	17	18	16	17	20	16	18	18	8	7	4	6
3/4	11	9	10	10	18	17	15	17	24	22	23	23	12	12	11	12
1	9	6	6	7	14	14	13	14	20	20	16	19	14	12	10	12
1½	4	5	3	4	8	7	8	8	12	10	9	10	4	4	3	4
2	1	0	1	1	3	4	4	4	4	5	5	5	3	2	2	2

Table 2-D

Torpedospora radiata colony diameters (mm); third run of Table 2-C after 14 days.

	Temperature (C)			
Strength AS-1 Base	20	25	30	35
1/16	4	4	4	0
1/4	10	12	12	3
1/2	14	20	24	5
3/4	12	21	26	12
1	10	17	24	11
1½	5	10	14	3
2	3	4	5	2

Table 2-E

Colony diameters (mm) of Halosphaeriopsis after 12 days on seven strengths of AS-1 at four temperatures; averages for each of three runs, and average of three runs.

Temperature (C)

Strength AS-1	20				25				30				35			
				(avg)				(avg)				(avg)				(avg)
1/16	5	5	6	4	5	6	6	6	9	6	6	7	5	4	3	4
1/4	8	11	9	10	10	13	10	11	13	12	12	12	9	6	8	8
1/2	9	13	10	11	17	17	17	17	21	18	16	18	6	2	3	4
3/4	11	12	10	11	16	18	16	15	18	20	19	19	15	8	12	12
1	7	10	8	8	16	14	13	14	13	16	14	14	17	13	13	14
1½	5	5	6	5	10	8	8	9	8	12	11	10	10	6	9	8
2	3	2	2	2	6	5	5	5	5	5	6	5	3	1	2	2

Table 2-F

Halosphaeriopsis colony diameters (mm); third run of Table 2-E after 14 days.

Temperature (C)

Strength AS-1	20	25	30	35
1/16	5	7	7	4
1/4	10	11	12	10
1/2	14	19	21	4
3/4	14	22	20	15
1	12	20	19	15
1½	8	12	11	9
2	5	6	6	2

Table 3-A

Colony diameters (mm) of *C. globosum* after six and 12 days on seven strengths of AS-1, less all vitamins, at four temperatures; average of four replicates.

Strength AS-1	Temperature (C) 20		25		30		35	
	6	12	6	12	6	12	6	12
1/16	17	30	24	38	24	45	12	14
1/4	19	36	31	53	39	61	17	21
1/2	9	13	15	45	49	70	35	38
3/4	9	15	15	33	34	54	35	39
1	6	18	15	30	32	49	25	33
1½	2	15	15	35	22	39	20	33
2	0	6	3	25	16	28	11	14

Table 3-B

Stacking phenomenon in *C. globosum*; 35 C plates of Table 3-A, after 12 days.

Strength AS-1	Top Plate	Middle Plate	Bottom Plate
1/16	17	14	11
1/4	23	20	20
1/2	41	40	34
3/4	43	40	35
1	36	34	30
1½	36	34	28
2	20	14	9

Table 3-C

Relative abundance of C. globosum perithecia after 12 days on seven strengths of AS-1, less vitamins, at four temperatures; average of four replicates.

	Temperature (C)			
Strength AS-1	20	25	30	35
1/16	2	5	3	0
1/4	?	3	1	0
1/2	0	2	1	0
3/4	0	1	?	0
1	0	0	0	0
1½	0	0	0	0
2	0	0	0	0

Table 3-D

Colony diameters (mm) of T. radiata on seven strengths of AS-1, less vitamins, at four temperatures; average of four replicates.

	Temperature (C)			
Strength AS-1	20	25	30	35
1/16	2	3	1	2
1/4	8	4	3	2
1/2	7	3	3	1
3/4	4	3	2	2
1	3	3	3	1
1½	2	2	4	2
2	0	0	2	2

Table 3-E

Colony diameters (mm) of *Halosphaeriopsis* after 12 and 15 days on seven strengths of AS-1, less vitamins, at four temperatures; average of four replicates.

	Temperature (C)							
Strength AS-1	20		25		30		35	
	12	15	12	15	12	15	12	15
1/16	5	6	6	8	9	11	3	3
1/4	10	12	13	20	17	20	7	10
1/2	8	10	12	16	16	19	0	0
3/4	6	8	8	11	9	12	7	8
1	6	7	7	8	8	10	10	11
1½	3	3	3	5	5	7	4	6
2	1	2	2	2	3	2	1	0

Table 3-F

Descriptions of T. radiata from AS-1, at various strengths and temperatures, with and without vitamins, after 15 days. Each description is the record of at least three observations.

20 C, vitamins present

1/16 - dense white colony, somewhat crumbly and raised in the center. The perimeter of the colony was composed primarily of thin vegetative hyphae, and some of these were present toward the center of the colony. The center of the colony was composed largely of a mass of thin, as well as thick stubby vegetative hyphae, and chlamydospores. See Figure 3-F (1).

1/4 - white to cream disk-shaped colony with a tough raised center. The periphery was composed of thin vegetative hyphae, and the center of chlamydospores and white to light-brown parallel vegetative hyphae.

1/2 - The periphery was as above, in 1/4, but the center was more raised, white, with a few dark flecks. In the center, parallel hyphae and short, thicker hyphae were more abundant than in 1/16 and 1/4.

3/4, 1 and 1½ - in the white, disk-shaped colony was a dark, somewhat raised center. Parallel hyphae in 3/4 and 1 were more abundant than in 1/16 and 1/4. No spores were observed.

2 - white colony with dark center, composed of fine vegetative hyphae, none of which grew parallel.

20 C, vitamins absent

1/16 through 2 - all colonies white and very sparse; 1/4 was probably the heaviest, and 1/16 the second heaviest colony. Only 1/16 and 1/4 contained chlamydospores.

Table 3-F (continued)

25 C, vitamins present

1/16 - cream colored colony, somewhat raised and irregular

1/4 - low, white, disk-shaped colony; dense

1/2 - as 1/4 25 C, above, but dark flecks were in the center of the colony.

3/4, 1, and 1½ - white disk, dense, and a dark-brown center comprised 1/2, 2/3, and 1/2 of the diameter of 3/4, 1, and 1½ colonies, resp.

1/16 - raised, cream colored colony; second heaviest of the series.

1/4 - fairly dense cream center and sparse white periphery; heaviest of this series.

1/2 through 2 - sparse, with occasional dark fleck in the center.

30 C, vitamins present

1/16 - white, irregular, and raised; composed of a mass of chlamydospores and perhaps other asexual spores. Filamentous hyphae were rare in some colonies and absent in others. The proportion of the mycelium devoted to sporulation, or fragmentation was greatest in this series. See Figure 3-F (3).

1/4 - disk-shaped; dense white periphery and raised white center. Some thin filamentous hyphae were in the center, and cells became shorter and thicker toward the periphery. Sporulation or fragmentation was second greatest in this series. A mass of chlamydospores was present. See Figure 3-F (4).

1/2 - flat white colony with raised center; center composed of thin filamentous hyphae, becoming somewhat shorter and thicker toward the periphery; a few parallel hyphae and chlamydospores were in the periphery. See Figure 3-F (5).

Table 3-F (continued)

3/4 through 2 - a white disk with dark-brown center comprising, resp., 1/3, 1/2, 1/2, and 1/3 of the diameter of 3/4, 1, 1½, and 2 colonies; 3/4 contained the greatest number of parallel hyphae in this series. These diminished in number in 1½ and were not present in 2.

30 C, vitamins absent

1/16 through 2 - sparse growth; somewhat orange in 1/4. Production of chlamydospores was greatest in 1/16 and 1. Spores resembling microconidia were present. See Figure 3-F (2).

35 C, vitamins absent

1½ - apparently heaviest colony in this series; pink to peach, and raised.

Table 3-G

Descriptions of Halosphaeriopsis on AS-1 at various strengths and temperatures, with and without vitamins, after 15 days growth. Each description is the record of at least three observations.

20 C, vitamins present

1/16 through 2 - dark, dense colony with aerial hyphae most abundant in the center; gray to white at the periphery and gray to black in the center; abundance of conidia in 1/16 and 1/4; the number of conidia decreased as salinity increased.

20 C, vitamins absent

1/16 - abundance of conidia in dark center of sparse colony; light brown sparse periphery.

1/4 - as 20 C 1/16 above, but more sparse.

Table 3-G (continued)

20 C, vitamins absent (continued)

1/2 through 2 - very sparse, light brown to white

25 C, vitamins present

1/16 through 2 - dense, dark green in lower salinities, light green in middle range, and dark gray at highest salinities; aerial hyphae and conidia abundant; number of conidia decreased as salinity increased.

25 C, vitamins absent

1/16 - dark gray-green, somewhat raised; heaviest in this series.

1/4 through 2 - sparse, with brownish center and lighter periphery.

30 C, vitamins present

1/16 through 2 - dense, with many aerial hyphae; periphery light green to dark green; center gray to black; little to no green coloration at highest salinities; high salinity colonies were less dense, almost black-centered, with gray to white periphery. Number of spores decreased as salinity increased; 1/16 contained a number of asexual cells: conidia, chlamydospores, and perhaps others.

30 C, vitamins absent

1/16 - dense black colony composed of dark vegetative hyphae and conidia.

1/4 - dark center; sparse periphery; fewer conidia than 1/16 above.

1/2 through 2 - number of conidia decreased as salinity increased; dark center and sparse periphery.

35 C, vitamins present

1/16 through 2 - dark green to black center, and lighter periphery. Conidia decreased as salinity increased.

Table 3-G (continued)

35 C, vitamins absent

1/16 - dense black colony composed of short, dark hyphae and conidia.

1/4 - black center and sparse periphery; heaviest of this series.

1/2 through 2 - dark center and sparse periphery.

Table 4-A

Colony diameters (mm) of T. radiata after 13 days on AS-1 strengths 1/4 and 3/4, at four temperatures; single vitamin deletions; averages of four replicates; S denotes sparse growth.

	1/4 AS-1				3/4 AS-1			
Vitamin mixture	20 C	25 C	30 C	35 C	20 C	25 C	30 C	35 C
complete	10	14	14	4	10	18	25	8
no pyridoxine	8	15	15	5	9	19	24	7
no nicotinic acid	10	14	14	3	9	18	24	6
no inositol	9	14	13	4	9	18	24	7
no thiamin	11S	16S	15S	1S	5S	15S	24S	2S
no biotin	8S	5S	4S	2S	5S	4S	3S	2S
no vitamins	8S	6S	4S	1S	3S	2S	3S	1S

Table 4-B

Colony diameters (mm) of Halosphaeriopsis after 13 days on AS-1 strengths 1/4 and 3/4 at four temperatures; single vitamin deletions; averages of four replicates; S denotes sparse growth.

	1/4 AS-1				3/4 AS-1			
Vitamin mixture	20 C	25 C	30 C	35 C	20 C	25 C	30 C	35 C
complete	12	14	14	10	13	19	20	13
no pyridoxine	11	13	13	9	12	18	20	13
no nicotinic acid	11	14	13	10	11	17	20	12
no inositol	12	14	13	8	11	19	22	13
no thiamin	12S	14S	16S	9S	10S	12S	14S	8S
no biotin	10	13	12	7	10	15	20	12
no vitamins	6S	10S	10S	5S	2S	3S	2S	2S

Table 5-A

Colony diameters (mm) of Halosphaeriopsis after 13 days on 1/4 and 3/4 AS-1 at 30 C; vitamin content variable; averages of four replicates.

Vitamins in medium	1/4 AS-1		3/4 AS-1	
thiamin, biotin	12	gray aerial, dark green beneath	20	
thiamin	13	white to gray aerial, most abundant; dark green beneath	19	
complete	13	gray aerial; dark green beneath	20	
no vitamins	13S		7S	
biotin	19	very sparse	14	very sparse
inositol	16S		6S	
nicotinic acid	17S		10S	
pyridoxine	20S		10S	
no thiamin	16S		17S	
no thiamin or biotin	20S		16S	

Table 6-A

Torpedospora radiata cross inoculation test; diameters (mm); S denotes sparse growth.

Temperature	Strength AS-1	Inoculum		Medium	
		no vitamins	vitamins	no vitamins	vitamins
20	1/4		X	8S	10
20	1/4	X		0	10
20	3/4		X	3S	11
20	3/4	X		0	11
25	1/4		X	7S	14
25	1/4	X		0	13
25	3/4		X	2S	18
25	3/4	X		0	17
30	1/4		X	4S	12
30	1/4	X		0	11
30	3/4		X	4S	25
30	3/4	X		0	23
35	1/4		X	1S	4
35	1/4	X		0	3
35	3/4		X	2S	11
35	3/4	X		0	10

Table 6-B

Halosphaeriopsis cross inoculation test; diameters (mm); S denotes sparse growth.

Temperature	Strength AS-1	Inoculum		Medium	
		no vitamins	vitamins	no vitamins	vitamins
20	1/4		X	8S	12
20	1/4	X		6S	12
20	3/4		X	4S	13
20	3/4	X		2S	12
25	1/4		X	14S	11
25	1/4	X		10S	10
25	3/4		X	14S	15
25	3/4	X		1S	12
30	1/4		X	11S	14
30	1/4	X		11S	14
30	3/4		X	8S	22
30	3/4	X		1S	20
35	1/4		X	5S	10
35	1/4	X		3S	9
35	3/4		X	5S	17
35	3/4	X		1S	15

Table 7-A

Dry weights (mg) of T. radiata from liquid AS-1, 1/4 and 3/4 strengths, vitamins variable, at 20 C and 30 C, after two, four, and six weeks; averages of four replicates. (When present, biotin 5 ug, thiamin 100 ug/liter.

Vitamin Mix	1/4	3/4	20 C	30 C	2wks	4wks	6wks
none	X		X		0.2	0.3	0.2
none	X			X	0.5	0.3	0.3
none		X	X		0.8	0.2	0.3
none		X		X	0.1	0.2	0.4
thiamin-biotin	X		X		4.6	9.7	7.8
thiamin-biotin	X			X	4.1	7.2	7.1
thiamin-biotin		X	X		5.0	10.0	17.1
thiamin-biotin		X		X	23.8	88.3	85.5
biotin	X		X		2.7	3.2	2.0
biotin	X			X	3.0	2.7	1.0
biotin		X	X		3.2	3.5	1.3
biotin		X		X	1.8	1.2	0.8
thiamin	X		X		0.8	0.4	0.4
thiamin	X			X	0.4	0.3	0.5
thiamin		X	X		0.7	0.3	0.2
thiamin		X		X	0.4	0.5	0.7

Table 8-A

Dry weights (mg) of *T. radiata* after 20 days in three strengths of AS-1 with tris constant 0.1 %, thiamin constant 100 ug/liter, and biotin varied; 25 C. Initial pH 8.4-8.6, and final pH plus or minus 0.2 recorded by weight. Averages of each of two runs, and average of two runs.

Biotin ug/liter	Strength AS-1											
	1/4				1/2							
	pH	1	2	avg	pH	1	2	avg	pH	1	2	avg
5	4.5	28.5	33.2	30.8	5.5	31.3	39.0	35.1	4.7	18.7	15.0	16.8
0.5	4.2	14.4	12.6	13.5	4.2	19.4	18.7	19.1	4.3	12.4	11.3	11.9
0.05	4.2	7.8	6.9	7.4	4.5	6.1	5.5	5.8	4.3	5.8	5.4	5.6
0.005	6.0	3.9	4.6	4.2	6.8	2.3	1.5	1.9	6.3	2.0	1.8	1.9
0.000	7.6	0.1	0.2	0.1	8.0	0.2	0.2	0.2	7.6	0.2	0.2	0.2

Table 8-B

As Table 8-A, but biotin constant (5 ug/liter) and thiamin varied.

Thiamin ug/liter	Strength AS-1											
	1/4				1/2							
	pH	1	2	avg	pH	1	2	avg	pH	1	2	avg
10	4.0	15.9	15.8	15.8	4.0	11.6	12.4	12.0	4.0	10.7	9.8	10.2
1.0	4.0	4.5	4.3	4.4	4.0	3.6	3.4	3.5	4.0	3.2	4.0	3.6
0.1	7.0	0.6	0.5	0.5	7.2	0.5	0.8	0.6	7.2	1.0	1.0	1.0
0.0	7.0	0.5	0.6	0.5	7.4	0.5	0.5	0.5	7.6	0.8	0.9	0.8

Table 8-C

Dry weights (mg) of <u>Halosphaeriopsis</u> after 20 days in three strengths of AS-1, tris constant 0.1 %, thiamin varied, at 25 C. Initial pH 8.4-8.6, final pH plus or minus 0.2 recorded. Averages of each of two runs, and average of two runs.

