HANDBOOK OF PHYCOLOGICAL METHODS:

Culture Methods & Growth Measurements

EDITED BY

JANET R. STEIN

Introduction

JANET R. STEIN
Department of Botany, University of British Columbia, Vancouver 8, British Columbia, Canada

The purpose of the *Handbook of Phycological Methods* is to present a compendium of techniques for culturing and measuring growth of those organisms collectively termed 'algae'. The audience for whom it is intended is diverse and includes experienced researchers, novices, phycologists, and/or non-phycologists. In general, each chapter is concerned primarily with the method rather than theories behind the method. Whenever possible methods based on original research are given. The methods are presented so they can be followed easily, although not necessarily in stepwise fashion. Minor variations are possible depending upon a variety of factors, local circumstances and training of those using the techniques.

Editorial revisions have attempted to present the methods in a consistent fashion. However, as the subject matter is not uniform, this has not always been possible.

The materials and equipment listed serve only for reference, and are not necessarily endorsements of given products or manufacturers. United States and Canadian suppliers are mentioned only as guidelines, but unfortunately these change. Sources of equipment are published annually in the United States in *Science* (American Association for the Advancement of Science, 1515 Massachusetts Ave., N.W., Washington, D.C. 20005) and in Canada in *Research and Development* (Maclean Hunter, 418 University Ave., Toronto 101, Ontario) and *Laboratory Product News* (Southam Business Publications Ltd, 1450 Don Mills Rd, Don Mills, Ontario). These publications should be consulted for up to date addresses of suppliers.

In many chapters, specific test organisms have been designated so that a 'known' taxon is available upon which to try the method. Sources for these organisms are given in the individual chapters and in the general listing on Culture Collections at the end of this Introduction. Other methods and technique books pertinent to the algae are also listed and should be consulted for additional information.

CULTURE COLLECTIONS

(Abbreviations used in text in parentheses)

United States

1. Indiana University Culture Collection (IUCC)
   Department of Botany
   Indiana University
   Bloomington, Indiana 47401
   Starr, R. C. 1964. The culture collection of algae at Indiana University. *Amer. J. Bot.* **51**, 1013–44.
   Starr, R. C. 1966. *Culture collection of algae. Additions to the collection July 1, 1963 – July 1, 1966*, pp. 1–8, Department of Botany, Indiana University, Bloomington, Indiana 47401.
   Starr, R. C. 1971. The culture collection of algae at Indiana University – additions to the collection July 1966 – July 1971. *J. Phycol.* **7**, 350–62.
   Rosowski, J. R. and Parker, B. C. 1971. Sources of algal cultures. *Selected Papers in Phycology*, pp. 816–51. Univ. Nebraska Press, Lincoln.
2. American Type Culture Collection (ATCC)
   12301 Parklawn Drive
   Rockville, Maryland 20852
   American Type Culture Collection 1972. *Catalogue of Strains*, 10th ed. American Type Culture Collection, Washington, D.C.
3. Culture Collection of Algae (WHOI)
   Woods Hole Oceanographic Institution
   Woods Hole, Massachusetts 02543
4. Culture Collection of Thermophilic Bluegreen Algae
   c/o Dr R. W. Castenholz
   Department of Biology
   University of Oregon
   Eugene, Oregon 97403
   Castenholz, R. W. 1970. Laboratory culture of thermophilic cyanophytes. *Schweiz. Z. Hydrol.* **32**, 538–51.
5. Carolina Biological Supply Company
   Burlington, North Carolina 27215
   James, D. E. 1969*a*. Unialgal cultures. *Carolina Tips*, **32**, 33–6.
   James, D. E. 1969*b*. Maintenance and media for marine algae. *Carolina Tips*, **32**, 45–6.