Thiamin ug/liter	Strength AS-1											
	1/4				1/2							
	pH	1	2	avg	pH	1	2	avg	pH	1	2	avg
100	6.0	32.0	23.0	27.0	5.0	16.6	18.4	17.0	5.0	15.0	17.9	16.0
10	6.2	9.6	11.0	10.3	6.2	11.6	10.1	10.8	6.2	10.9	9.4	10.1
1.0	7.5	1.7	2.0	1.8	7.5	1.5	1.9	1.7	7.5	1.2	1.5	1.3
0.1	8.0	0.2	0.0	0.1	8.0	0.2	0.2	0.2	8.0	0.2	0.2	0.2
0.0	8.4	0.1	0.1	0.1	0.4	0.1	0.0	0.1	8.4	0.1	0.1	0.1

Table 9-A

Dry weight (mg) of <u>Halosphaeriopsis</u> and <u>T. radiata</u> after 21 days in 1/4 AS-1, all tris replaced by $CaCO_3$, vitamins varied, at 25 C; average of six replicates.

Torpedospora radiata				Halosphaeriopsis	
Thiamin 100 ug/liter		Biotin 5 ug/liter			
Biotin ug/liter	Wt.	Thiamin ug/liter	Wt.	Thiamin ug/liter	Wt.
5	34.9	100	34.9	100	29.7
0.5	26.9	10	21.6	10	15.6
0.05	8.6	1	5.6	1	4.7
0.005	3.0	0.1	2.3	0.1	1.3
0.000	0.3	0.0	2.0	0.0	0.8

Table 10-A

Dry weights (mg) of T. radiata from three strengths of AS-1, tris constant 0.1 %, initial pH 8.4 to 8.6, after 20 days at 25 C, using three levels of nitrogen and phosphorus; final pH plus or minus 0.2 recorded; average of each of two runs, and average of two runs.

N:P/liter added	Strength AS-1											
	1/4				1/2				1			
	pH	1	2	avg	pH	1	2	avg	pH	1	2	avg
7:1 mg	7.0	8.4	9.5	8.9	6.6	13.0	11.8	12.5	6.6	12.7	11.0	11.8
10:1 ug	7.4	2.8	2.4	2.6	7.1	5.9	6.5	6.2	7.1	3.6	2.5	3.0
none	7.6	1.2	1.7	1.5	7.3	2.0	1.8	1.9	7.3	2.2	2.4	2.3

Table 11-A

Dry weights (mg) of T. radiata from AS-1 strength 1, after 20 days at 25 C; thiamin and nitrogen levels constant at 100 ug and 140 mg per liter, resp.; nitrogen sources and biotin level varied. Averages for each of two runs, and average of two runs.

	Nitrogen Sources								
	all NO_3			NO_3-aspartic			NO_3-casein hydrol.		
Biotin ug/liter	1	2	avg	1	2	avg	1	2	avg
0.5	46.6	44.7	45.6	39.1	33.6	36.3	45.3	46.7	46.0
0.05	30.2	24.1	27.1	11.4	7.2	9.3	17.0	19.3	18.1
0.005	3.9	3.2	3.5	3.6	3.8	3.7	6.2	5.1	5.6
0.000	0.0	0.1	0.1	0.5	0.3	0.4	3.9	3.0	3.4

Table 12-A

Relative amounts of growth of T. radiata and Halosphaeriopsis (in parentheses) in strength 1 AS-1 liquid after 20 days at 25 C, using various vitamin contents and carbon sources; average of ten replicates from two runs. S denotes sparse growth. N denotes no growth.

Carbon Source	Vitamins Added: Biotin-thiamin	Biotin	Thiamin	None
balsa	2	2	2 (2)	2 (2)
pine	3	3	3 (3)	3 (3)
Whatman paper	2	S	S (3)	N (S)
Whatman cellulose	2	S	N (2)	N (N)
glucose 5 g/liter	4	S	N (4)	N (N)

Table 13-A

Diameters (mm) of three Pyrenomycetes on altered AS-1 base; AS-1 nutrients const.

Strength of System				C. globosum			T. radiata			Halosphaeriopsis		
Tris	Metals	Minor	Major	20 C	30 C	35 C	20 C	30 C	35 C	20 C	30 C	35 C
1/4	1/4	1/4	1/4	49	61	23	5	12	3	9	12	6
1/2	1/2	1/2	1/2	50	69	30	6	18	6	10	16	4
1	1	1	1	42	66	49	4	16	15	8	14	12
1/4	1/2	1/2	1/2	41	59	24	5	16	9	10	14	12
1/4	1	1	1	45	56	36	4	16	13	8	14	16
1/2	1/4	1/2	1/2	47	67	35	5	18	6	10	16	12
1	1/4	1	1	46	68	48	4	17	15	8	15	14
1/2	1/2	1/4	1/2	54	68	22	6	18	6	10	16	4
1	1	1/4	1	49	60	48	5	15	15	8	15	10
1/2	1/2	1/2	1/4	40	71	25	5	14	7	11	15	11
1	1	1	1/4	51	58	4	5	12	3	10	15	10

Table 14-A

Diameters (mm) of three Pyrenomycetes on strength 1/4 AS-1 base made strength 1 in major system ingredients only. See experiment 14, Materials and Methods section, for key to additives 1-6.

	C. globosum		T. radiata		Halosphaeriopsis	
Additives	20 C	35 C	20 C	35 C	20 C	35 C
1.	46	22	9	6	8	8
2.	45	47	7	11	8	14
3.	36	49	7	12	6	13
4.	38	45	7	11	8	13
5.	9	10	3	7	7	11
6.	52	36	10	3	9	12

Table 15-A

Ascocarps of C. globosum per plate on three natural sea water (salinity 26 o/oo) media containing seven concentrations of tris buffer, after four weeks.

% tris	#	A(NS-1)		B(NS-7)		C(inorg.N:P:paper)	
		pH	ascocarps	pH	ascocarps	pH	ascocarps
0.000	1	5.9	245	7.2	1620	6.7	1188
0.007	2	6.3	221	7.7	1260	7.0	1224
0.013	3	6.4	270	7.8	1280	7.5	1188
0.025	4	6.8	419	8.0	1080	7.7	1224
0.05	5	7.3	764	8.2	1044	7.9	1264
0.1	6	7.7	85	8.4	1080	8.3	1620
0.2	7	7.9	55	8.5	864	8.5	1224

Table 16-A

Dry weights (mg) of C. globosum and Halosphaeriopsis from strengths 1/4 and 1 AS-1, potassium variable; buffer $CaCO_3$ or $CaCO_3$ plus 0.1 % tris.

			C. globosum		Halosphaeriopsis	
1/4 AS-1	1 AS-1	K+mg/liter	$CaCO_3$-tris	$CaCO_3$	$CaCO_3$-tris	$CaCO_3$
X		1000	33.6	36.5	27.2	24.3
X		100	35.1	36.5	29.4	19.2
X		10	27.3	31.3	24.8	14.7
X		1	25.0	20.6	7.1	7.6
X		0	11.4	7.7	3.0	1.8
	X	400		29.1		9.8
	X	100		30.9		8.9
	X	40		29.8		7.6

Table 17-A

Relative numbers of T. radiata ascocarps on seven strengths of NS-7 at three temperatures; pH 6.6-7.2 and pH 8.2-8.4 (0.1 % tris).

	pH 6.6 - 7.2			pH 8.2 - 8.4		
Strength NS-7	20 C	25 C	30 C	20 C	25 C	30 C
0	2.5	4.0	4.0	0.5	3.0	1.0
1/4	5.0	6.0	7.0	2.5	3.0	2.0
1/2	4.0	6.0	7.0	2.5	3.3	2.0
3/4	2.0	4.5	5.5	2.5	4.0	2.8
1	1.5	4.0	3.0	2.0	3.0	1.0
1½	0.0	2.3	2.5	0.0	0.0	0.0
2	0.0	0.0	0.0	0.0	0.0	0.0

Table 18-A

Halosphaeriopsis conidia (cnda) and ascospores (asco) per HPF from seven strengths of NS-1 at three temperatures; average of 6 tubes.

Strength NS-1	20 C		25 C		30 C	
	cnda	asco	cnda	asco	cnda	asco
0	22.6	0.12	16.5	0.32	24.1	0.033
1/4	19.7	0.27	13.2	1.6	19.0	0.13
1/2	13.8	0.83	15.2	1.4	13.5	0.011
3/4	12.7	0.011	8.7	0.022	10.6	0.0
1	7.5	0.0	7.3	0.011	5.9	0.0
1½	1.5	0.0	0.5	0.0	0.7	0.0
2	0.0	0.0	0.0	0.0	0.03	0.0

Table 18-B

Halosphaeriopsis conidia (no ascospores observed) per HPF from seven strengths of NS-1 plus 0.1 % tris at three temperatures; average of six tubes.

Strength NS-1	20 C	25 C	30 C
0	0.07	0.0	0.0
1/4	0.17	0.0	0.0
1/2	0.22	0.0	0.0
3/4	1.0	0.9	0.41
1	0.77	1.5	0.70
1½	0.13	1.1	0.35
2	0.0	0.0	0.01

Table 19-A

Halosphaeriopsis conidia (cnda) and ascospores (asco) per HPF from four strengths of NS-1, modified to contain yeast extract 100, 10, and 0.0 mg/liter; glucose constant at 5 g/liter; averages of six tubes.

yeast, mg/liter	1/4		1/2		3/4		1	
	cnda	asco	cnda	asco	cnda	asco	cnda	asco
100	14.3	0.16	13.6	0.33	14.3	0.12	13.4	0.05
10	6.3	0.0	5.9	0.0	5.7	0.0	11.2	0.0
0.0	0.68	0.0	1.4	0.0	1.1	0.0	1.1	0.0

Table 20-A

Halosphaeriopsis conidia (cnda) and ascospores (asco) per HPF and carbonous perithecia (prth) per tube from four strengths NS-7, with and without 0.1 % tris, at 20 C and 25 C; averages six tubes.

	No tris						0.1 % tris					
	20 C			25 C			20 C			25 C		
NS-7	cnda	asco	prth	cnda	asco	prth	cnda	asco	prth	cnda	asco	prth
1/4	.78	.006	61	.94	.34	88	.82	none	13	.45	.05	27
1/2	.61	.011	49	.63	.29	71	.61	none	18	.45	.10	41
3/4	.57	none	37	.58	.011	29	.42	none	9	.33	.011	31
1	.24	none	11	.30	none	12	.011	none	1	.22	.005	16

Table 20-B

Comparison of NS-1 and NS-7 conidia (cnda), ascospore (asco), and carbonous perithecia (prth) ratios.

		20 C		25 C	
		NS-1	NS-7	NS-1	NS-7
Ratio cnda/HPF	1/4:1/2	1.4	1.3	.87	1.5
	1/2:3/4	1.1	1.1	1.7	1.1
	3/4: 1	1.7	2.4	1.2	1.9
Ratio asco/HPF	1/4:1/2	.33	.5	1.1	1.2
	1/2:3/4	75	Large?	64	26
	3/4: 1	Large?	-	2.0 (Larger?)	Large?
Ratio prth/tube	1/4:1/2		1.2		1.2
	1/2:3/4		1.3		2.4
	3/4: 1		3.4		2.4

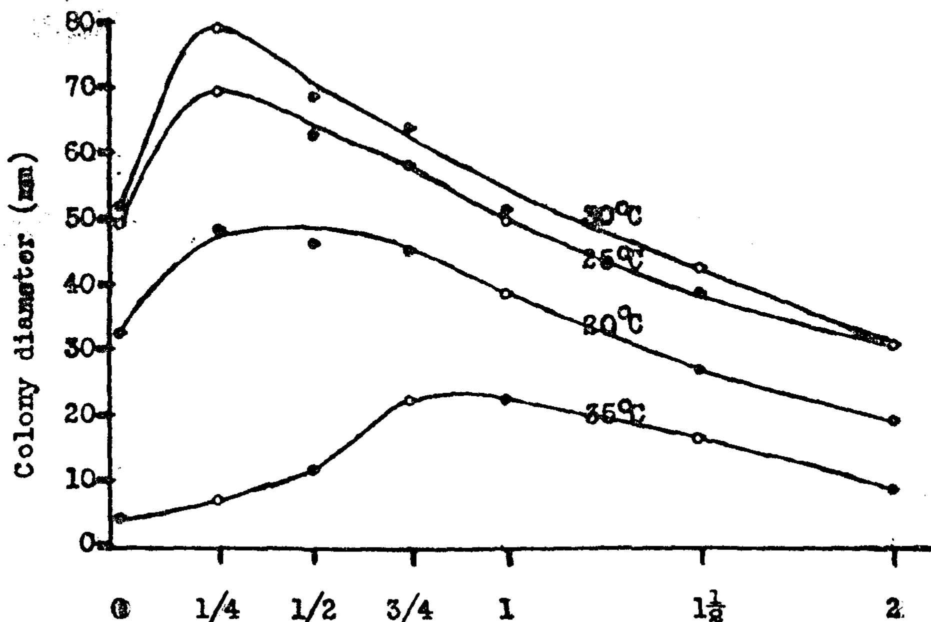
Table 21-A

Diameters (mm) T. radiata and Halosphaeriopsis (in parentheses) on seven strengths NS-1 at three temperatures; tris constant 0.1 %.

	Temperature (C)					
Strength NS-1	20		25		30	
0	3	(7)	5	(8)	7	(9)
1/4	5	(8)	8	(10)	14	(12)
1/2	5	(8)	12	(11)	18	(13)
3/4	5	(7)	12	(13)	19	(14)
1	3	(5)	11	(11)	18	(12)
1½	2	(2)	7	(8)	10	(9)
2	1	-	2	(4)	3	(4)

Figure 1-A

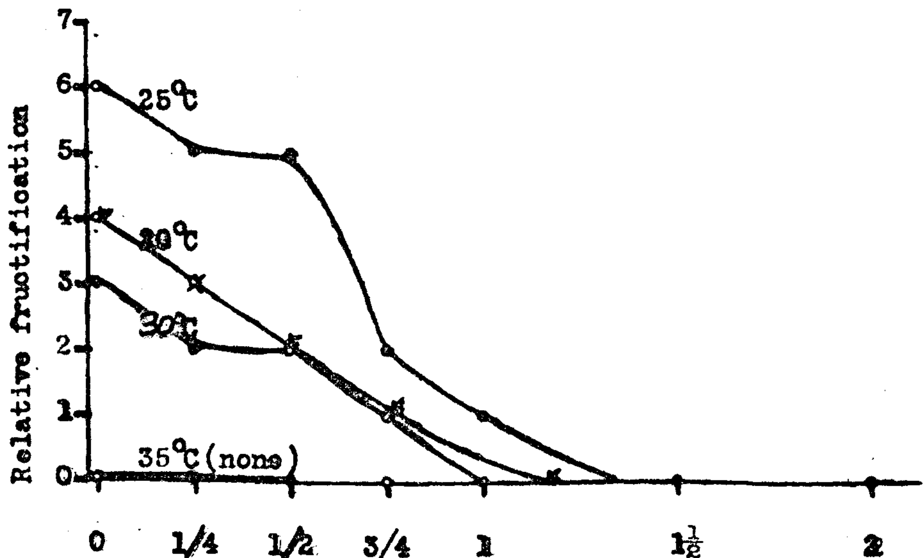
Colony diameters (mm) of C. globosum after six days on seven strengths of NS-1 at four temperatures; average of three runs.



Strength of NS-1; strength 1 equals salinity 34 o/oo

Figure 1-C

Relative abundance of C. globosum perithecia after 12 days on seven strengths of NS-1 at four temperatures; average of three runs.



Strength of NS-1; strength 1 equals salinity 34 o/oo

Figure 1-D

Colony diameters (mm) of T. radiata after 12 days on seven strengths of NS-1 at four temperatures; average of three runs.