6. Ward's Natural Science Establishment, Inc.
   P.O. Box 1712
   Rochester, New York 14603

United Kingdom

1. Culture Centre of Algae and Protozoa (Cambridge)
   The Director
   36 Storeys Way
   Cambridge, CB3 ODT
   Natural Environment Research Council. 1971. *Culture Collection of Algae and Protozoa – List of Strains*, pp. 1–73. Culture Centre of Algae and Protozoa, Cambridge.
2. Marine Biological Association of the UK (MBA)
   The Laboratory
   Citadel Hill
   Plymouth, PL1 2PB
3. Freshwater Biological Association
   Supply Department
   Windermere Laboratory
   The Ferry House
   Ambleside, Westmorland

Germany

1. Sammlung von Algenkulturen
   Universität Göttingen
   18 Nikolausbergerweg
   Göttingen
   Koch, W. 1964. Verzeichnis der Sammlung von Algenkulturen am Pflanzenphysiologisches Institut der Universität Göttingen. *Arch. Mikrobiol.* **47**, 402–32.

Japan

1. Algal Culture Collection
   Institute of Applied Microbiology
   University of Tokyo
   Tokyo
   Watanabe, A. and Hattori, A. 1966. Cultures and collections of algae. *Proc. U.S.–Japan Conf., Hakone, Japan. Japan Soc. Plant Physiol.* 100 pp.

Czechoslovakia

1. Culture Collection of Algae
   Department of Botany
   Charles University of Prague
   Prague
   Fott, B. and Trucova, E. 1968. List of species in the culture collection of algae at the Department of Botany, Charles University of Prague. *Acta Univ. Carol. Biol.* **1967**, 197–221.

GENERAL REFERENCES

Brunel, L., Prescott, G. W. and Tiffany, L. H., eds. 1950. *The Culturing of Algae.* Charles F. Kettering Foundation, Antioch Press, Yellow Springs, Ohio. 114 pp.

Burlew, J. S., ed. 1953. *Algae Culture, From Laboratory to Pilot Plant.* Carnegie Institution of Washington, Washington, D.C. Publ. 600. 357 pp.

Carr, N. G. 1969. Growth of phototrophic bacteria and blue-green algae. In Norris, J. R. and Ribbons, D. W., eds., *Methods in Microbiology*, **3B**, 53–76. Academic Press, New York.

Droop, M. R. 1969. Algae. In Norris, J. R. and Ribbons, D. W., eds. *Methods in Microbiology*, **3B**, 269–313. Academic Press, New York.

Klein, R. M. and Klein, D. T. 1970. *Research Methods in Plant Science.* Natural History Press, Garden City, New York. 756 pp.

Lapage, S. P., Shelton, J. E. and Mitchell, T. G. 1970. Media for the maintenance and preservation of bacteria. In Norris, J. R. and Ribbons, D. W., eds., *Methods in Microbiology*, **3A**, 1–333. Academic Press, New York.

Lapage, S. P., Shelton, J. E., Mitchell, T. G. and MacKenzie, A. R. 1970. Culture collections and the preservation of bacteria. In Norris, J. R. and Ribbons, D. W., eds., *Methods in Microbiology*, **3A**, 135–228. Academic Press, New York.

Pringsheim, E. G. 1946. *Pure Cultures of Algae.* Cambridge Univ. Press, London. 119 pp.

Venkataraman, G. S. 1969. *The Cultivation of Algae.* Indian Council of Agricultural Research, New Delhi. 319 pp.

Section 1

Isolation and purification

1: Growth media – freshwater

H. WAYNE NICHOLS
Department of Biology, Washington University, St Louis, Missouri 63130

CONTENTS

I. INTRODUCTION

There are perhaps as many media and modifications as there are active phycologists today. Each investigator generally employs his or her own particular medium for successful cultivation of freshwater algae. I have attempted to include in this chapter those media generally employed by several investigators, as well as having general applicability to culturing of a variety of species or algal groups. Further, the media selected represent a wide diversity of pH and dilution. Among the major considerations are: pH; concentration of major nutrients; nitrogen source; possible organic or growth factors for enrichment; and micronutrient composition.