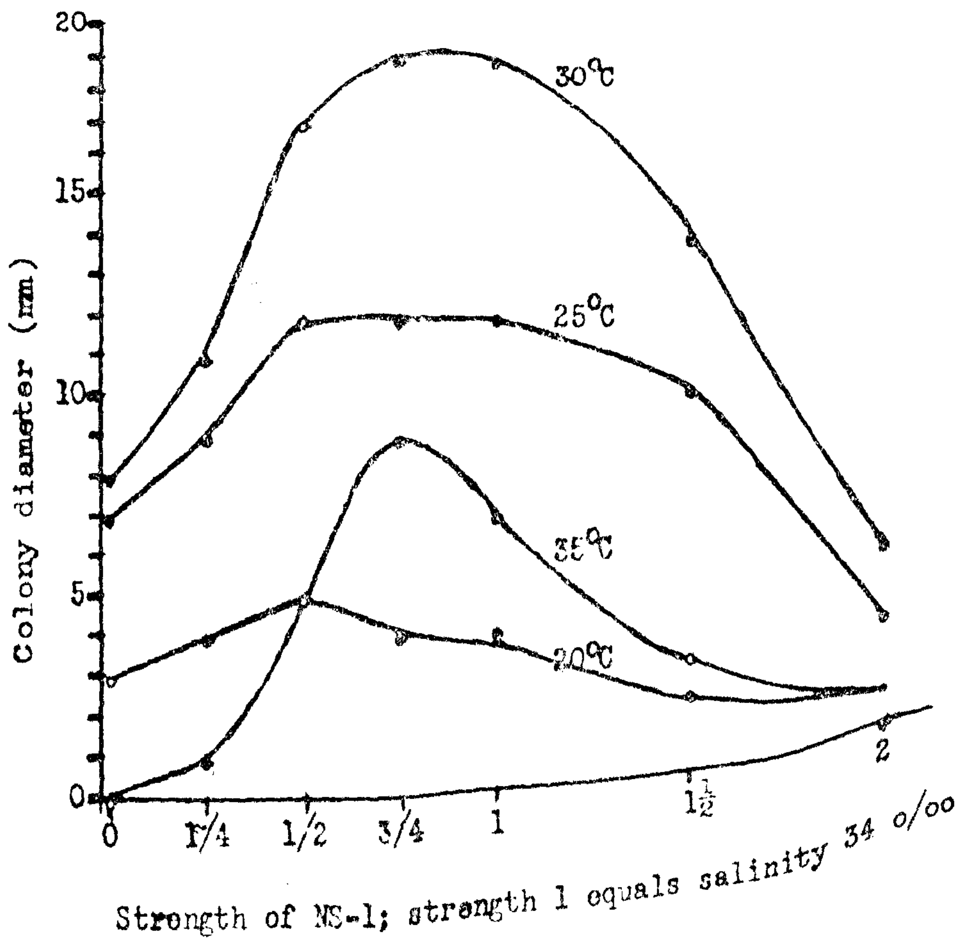


Figure 1-E

Torpedospora radiata; first run of Table and Graph 1-D after 14 days.

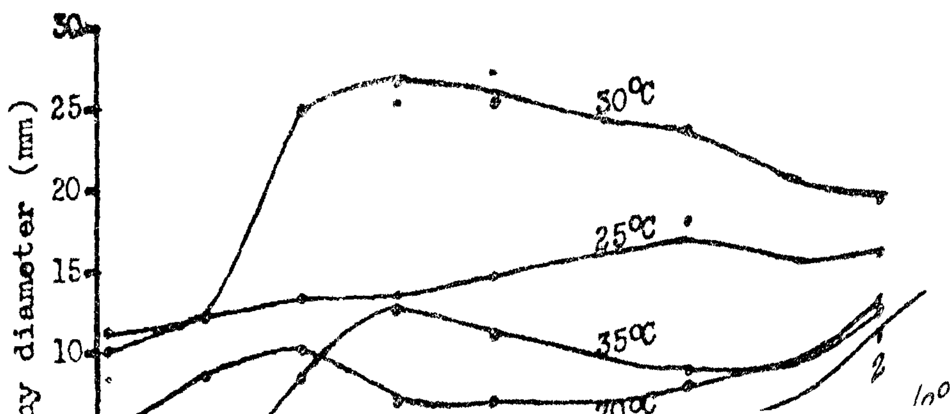


Figure 1-F

Halosphaeriopsis colony diameters (mm) after 12 days on seven strengths of NS-1 at four temperatures; average of three runs.

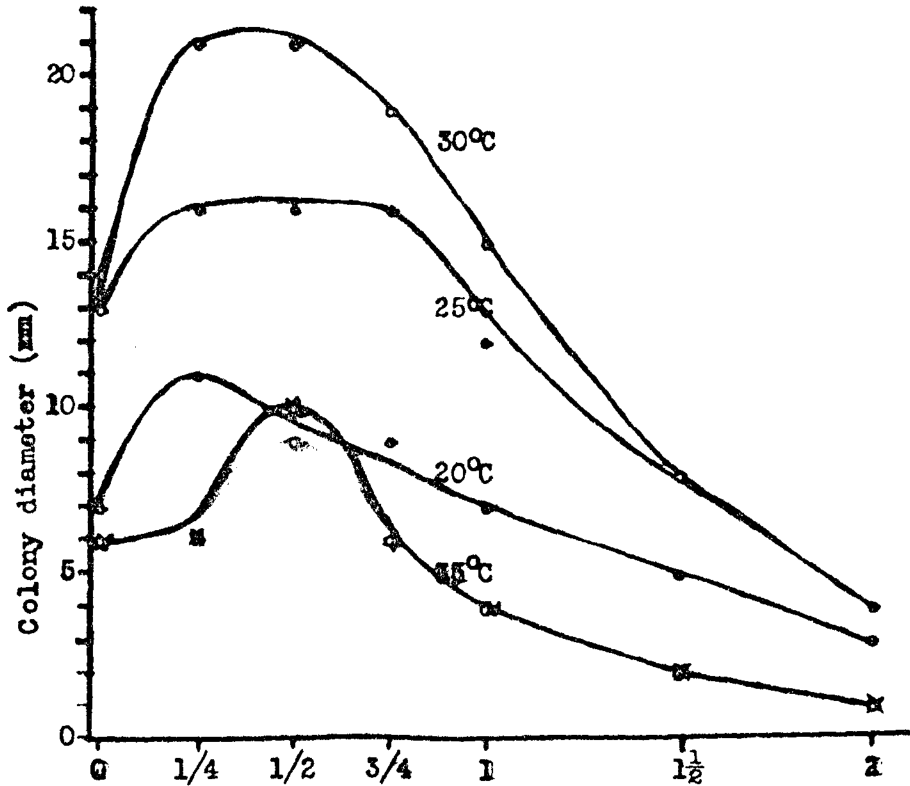
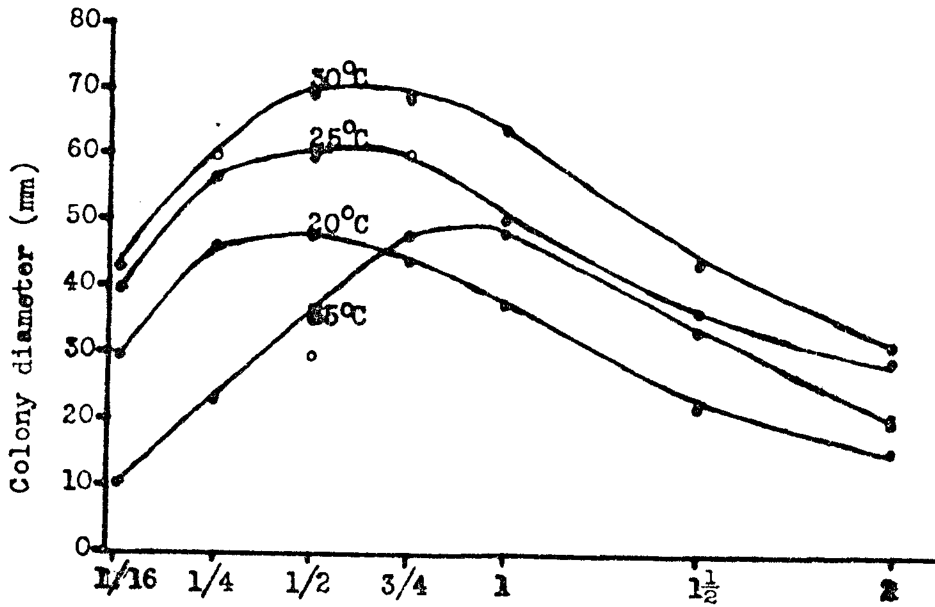


Figure 2-A

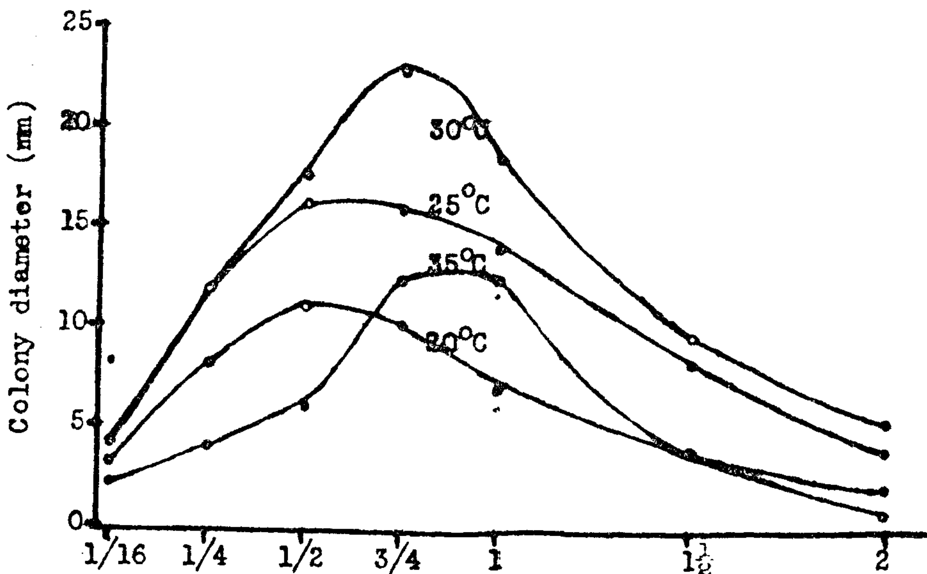
Colony diameters (mm) of C. globosum after six days on seven strengths of AS-1 at four temperatures; average of three runs.



Strength of AS-1; strength 1 equals salinity 34 o/oo

Figure 2-C

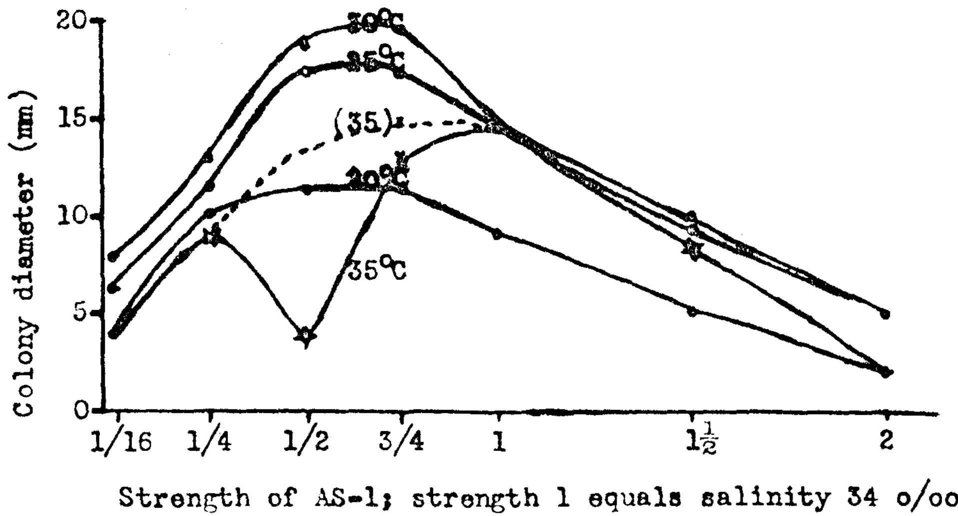
Colony diameters of T. radiata (mm) after 12 days on seven strengths of AS-1 at four temperatures; average of three runs.



Strength of AS-1; strength 1 equals salinity 34 o/oo

Figure 2-E

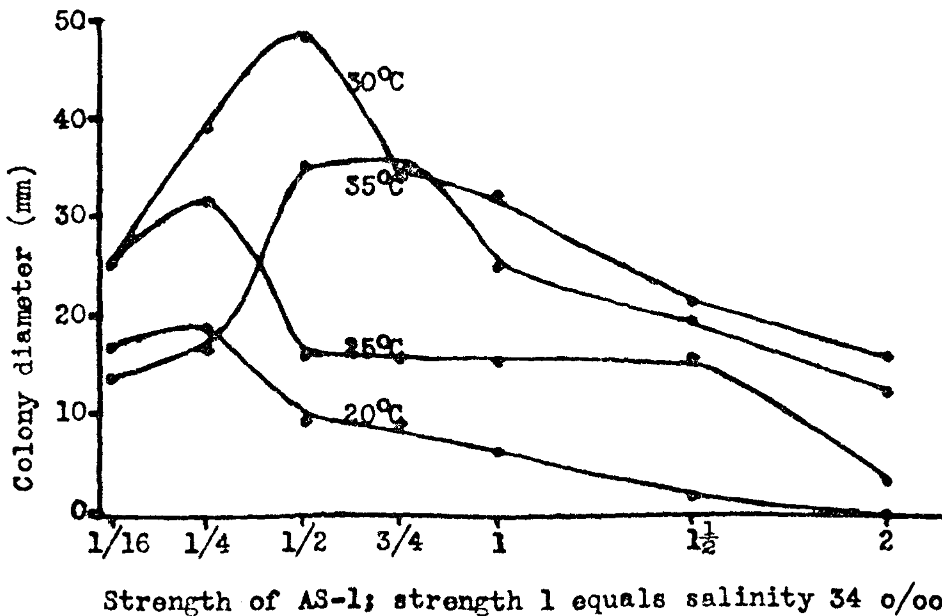
Colony diameters (mm) of Halosphaeriopsis after 12 days on seven strengths of AS-1 at four temperatures; average of three runs.



Strength of AS-1; strength 1 equals salinity 34 o/oo

Figure 3-A

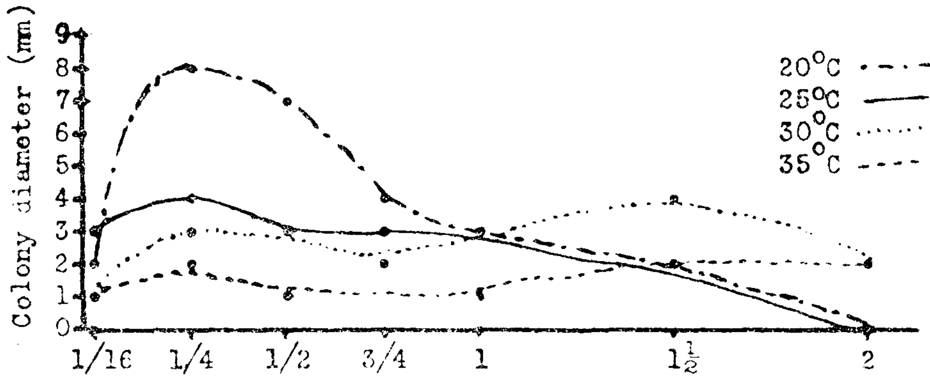
Colony diameters (mm) of C. globosum after six days on seven strengths of AS-1, less all vitamins, at four temperatures; average of four replicates.



Strength of AS-1; strength 1 equals salinity 34 o/oo

Figure 3-D

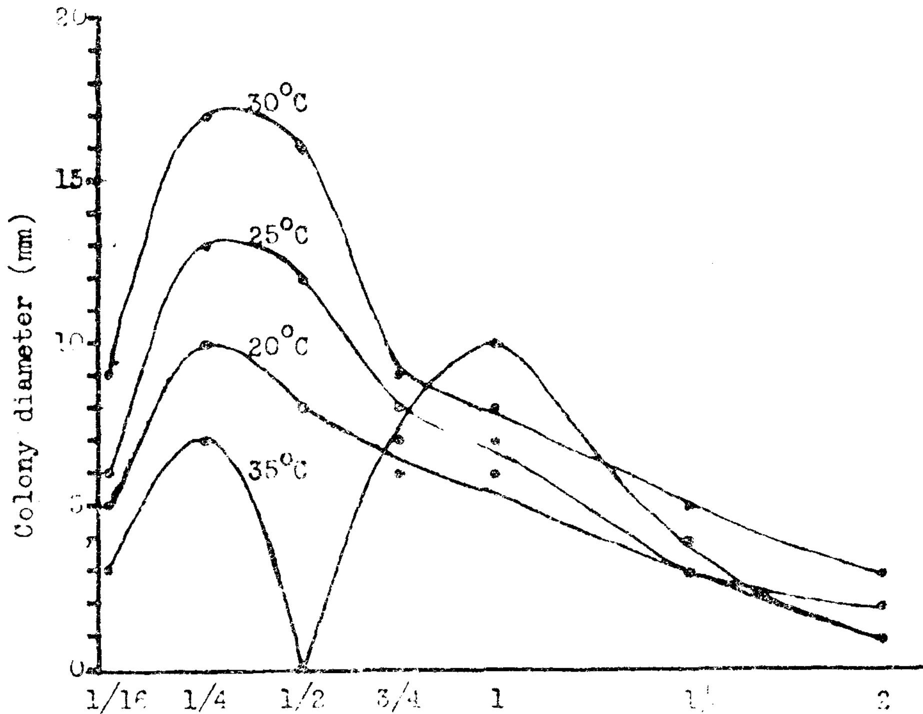
Colony diameters (mm) of *T. radiata* on seven strengths of AS-1, less vitamins, at four temperatures; average of four replicates after 12 days.



Strength of AS-1; strength 1 equals salinity 34 o/oo

Figure 3-E

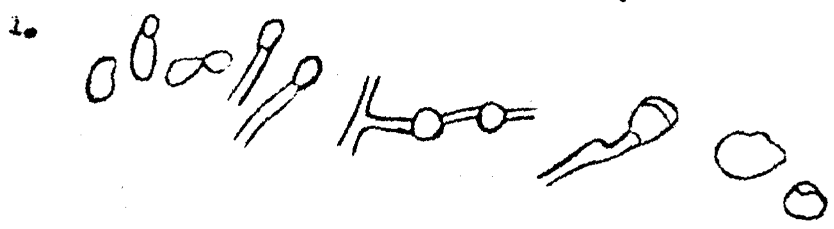
Colony diameters (mm) of *Halosphaeriopsis* after 12 days on seven strengths of AS-1, less vitamins, at four temperatures; average of four replicates.



Strength of AS-1; strength 1 equals salinity 34 o/oo

Figure 3-F

1.



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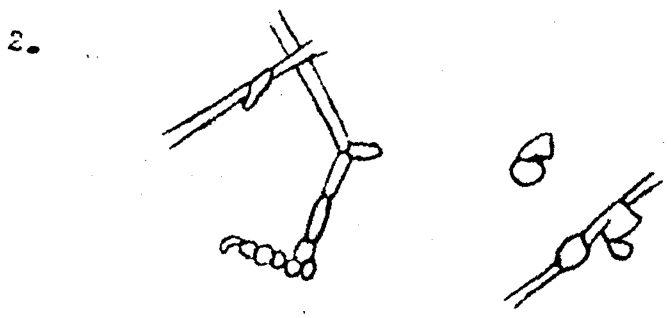

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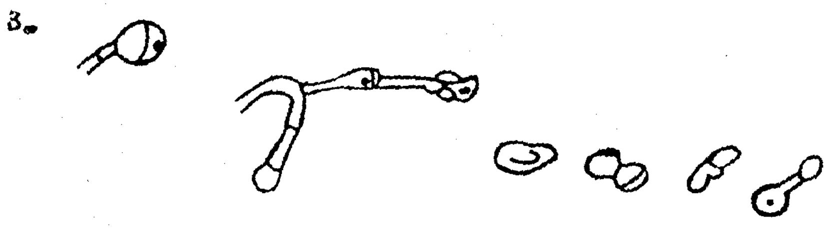

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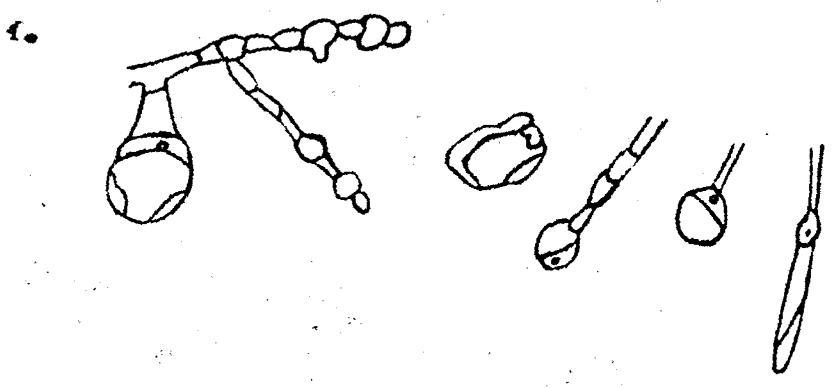

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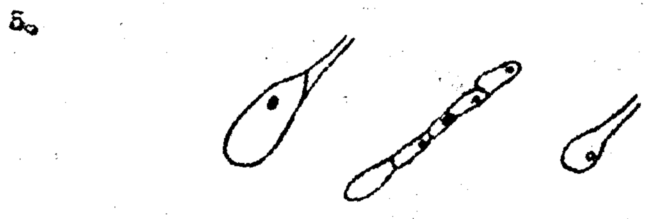


Figure 3-F

Torpedospora radiata from various strengths of AS-1 at 20 and 30 C, vitamins varied

1. Strength 1/16, vitamins present, 20 C

2. Strength 1/16, vitamins absent, 30 C

3. Strength 1/16, vitamins present, 30 C

4. Strength 1/4, vitamins present, 30 C

5. Strength 1/2, vitamins present, 30 C

Figure 4-A

Colony diameters (mm) of *T. radiata* after 13 days on 1/4 and 3/4 AS-1 at four temperatures; average of four replicates; T-bar denotes sparse growth. Single vitamin deletions are: 1) complete, no deletion; 2) pyridoxine; 3) nicotinic acid; 4) inositol; 5) thiamin; 6) biotin; 7) no vitamins added.

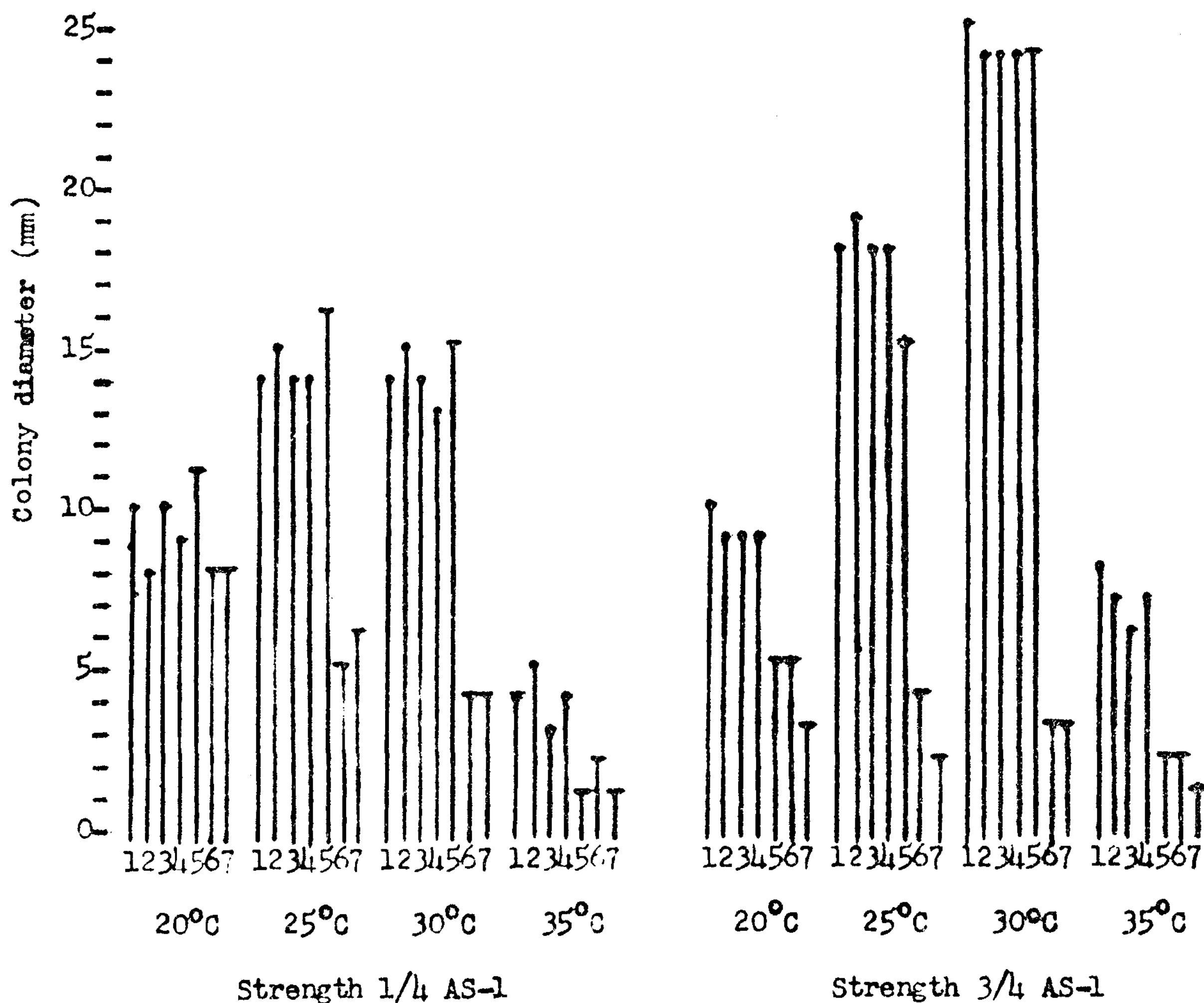


Figure 4-B

Colony diameters (mm) of Halosphaeriopsis after 13 days on 1/4 and 3/4 AS-1 at four temperatures; average of four replicates; T-bar denotes sparse growth. Single vitamin deletions are: 1) complete, no deletion; 2) pyridoxine; 3) nicotinic acid; 4) inositol; 5) thiamin; 6) biotin; 7) no vitamins added.

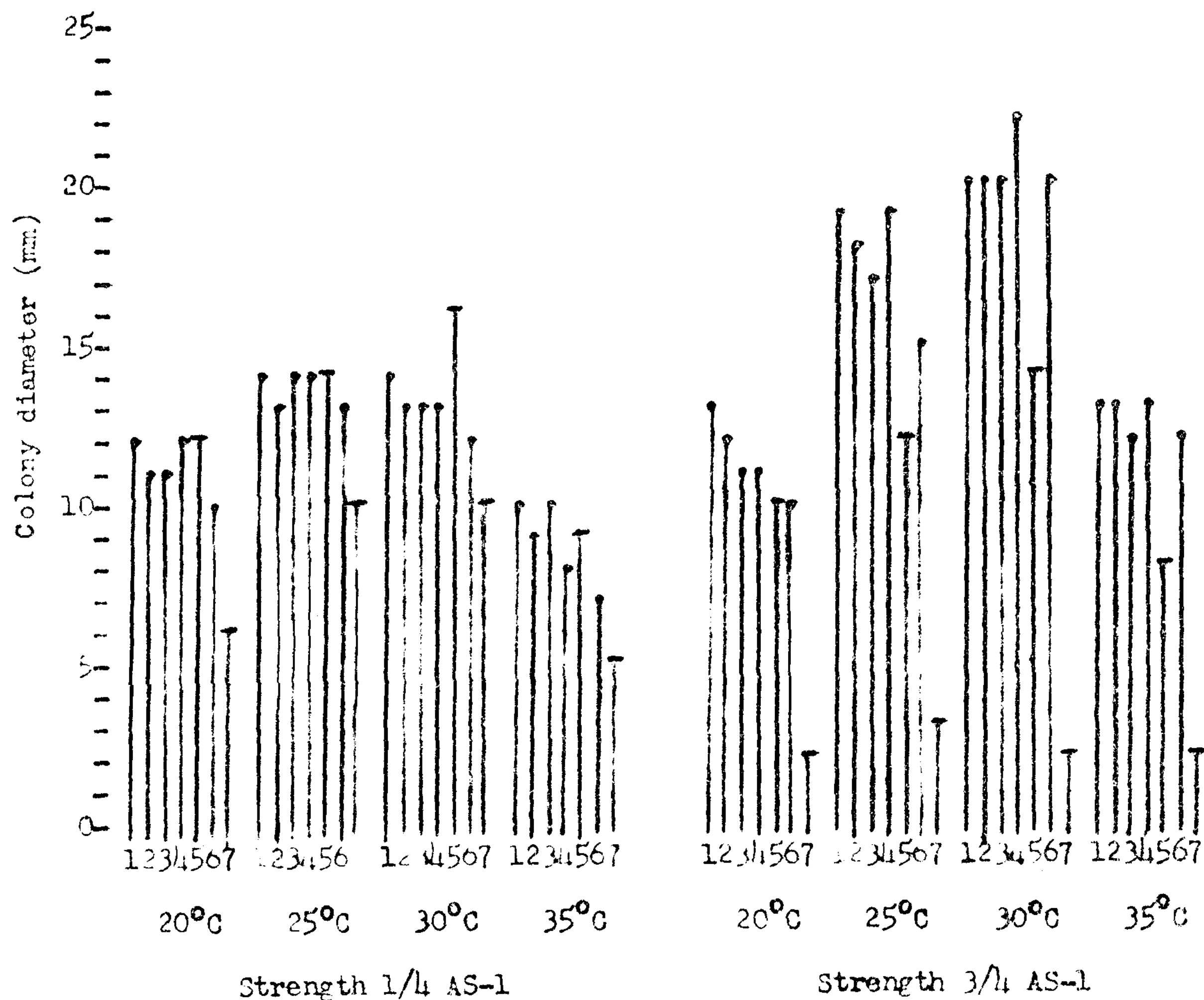
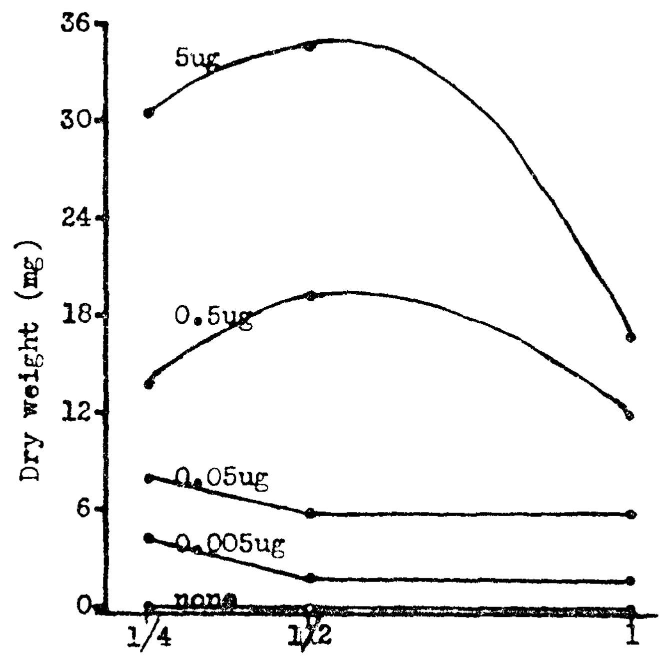


Figure 8-A

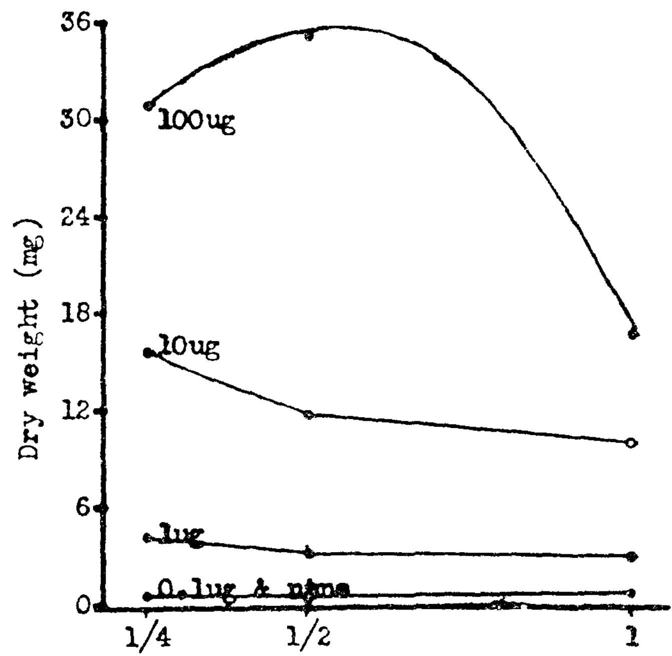
Dry weights (mg) of T. radiata from three strengths AS-1, biotin variable.