Freshwater media may be designed from several viewpoints. They are generally selected because they possess characteristics similar to the natural environment or they differentially select for a specific algal component of the habitat. Media of an artificial nature, i.e., known chemical composition, are often employed as additives to natural media with an unknown chemical composition, such as lakewater. These then function as enrichment media and are often used to simulate diverse nutritional or physical requirements of a particular species or groups of species, especially when the exact nutritional requirements are unknown.

II. MATERIALS

A. *Chemicals*

The chemical constituents used for the preparation of media should generally be of the highest quality available to the investigator. Quality is determined by the manufacturer and reflects certain specifications established by the Committee on Biological Chemistry of the National Research Council (National Academy of Sciences 1960). The quality is determined by the purity of the compounds and highest purity is confirmed by chromatographic or electrophoretic analysis. These analyses are generally supplied to the investigator routinely or on request. Each manufacturer uses his own code for designation of grade and catalogs should be consulted for code designation and purity of constituents to be used in growth media.

One of the most important points to consider in the preparation of media is the accuracy in weighing the chemicals to be used. Therefore, one should be particularly careful that accurate analytical procedures are followed when weighing small quantities of reagents.

B. *Agar*

Agar is a neutral polymer of galactose which has: (1) low viscosity in water solution; (2) a sharp, stable gelling temperature; and (3) a strong gel structure. It has a melting point *ca.* 95 °C and solidifies *ca.* 45 °C. It is routinely used to solidify growth media and is added after the medium has been mixed, prior to autoclaving. However, as a general rule it is unsafe to autoclave agar in media with very acid pH. Hutner *et al.* (1966) suggests that a slight hydrolysis of the agar liberates acid which accelerates further hydrolysis and liberation of additional acid. They suggest autoclaving the agar and the liquid separately (both at double strength) and mixing them aseptically while hot.

Agar can generally be obtained from a variety of suppliers and is coded by the degree of purity of the agar. The purity is generally based on washing procedures and it has been my experience that a medium to high grade agar (Difco 0560, or 0142, Difco Laboratories, P.O. Box 1058A, Detroit, Michigan 48232) has been satisfactory. The most highly purified agars generally available also may be used not only in solidifying growth media but also for preparing gels for immunodiffusion and electrophoretic techniques.

Agar is generally used at concentrations of 1–2 %. It has been successfully used at lower concentrations. However, lower concentrations were generally used in purification procedures (see Chp. 3, VI.B) or in bacteriological work for cultivation of anaerobes.

1. Heat to *ca.* 95 °C 1 l of medium in a 2 l flask in a water bath. (The medium may be heated directly, then placed in the water bath.)
2. Slowly, add the desired quantity of agar while stirring continuously.
3. When all the agar is dispersed and in solution, quickly dispense into containers as desired.
4. Sterilize at 121 °C, 20 lb/in^2 for 20–35 min (see Chp. 12, II.C.2).
5. Sterile petri plates should be filled aseptically after sterilization. Test tubes and flasks may be filled before sterilization. Petri plates should be poured when the temperature of the medium is *ca.* 50 °C (warm to the inside wrist), otherwise large amounts of condensation occur (see C following).

C. *Glassware*

A variety of sizes and types of glassware is available, including disposable glassware. Glassware is generally prepared from either alumina silicate glass or borosilicate. Either of these types of glass is satisfactory for initial

use in preparation and dispensing of growth media. A variety of special glassware to meet particular investigators' problems can be obtained from Corning Glass Works (Science Products Division, Corning, New York 14830; 135 Vanderhoof Ave., Toronto, Ontario). Media are generally dispensed into test tubes of a variety of sizes, depending upon the specific future uses of the media (i.e., stock cultures, nutritional experiments or the like). The tubes are plugged with cotton, plastic closures, or screw caps and sterilized. Screw cap tubes or flasks are generally used to maintain sterile cultures for a long period of time and appear to retard evaporation, although they are generally more expensive. More rapid growth can often be obtained using the media given here by dispensing the media in petri plates. However, media used in petri plates are often difficult to maintain in sterile condition unless given particular types of treatment.