Strength of AS-1; strength 1 equals salinity 34 o/oo

Figure 8-B

Dry weights (mg) of T. radiata from three strengths AS-1, thiamin variable.



Strength of AS-1; strength 1 equals salinity 34 o/oo

Figure 8-C

Dry weights (mg) of Halosphaeriopsis from three strengths AS-1, thiamin varied.

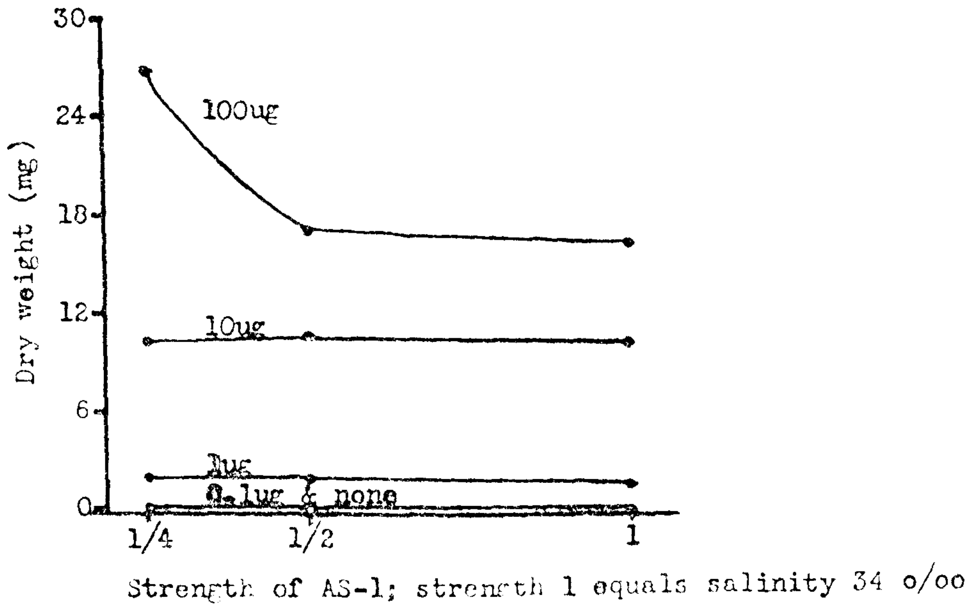
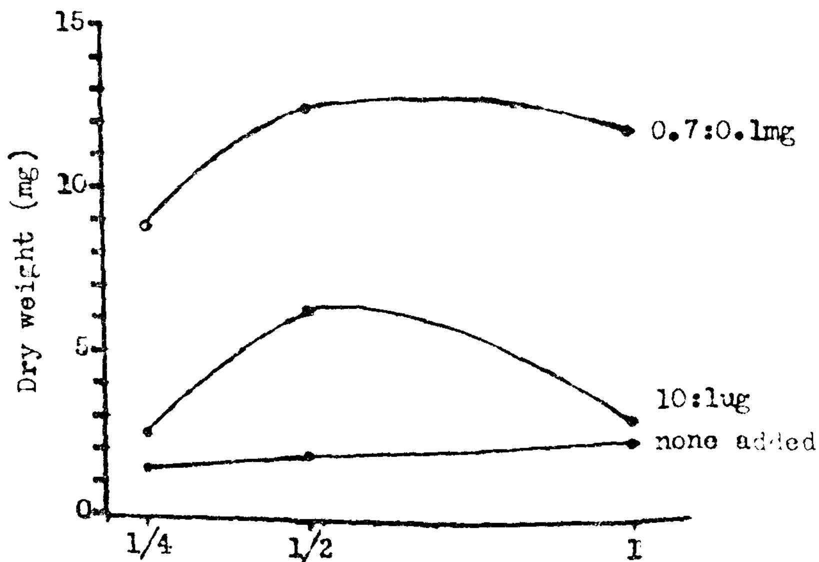


Figure 10-A

Dry weights (mg) of T. radiata from three strengths AS-1, nitrogen-phosphorus level variable.



Strength AS-1; strength 1 equals salinity 34 o/oo

FIGURE 12-A

<u>Torpedospora</u> <u>radiata</u> in AS-1, from left to right; glucose-biotin-thiamin; glucose-biotin; glucose-thiamin; glucose-no vitamins added.

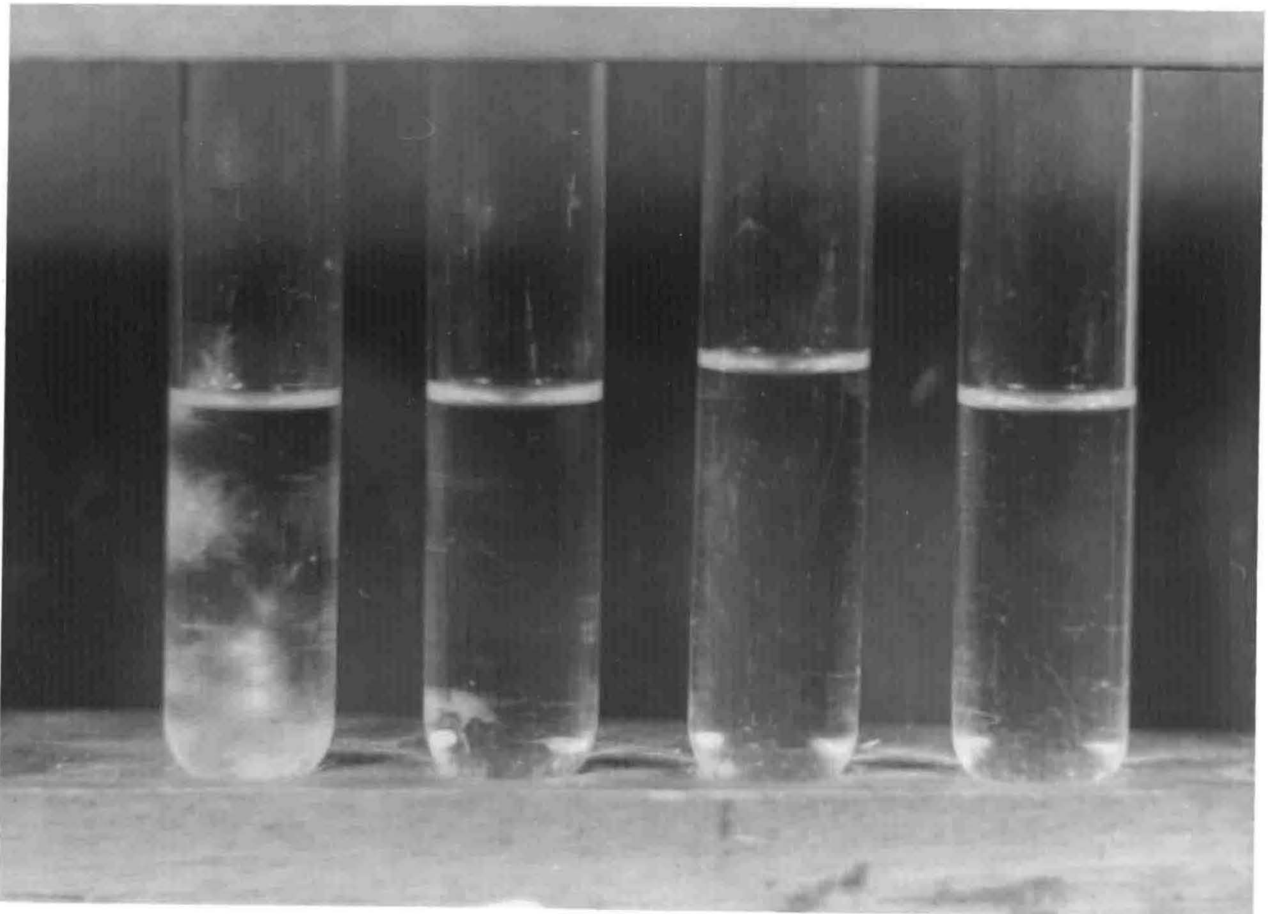


FIGURE 12-B

Torpedospora radiata in AS-1, from left to right: Whatman paper-biotin-thiamin; Whatman paper-biotin; Whatman paper-thiamin; Whatman paper-no vitamins added.

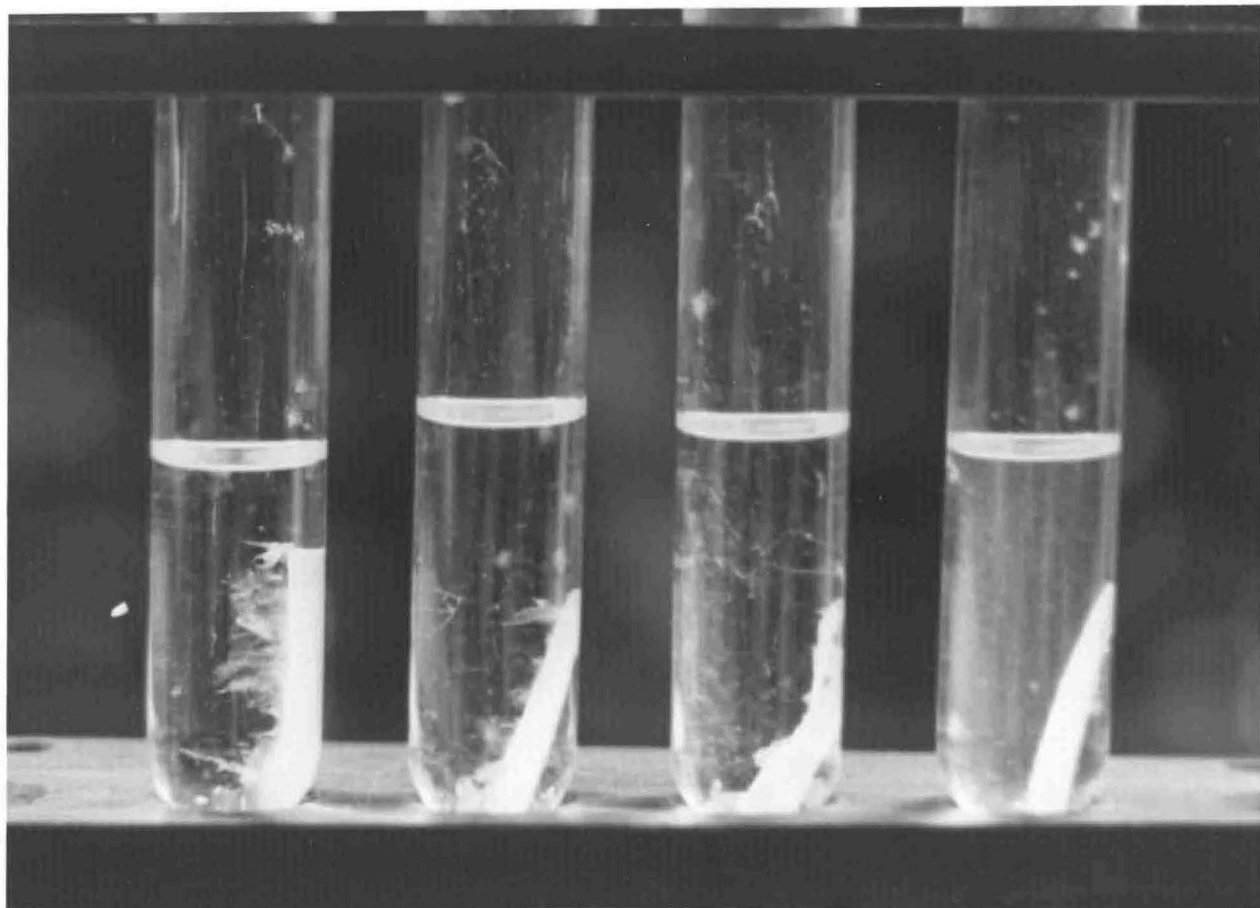


FIGURE 12-C

Torpedospora radiata in AS-1, from left to right: balsa-biotin-thiamin; balsa-biotin; balsa-thiamin; balsa-no vitamins added.

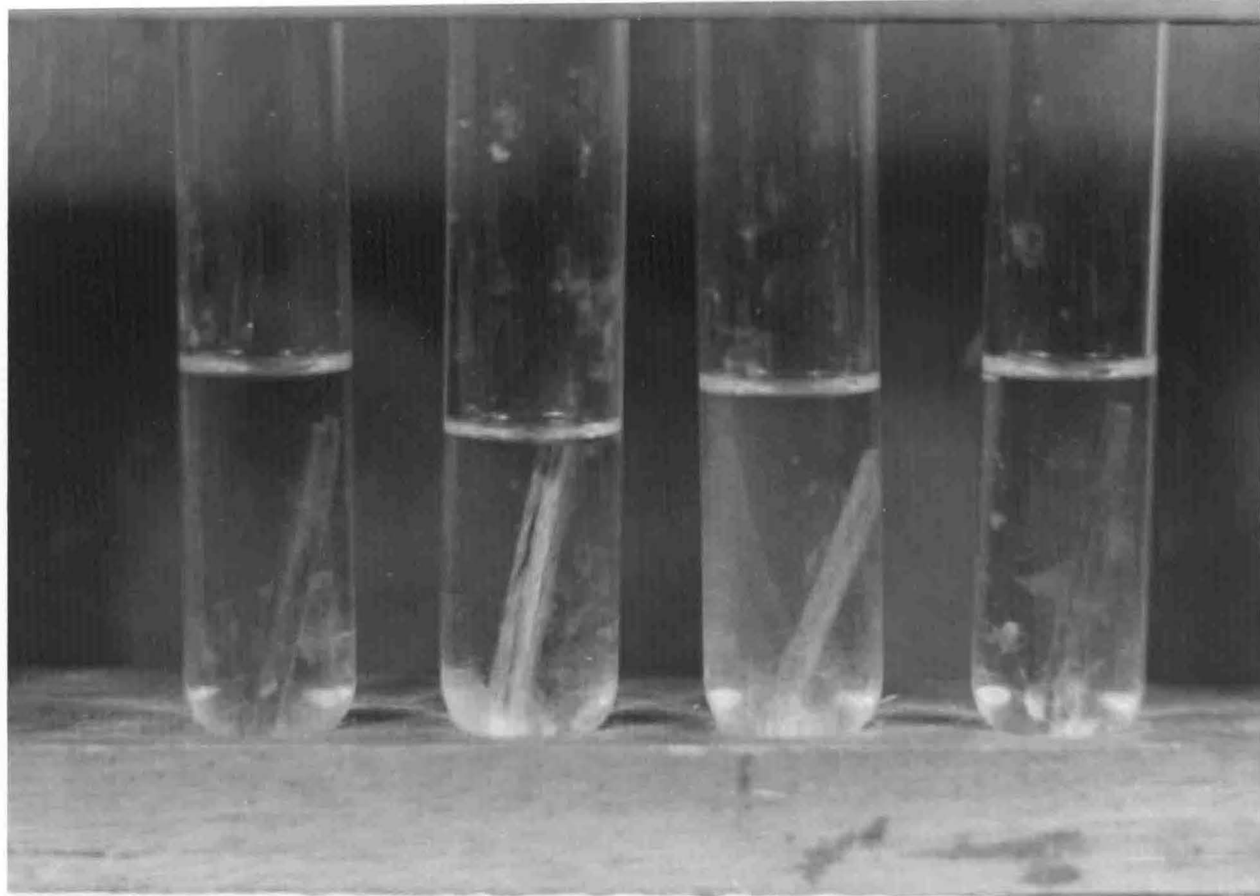


FIGURE 12-D

Torpedospora radiata in AS-1, from left to right: pine-biotin-thiamin; pine-biotin; pine-thiamin; pine-no vitamins added.

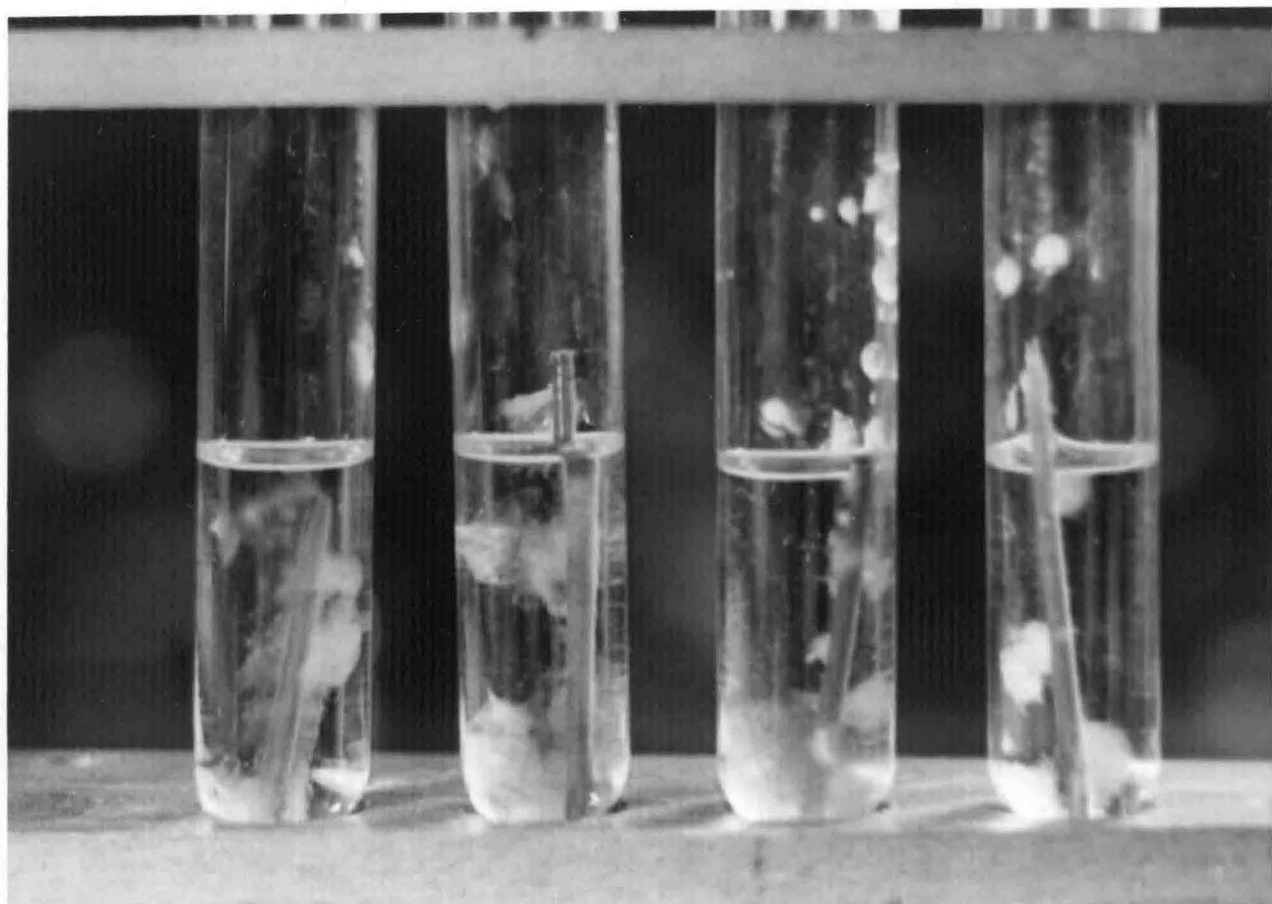


FIGURE 12-E

Halosphaeriopsis in AS-1, from left to right: balsa-thiamin; balsa-no thiamin; pine- thiamin; pine-no thiamin (note perithecia at base and at top of stick).

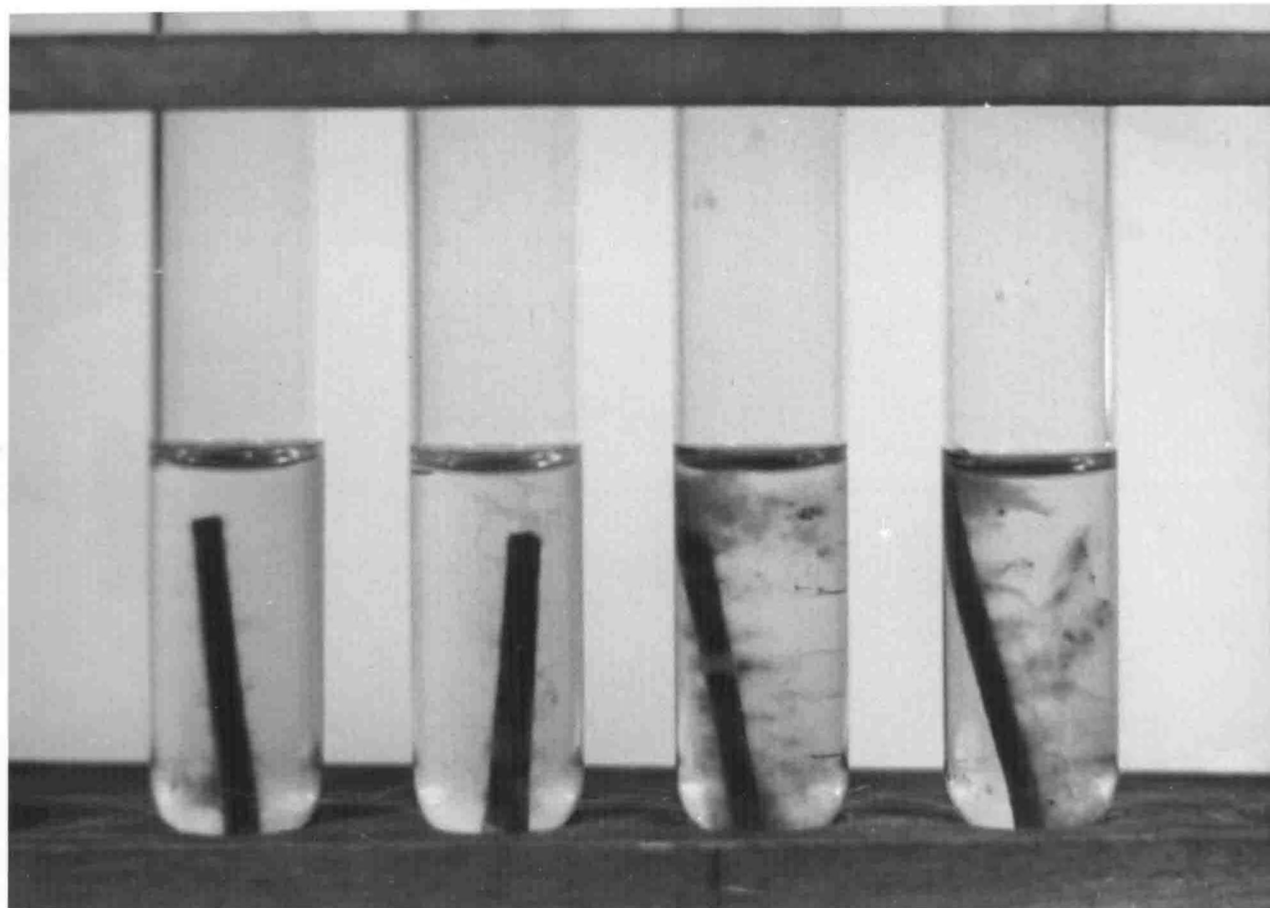


FIGURE 12-F

Halosphaeriopsis is AS-1, from left to right: glucose-thiamin; glucose-no thiamin; Whatman paper-thiamin; Whatman paper-no thiamin.

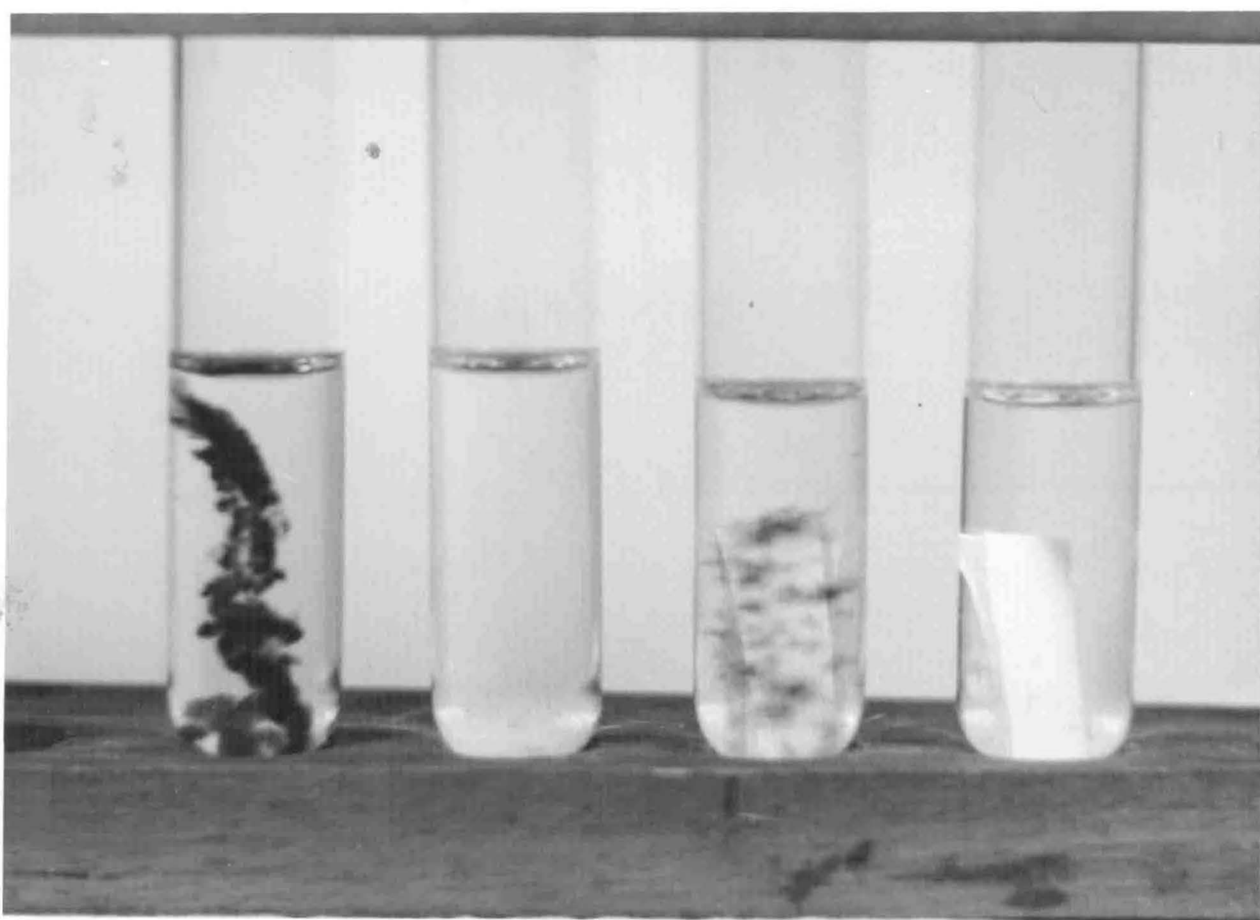
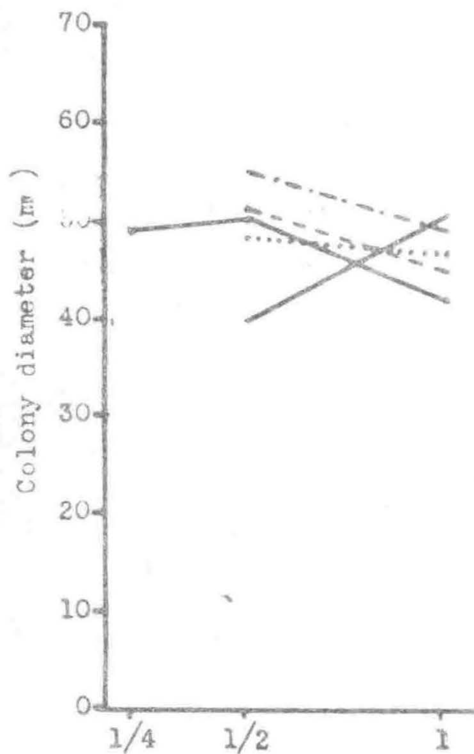


Figure 13-A, Parts 1 - 3

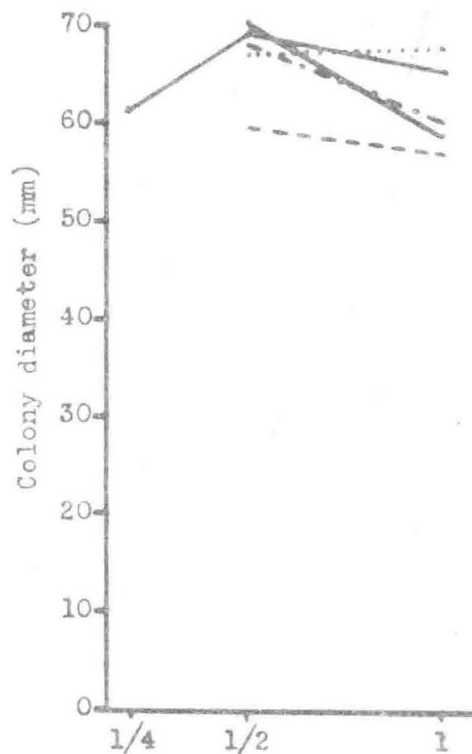
Colony diameters (mm) of C. globosum after six days on altered AS-1 media

Part 1 - 20°C



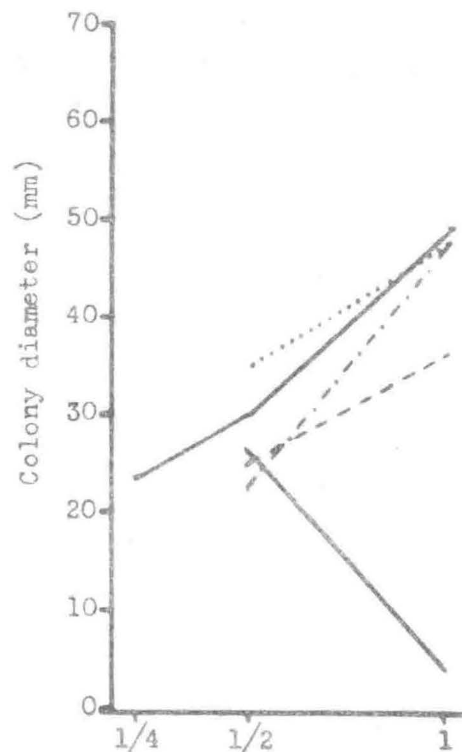
Strength of variable system; strength 1 equals salinity 34 o/oo

Part 2 - 30°C



Strength of variable system; strength 1 equals salinity 34 o/oo

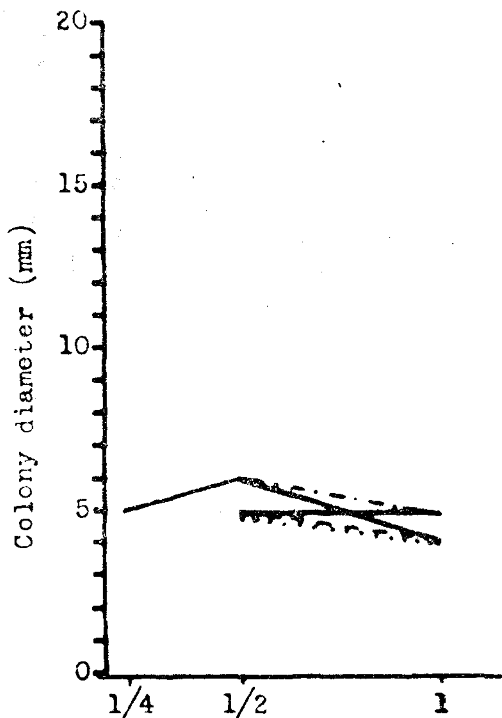
Part 3 - 35°C



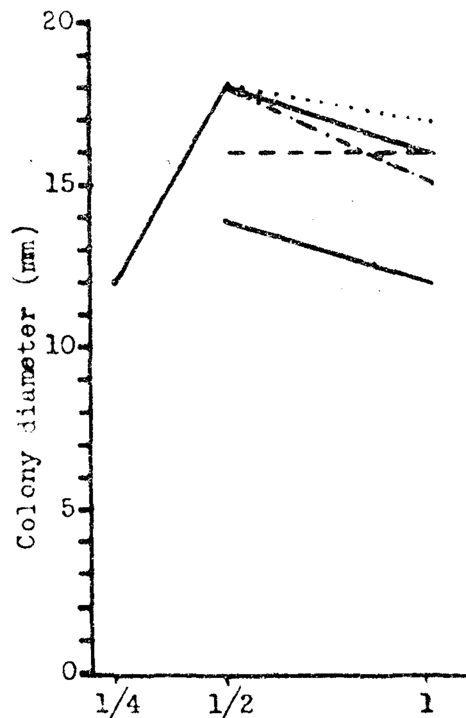
Strength of variable system; strength 1 equals salinity 34 o/oo

Figure 13-A. Parts 4 - 6

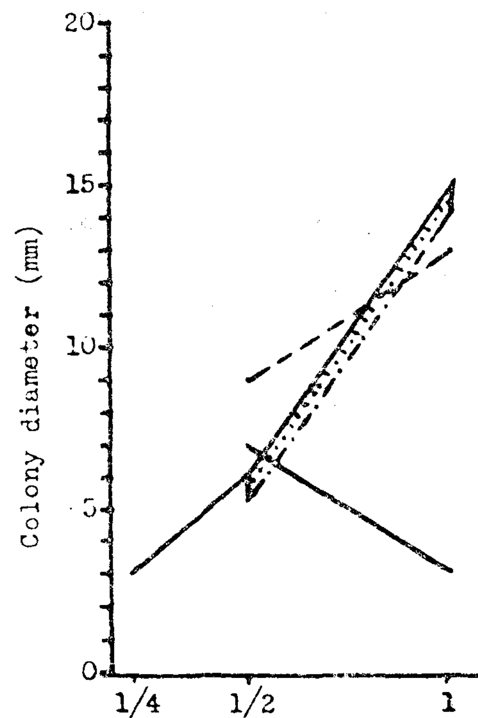
Colony diameters (mm) of T. radiata after twelve days on altered AS-1 media.



Strength of variable system; strength 1 equals salinity 34 o/oo



Strength of variable system; strength 1 equals salinity 34 o/oo

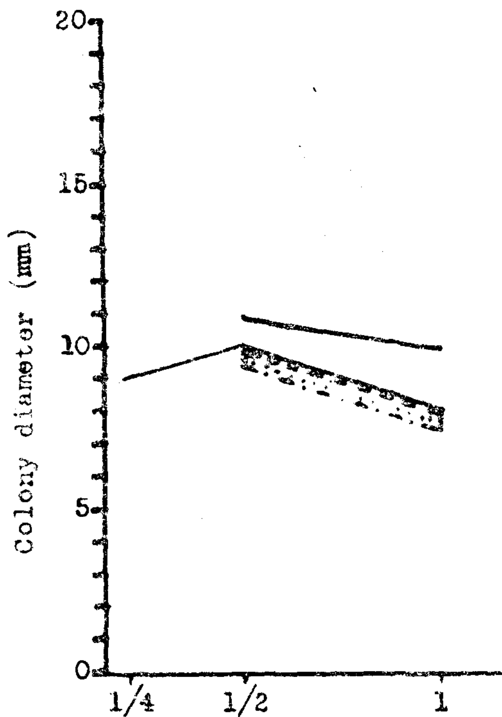


Strength of variable system; strength 1 equals salinity 34 o/oo

Figure 13-A, Parts 7 - 9

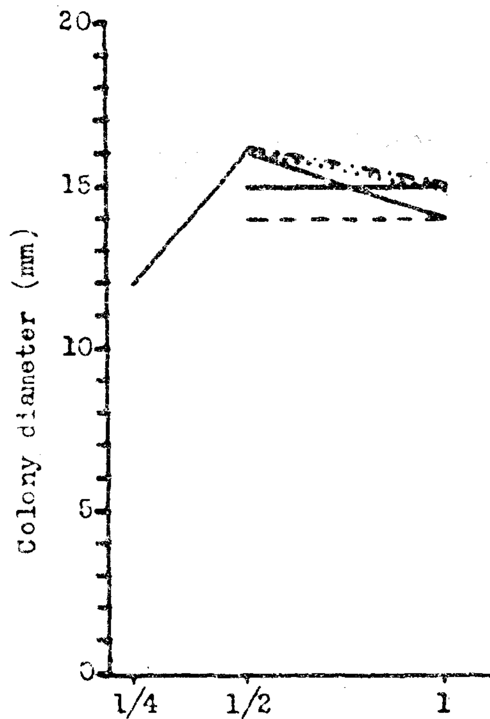
Colony diameters (mm) of Halosphaeriopsis after twelve days on altered AS-1 media

Part 7 - 20°C



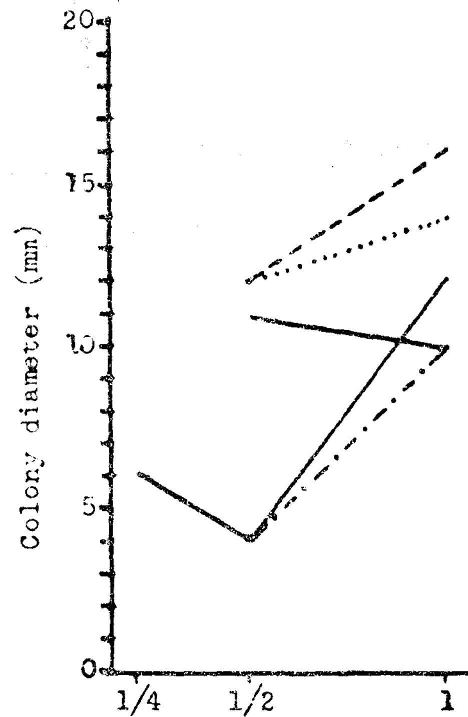
Strength of variable system; strength 1 equals salinity 34 o/oo

Part 8 - 30°C



Strength of variable system; strength 1 equals salinity 34 o/oo

Part 9 - 35°C



Strength of variable system; strength 1 equals salinity 34 o/oo

Figure 13-A, Explanation

This figure is a graphic representation, in nine parts, of the data in Table 13-A.

The ordinates of all points are colony diameters in millimeters, and the horizontal scale of all graphs is in strengths of variable systems of altered AS-1. The long solid line connecting strengths 1/4, 1/2, and 1 points is the control curve. The short solid line connects diameters of colonies which grew in strengths 1/2 and 1 media with strength 1/4 major salts constant. The dash line connects diameters from media with a constant strength 1/4 buffer. The dot line connects diameters from constant metals-chelator media, and the dash-dot-dash line is that of the constant minor salts media.

Parts 1-3 are for *C. globosum* at 20, 30, and 35 C, respectively. Parts 4-6 and 7-9 are for *T. radiata* and *Halosphaeriopsis*, respectively.

Figure 17-A-

Fructification of T. radiata on seven strengths NS-7 at three temperatures.

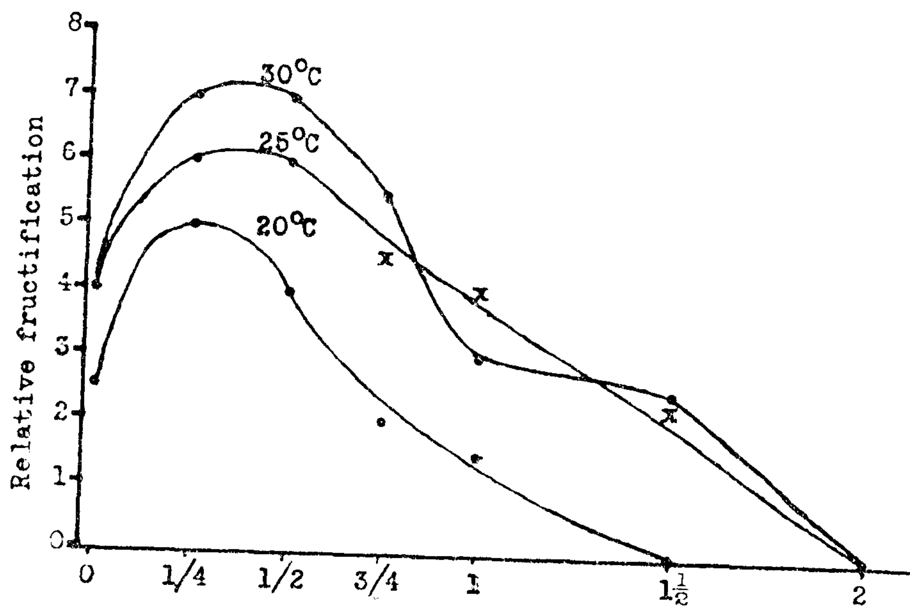


Figure 18-A

Halosphaeriopsis ascospores per HPF from seven strengths NS-1 at three temperatures, and from four strengths with reduced nutrient level at 25°C.

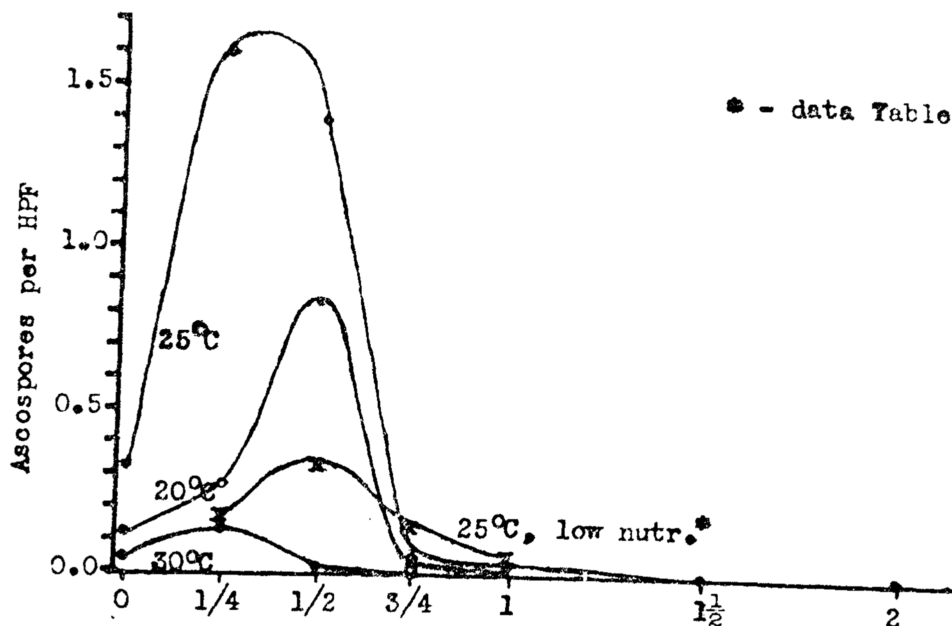


Figure 18-C

Halosphaeriopsis conidia per HPF from seven strengths NS-1 at three temperatures, and four strengths with reduced nutrient levels at 25°C.

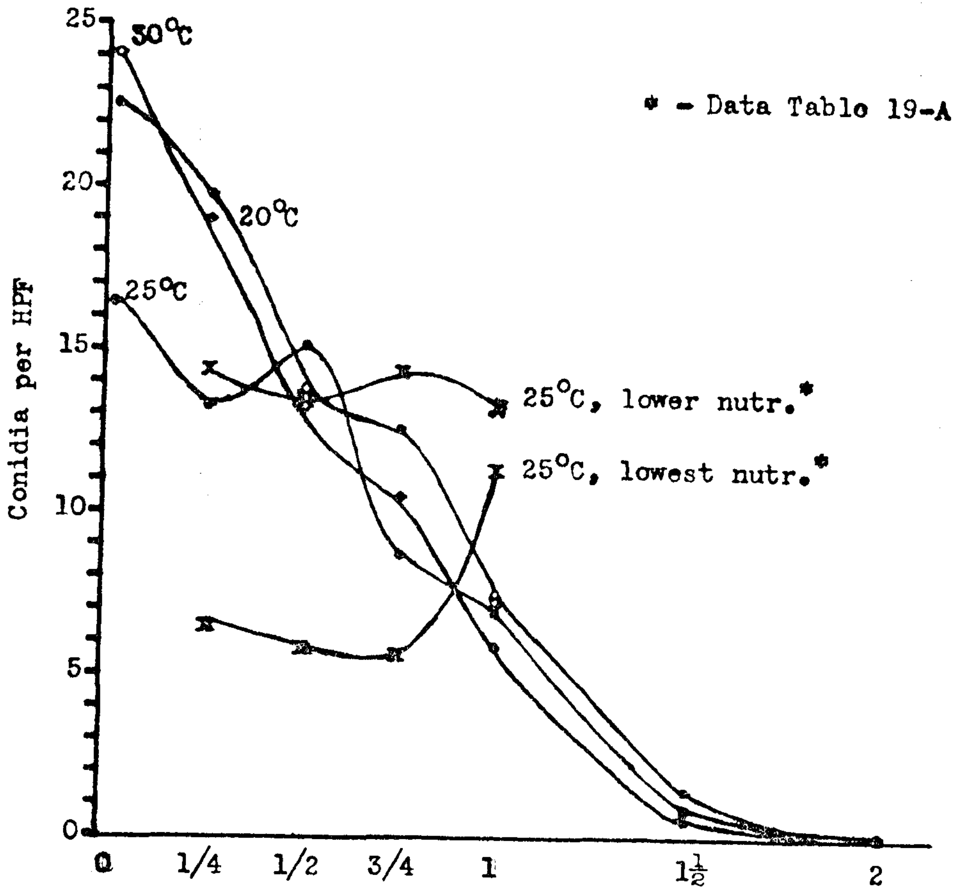


Figure 18-B

Conidia of _Halosphaeriopsis_

Figure 22-A

Rhizomorph-like _Halosphaeriopsis_ on balsa

VITA

Paul W. Kirk, Jr. was born February 23, 1931, in Jacksonville, Florida. He attended the College of William and Mary - V.P.I. in Norfolk, Virginia for two years on scholarship, and there acquired an interest in biology and chemistry. He transferred to the University of Richmond in September, 1951, and there majored in chemistry and minored in biology. His undergraduate education was interrupted by service in the U.S. Army in Verdun, France, where he functioned as a medical laboratory technician, medical bacteriologist, and veterinary bacteriologist. Upon honorable discharge from the armed service he continued to pursue his Bachelor of Science Degree in Chemistry, which he received from the University of Richmond in August, 1957.

Prior to 1957 he had worked several summers and after classes as a public health bacteriologist, medical laboratory technician, and bloodbank technician for the Medical College of Virginia, and both Norfolk and Richmond, Virginia City Public Health Laboratories. After receiving his Bachelor's degree, he completed his major in biology and taught this, and other subjects, in Thomas Jefferson High School of Richmond, Virginia.

He re-entered the University of Richmond in September, 1959, as an A.D. Williams Fellow, to begin work on the Master of Science Degree in Biology. From a course in general mycology taught by Dr. Wilton R. Tenney, the author became interested in the physiology of fungi. He became interested in the physiology and ecology of marine lignicolous fungi from the publications of Dr. Terry W. Johnson, Jr., of the Duke University, and later those of Dr. Samuel P. Meyers of the University of Miami and Dr. Don Ritchie of Barnard College. Mr. Kirk will enter Duke University in September, 1961, to begin work on the Doctor of Philosophy Degree in Botany, with specialization in marine microbiology.

APPENDIX I

HPF tabulations from which averages, Table 18-A, were derived; 20 C cultures only; six tubes per salinity-temperature combination; three cover-glass preparations per tube; ten HPF per cover-glass for a total of 180 HPF per recorded average.

Salinity "0"

Tube # 1			Tube # 2			Tube # 3			Tube # 4			Tube #5			Tube # 6		
15	29	16	18	49	25	45	40	22	33-1	25-1	8	23	24	17	27	24	8
24	10	27	25	50	48	23	34	31	6	11	17	21	18	9	19	23	15
30	18	27	19-1	41	19	22	13	20	28	21	10	29	15	21	18	19	32
25	40	21	13	36	17-1	21	21	21	43	24	9	29	36	17	15	17	15
20	25	36	29	20	22	24	19	15	29	18	20-1	19	12	12	29	18	16
8	21	16	23	21	10	25	29	24	27	28	15-1	21	17	11	46	15	18
26	25	18	30	34-1	31	30-1	22	38	32	27-1	20	35	10	13-1	10	31	25-1
25	7	13	18	30	45	15	29	11-1	27	10	24	27	15	19	11	10	18
25	21-1	20	46	26	46	23	11-1	28	31	17	11	23	24-1	23	35	20	11
21	20	18	40	41	10	30	28	25	10	15	15	23-7	25	21	21	20	15
Tot		avg	Tot		avg	Tot		avg	Tot		avg	Tot		avg	Tot		avg
647		21.5	882		29.4	734		24.4	611		20.3	609		20.3	601		20.0

Total Conidia - 4084 Average - 22.6
Total ascospores - 21 Average - 0.12

Salinity 8.5

Tube # 1			Tube # 2			Tube # 3			Tube # 4			Tube # 5			Tube # 6		
22	17	17	28	20	15	19	17	14	15-1	14	15	18	23	17-1	42	36-1	28-1
18	17	17	29	6	18	25	14	19	20-1	14-3	15	23	19	11	13-1	31	18
30	35	27	28	40	8	17	18	20	16	15-1	19-2	13	15	28	15	21	19-1
25	17	17	35	36	13	29	23	15	13-1	18-2	15	17	21	25	13-1	26-1	20
26	16	12	22	18	24	14	23	13	7	18	17	7-1	11	14	13	24	16
14	20	20	21	27	23	22	45	18	10-2	25	18	9	22	11	32	27	8
16	10-1	17	18	30	21	18	14	21	18-2	21-1	10-3	31	21-1	21-1	29-2	21	18-1
15	20	16	18	7	31-1	27	18-1	30	15-1	15	21-2	13	31	18	25-1	24	16
24	40-2	8	27	16	20	20-1	15	23	15-1	19-1	19	18	21	21-1	10	17-1	23
12	25	17	25	26	21	9	25	18	17-1	17-1	21-1	20	31	18	21	13	24
Tot		avg	Tot		avg	Tot		avg	Tot		avg	Tot		avg	Tot		avg
587		19.5	666		22.2	603		20.1	492		16.4	567		18.9	643		21.4

Total Conidia - 3558 Average - 19.7
Total ascospores 49 Average - 0.27

Salinity 17

Tube # 1			Tube # 2			Tube # 3			Tube # 4			Tube # 5		
10-1	19-1	15-1	10-1	11-3	18-1	12	17	9	23-2	17-1	13	25-1	22-3	21
7-1	14-2	10	10-1	12-7	7-2	19-1	14-1	10	23-1	14	12	12	22-1	17-1
19-1	15-2	9	6-4	6-1	14-2	14-1	9	11	21-1	11	17	22	15-1	17
20-1	6-1	9-1	8	23	12	15	9-1	18-1	19-1	14	27-3	12-2	18	16-3
10-1	10-2	8-2	16-1	8	10-1	8-3	14	12-1	11	12-2	25	15-2	22	24-1
13-1	11-1	8	8	11-2	7	18	11	18-1	16-2	14-1	12	12-4	25	17-1
10	9-1	10-1	17-1	12-2	8-1	8	13-2	12	16-1	16	17-2	13-3	18-1	23-1
4	12	12	12-3	8	12	11-1	17	15	15-1	16	22-1	16-1	17-4	19-4
5	15	15	14-2	12	4	10	18	7	19-3	19-1	21-1	19-1	18-1	14
9	7	10-1		7	12	13	10	10	11-1	21	9-1	10	16-1	17
Tot		avg	Tot		avg	Tot		avg	Tot		avg	Tot		avg
326		10.8	351		11.7	382		12.7	503		16.7	534		17.8

Tube # 6		
10-1	20	9
13-1	14-2	12
14-1	16	9-1
12	11-1	12
8	9	11
11-1	23-1	17-2
15	17	8
10-1	16	24
18	17	20
8	9-1	16-2
Tot		avg
409		13.6

Total conidia - 2505 Average - 13.8
Total ascospores - 150 Average - 0.83

Salinity 26

Tube # 1			Tube # 2			Tube # 3			Tube # 4			Tube # 5			Tube # 6		
14-1	25	7	17	20	13	12	12	14	18	13	11	7	8	10	9	10	14
12	10	10	18	17	11	18	14	13	21	3	3	11	10	16	11	10	15
9	23	14	7	23	19	11	11	16	6	14	4	12	6	11	15	11	11
19	26	16	16	19	19	12	12	12	14	4	12	2	10	13	9	10	14
15	21	18	9	9	14	13	19	23	11	12	7	15	15	13	12	9	9
18	13	17	11	7	18	21	9	18	9	10	15	19	5	11	10	15	13
22	18	14	16	16	6	18	15	12	6	12	10	14	9	7	7	21	14
18	9	6	11	19	7	10	22	9	7	9	8	7	11	12	14	7	7
27	20	12	14	26	10	10	13	15	13	6	11	15	12	14	8	9	8
27	12	14	17	14	13	10-1	12	27	11	6	5	8	13	9	9	18	10
Tot		avg	Tot		avg	Tot		avg	Tot		avg	Tot		avg	Tot		avg
486		16.2	427		14.2	433		14.4	291		9.7	325		10.8	335		11.1

Total conidia - 2297 Average - 12.7
Total ascospores - 2 Average - 0.011

Salinity 34

Tube # 1			Tube # 2			Tube # 3			Tube # 4			Tube # 5			Tube # 6		
10	11	7	6	4	5	9	6	7	10	7	5	7	7	5	4	10	11
7	10	8	9	12	8	4	10	9	10	10	4	6	7	4	8	9	12
6	9	7	5	8	17	4	12	6	7	12	10	6	5	9	12	7	8
4	5	11	5	12	11	5	10	5	5	17	5	4	10	11	12	4	3
7	6	4	4	9	11	10	12	3	12	20	7	4	3	5	10	9	8
8	10	4	5	12	7	4	11	9	11	4	3	6	2	4	4	13	7
8	7	6	6	13	10	8	10	3	7	3	8	7	6	4	8	13	5
4	5	3	19	11	13	7	8	3	8	2	8	8	4	9	6	10	4
5	3	2	8	12	8	8	19	6	8	6	10	8	3	10	13	11	8
5	6	6	6	9	7	7	6	10	6	3	4	8	9	5	10	4	11
Tot		avg	Tot		avg	Tot		avg	Tot		avg	Tot		avg	Tot		avg
194		6.4	272		9.0	231		7.7	232		7.7	186		6.2	254		8.4

Total conidia - 1369 Average - 7.5
No ascospores observed.

Salinity 51

Tube # 1			Tube # 2			Tube # 3			Tube # 4			Tube # 5			Tube # 6		
1	0	1	1	2	1	0	1	4	0	2	1	4	1	1	1	0	0
0	1	0	2	1	4	0	1	6	0	4	0	1	2	2	1	0	1
1	0	0	4	3	4	1	0	2	0	0	0	1	4	0	4	2	3
2	0	0	1	4	0	4	2	0	4	2	1	0	1	0	1	1	0
0	3	0	4	1	4	2	1	1	1	1	0	0	0	2	6	0	0
1	4	2	6	0	1	3	0	3	4	0	1	1	4	6	4	0	4
0	6	1	1	3	3	1	0	0	1	0	1	2	1	3	0	1	3
1	3	2	6	6	1	0	3	2	0	3	2	1	3	2	0	4	0
1	2	3	3	2	1	1	0	1	1	6	3	2	2	0	0	4	2
0	0	0	2	1	2	2	1	0	1	1	1	1	0	1	1	1	2
Tot		avg	Tot		avg	Tot		avg	Tot		avg	Tot		avg	Tot		avg
35		1.1	74		2.4	43		1.4	42		1.4	48		1.6	46		1.5

Total conidia - 286 Average - 1.5
No ascospores observed.

Salinity 68

No conidia or ascospores observed in 180 fields.

APPENDIX II

Tube averages, conidia per HPF, ascospores per 30 HPF, 25 C and 30 C of Table 18-A.

25 C

	Tube Number						
Salinity	1	2	3	4	5	6	avg
0	21.7-19	18.9-1	16.7-8	11.3-15	14.6-6	15.9-9	16.5- .32
8.5	13.8-70	14.7-64	11.6-57	15.2-40	10.4-28	13.6-45	13.2-1.6
17	15.4-55	13.8-35	15.9-59	13.9-31	17.9-34	14.4-43	15.2-1.4
26	7.8-1	7.2	11.0-1	8.6	8.9-1	8.7-1	8.7- .022
34	6.1	9.5-1	8.3	8.0	5.2	6.7-1	7.3- .011
51	.63	.63	.36	.36	.63	.33	.5-0
68	0.0	0.0	0.0	0.0	0.0	0.0	0.0

30 C

	Tube Number						
Salinity	1	2	3	4	5	6	avg
0	29.7	23.6-1	18.8	16.0-3	25.9	30.6-2	24.1-.033
8.5	16.2-6	19.0-5	21.6	24.9-12	16.6	15.9-2	19.0-.13
17	16.4	7.8	12.5	16.1	12.3-1	16.2-1	13.5-.011
26	12.2	9.8	11.9	10.9	7.1	11.8	10.6-0
34	8.0	6.5	4.9	4.8	5.2	6.3	5.9
51	1.0	.7	.9	.8	.7	.6	.7
68	0.0	0.0	.03	0.0	0.0	0.0	0.0

APPENDIX III

Tube averages, conidia (cnda) per HPF and ascospores (asco) per 30 HPF, Table 19-A.

Yeast Extract 100 mg/liter

Strength NS-1	1/4		1/2		3/4		1	
Tube #	cnda	asco	cnda	asco	cnda	asco	cnda	asco
1	13.1	3	12.1	5	12.0	1	14.6	0
2	17.4	11	11.2	6	11.3	15	8.2	0
3	11.5	2	11.5	7	15.8	1	15.3	8
4	12.7	1	13.8	16	15.8	1	14.8	1
5	13.9	1	21.5	18	15.9	1	14.3	0
6	16.9	10	11.2	8	15.2	3	13.3	0
	(avg 14.3 -	0.16)	(avg13.6 -	0.33)	(avg14.3 -	0.12)	(avg13.4 -	0.05)

Yeast Extract 10 mg/liter (no ascospores mature)

Strength NS-1	1/4	1/2	3/4	1
Tube #	cnda	cnda	cnda	cnda
1	5.6	4.6	6.2	15.5
2	6.5	6.1	4.7	14.2
3	6.0	7.5	5.7	10.0
4	5.5	6.9	6.0	7.5
5	8.2	5.9	5.4	7.8
6	5.8	5.9	5.4	12.0
	(avg 6.3)	(avg5.9)	(avg 5.7)	(avg 11.2)

No Yeast Extract Added (no ascospores observed)

Strength NS-1	1/4	1/2	3/4	1
Tube #	cnda	cnda	cnda	cnda
1	0.83	0.96	0.7	1.3
2	1.4	1.7	1.6	2.0
3	0.73	0.66	0.93	0.33
4	0.6	1.6	0.7	1.6
5	0.2	1.7	1.3	1.3
6	0.33	1.6	1.1	1.3
	(avg 0.68)	(avg 1.4)	(avg 1.1)	(avg 1.1)

APPENDIX IV

Inorganic constituents of sea water, Cl 19.00 o/oo, salinity 34.325 o/oo, total solids 34.4816 o/oo, after Sverdrup et al (1942). Per kilogram basis.

Primary cations		Primary anions	
sodium	10.5561 g	chloride	18.9799 g
magnesium	1.2720 g	sulphate	2.6486 g
calcium	0.4001 g	bromide	0.0646 g
potassium	0.3800 g	flouride	0.0013 g
strontium	0.0133 g	boron as 0.260 g of boric acid	

Other

silicon	0.02-4 mg
aluminum	0.5 mg
lithium	0.1 mg
iron	2-20 ug
manganese	1-10 ug
copper	1-10 ug
zinc	5 ug
molybdium	0.5 ug
cobalt	present
nitrogen, inorg.	0.01-0.7 mg
phosphorus, inorg.	1-100 ug

Inorganic nitrogen as:

nitrate	1-600 ug
ammonium	5-50 ug
nitrite	0.1-50 ug

Organic carbon (Friday Harbor)

dissolved	1.6-3.3 mg
total	1.7-5 mg

C:N:P ratio (by weight)

approx. 41:7.2:1

APPENDIX V

Composition of stock solutions of vitamins and trace metals for medium AS-1

A. Vitamins

Vitamin	Quantity/liter AS-1	g/300 ml stock
thiamin HCl	100 ug	0.03
biotin (free acid)	5 ug	(5 ug/ml)
inositol	5 mg	1.5
pyridoxine HCl	40 ug	0.012
nicotinic acid	100 ug	0.03

Use 1 ml stock per liter AS-1

B. Metals and chelator

Cation	mg/liter (cation)	mg/liter (cmpd)	Cl/liter	g/liter stock (cmpd)	cmpd
Fe 3+	1	4.84	2.00	4.84	$FeCl_3 \cdot 6H_2O$
Zn 2+	0.3	0.625	0.325	0.625	$ZnCl_2$
Mn 2+	0.5	1.78	0.6	1.78	$MnCl_2 \cdot 4H_2O$
Co 2+	0.01	0.04	0.015	0.04	$CoCl_2 \cdot 6H_2O$
Mo, Na free 6+	0.01	0.017	-	0.017	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$
Mo, Na cmpd 6+	0.01	0.025	-	0.025	$Na_2MoO_4 \cdot 2H_2O$
Cu 2+	0.02	0.07	0.02	0.07	$CuCl_2 \cdot 4H_2O$

EDTA free acid, 26 mg/liter AS-1; 26 g/liter metals stock

Na_2EDTA, 30 mg/liter AS-1; 30 g/liter stock; sodium 4 mg/liter AS-1

Metals and chelator stock, use 1 ml/liter strength 1 AS-1. Cl/ml is 3 mg.

APPENDIX VI

Major and minor marine salts, for AS-1 mineral nutrition studies; strength 1 AS-1 per liter basis. Sodium and chloride totals.

Minor	mg ion	mg cmpd	mg Na	mg K	mg Cl	cmpd
Sr	13.3	40.4	-	-	10.7	$SrCl_2 \cdot 6H_2O$
B	4.6	26.0	-	-	-	H_3BO_3
Si, as K	2	11	-	5.5	-	K_2SiO_3
Si, as Na	2	20.3	3.2	-	-	$Na_2SiO_3 \cdot 9H_2O$
F, as K	1.3	4	-	2.7	2.5 equiv.	KF
F, as Na	1.3	2.9	1.6	-	2.5 equiv.	NaF
Br, as K	64.6	96.1	-	31.5	28.4 equiv.	KBr
Br, as Na	64.6	83.1	18.5	-	28.4 equiv.	NaBr
Major						
Mg, all as Cl	1272	10634	-	-	3709	$MgCl_2 \cdot 6H_2O$
Mg, part as Cl	602	5033	-	-	1755	$MgCl_2 \cdot 6H_2O$
Mg, part as SO_4	670	6791	-	-	-	$MgSO_4 \cdot 7H_2O$
SO_4, as Mg	2649	6791	-	-	-	$MgSO_4 \cdot 7H_2O$
SO_4, as Na	2649	3917	1269	-	-	Na_2SO_4
K, all Cl	380	725	-	380	345	KCl
K, as F	2.7	4	-	2.7	2.5 equiv.	KF
K, as Br	31.5	96.1	-	31.5	28.5 equiv.	KBr
K, part as Cl	340.3	649	-	340.3	309	KCl
K, Si	5.5	11	-	5.5	-	$KSiO_3$
Na, all as Cl	10556.1	26837	10556.1	-	16281	NaCl
Ca	400.1	1467	-	-	707	$CaCl_2 \cdot 2H_2O$

Sodium, not all as Cl

mg Na	mg anion	mg Cl	mg cmpd	cmpd
1268	2699	-	3917	Na_2SO_4
18.5	64.6	28.4 equiv.	83.1	NaBr
1.6	1.3	2.5 equiv.	2.9	NaF
3.2	-	-	20.3	$Na_2SiO_3 \cdot 9H_2O$
4.1	26	-	30	Na_2EDTA
9260.7	-	14283.7	23544.4	NaCl

Chloride total, Na all as Cl

Chloride cmpd	mg Cl
$MgCl_2 \cdot 6H_2O$	1755
KCl	309
$SrCl_2 \cdot 6H_2O$	10.7
$CaCl_2 \cdot 2H_2O$	707
NaCl	16281
Vit. & Metals	3
	19.0657 o/oo
Br, equiv.	.0284
F, equiv.	.0025
Total Cl	19.0966 o/oo

Chloride, Na not all as Cl

Chloride cmpd	mg Cl
$SrCl_2 \cdot 6H_2O$	10.7
$CaCl_2 \cdot 2H_2O$	707
NaCl	14283.7
$MgCl_2 \cdot 6H_2O$	3709
KCl	345
Vit. & Metals	3
	19.089 o/oo
Br, F equiv.	.0309
Total Cl	19.089 o/oo

Difference - 7.3 mg Cl

AS-1 Cl approx. 19.1 o/oo

AS-1 Salinity approx. 34.5 o/oo