To prevent chemical contamination of media during sterilization, glassware may be washed or cleaned in particular ways. Glassware is often washed in a concentrated acid solution (see Chp. 2, II.C.2) after first washing with a detergent, such as 'Alconox' (Alconox Inc., 215 Park Ave. South, New York, New York 10003). A typical washing procedure is as follows: detergent washing in tap water; rinsing in tap water; washing with acid solution; rinsing in tap water; three rinses in glass-distilled, deionized water. Potassium dichromate is often difficult to remove from glassware by repeated washings. Thus, many investigators have discontinued the use of this chemical cleaning agent since it is toxic often in small quantities to most algal species (see Chp. 2, II.C.2).

D. *Water*

Water generally employed for freshwater growth media is as follows: (1) copper-distilled water; (2) single glass-distilled water; (3) double glass-distilled water; (4) membrane filtered water; and (5) deionized water.

Copper-distilled water has the disadvantage of residual copper which is toxic to many algal species. Single or double glass-distilled water is generally used in most laboratories and can be deionized by a simple attachment of a prepacked deionizing column. Deionized glass-distilled water is generally used, as glass stills (unless frequently and carefully cleaned) may impart undesirable contaminants to the medium and adversely affect the growth of algae to be cultured.

E. *Soil*

There is often some mystique regarding sources of soil for inclusion into those media which require it. Soils which have been successfully employed

for the growth of algae have been obtained from Tennessee, Vermont, California and a variety of other locations. A satisfactory soil is one containing small quantities of clay and one which will settle after it has been added to liquid. Pringsheim (1946; also see IV.B.4, 5, following) suggests that soil should not have too great a humus content and should not have commercial fertilizers added recently. It has been my experience that the selection of a suitable soil within these parameters is generally an experimental procedure which requires trial for successful cultivation of certain species by the investigator.

III. METHODS

Media are generally prepared from premixed stock solutions. Aliquots from these stocks are measured and added to a given volume of water. However, some media must be prepared by weighing or measuring the desired components and adding them directly to a given volume of liquid. Accuracy in measuring liquid aliquots from stock solutions or water, and weighing of chemicals is essential. Improper procedures may result in precipitation of one or more of the components of the medium (especially nitrates and phosphates) or a failure of some of the constituents to go into solution.

Stock solutions can be prepared and stored at low temperatures for lengthy periods of time. However, they should be stored, if possible, in tightly sealed glassware since evaporation alters initial concentrations. If crucial and accurate experiments are to be performed, it is best to mix fresh stock solutions of both macro- and micronutrients. All solutions are made using deionized or distilled water.

IV. MEDIA

Table 1-1 indicates the classes of algae that have been successfully grown in the media included in this chapter.

A. *Defined* (see Tables 1-2, 1-3)

1. Beijerinck pH 6.8 (Stein 1966). This medium is made from three stock solutions and the micronutrient solution.

a. Stock I – use 100 ml/l

NH_4NO_3	1.5 g/l
K_2HPO_4	0.2
$MgSO_4 \cdot 7H_2O$	0.2
$CaCl_2 \cdot 2H_2O$	0.1

TABLE 1-1. *Application of media*

Medium	Classes cultured[a]
Defined	
Beijerinck	C
Bold Basal	C, CR, CY, R
Bozniak Community	C, CR, CY, R (mixed)
Cg 10	CY
Chu no. 10	BA, C, CR, CY
Rodhe VIII	BA, C, CR, CY
Volvox	C
Waris	C
Woods Hole MBL	BA, C, CR, CY
Enrichment	
Modified *Porphyridium*	R
Polytomella	CO, E
Proteose	C, CR, CY, E
Soil extract agar	C, CR, CY, R
Soil water	BA, C, CO, CR, CY, E, R
Trebouxia agar	C

[a]BA Bacillariophyceae
C Chlorophyceae
CO Colorless flagellates
CR Chrysophyceae
CY Cyanophyceae
E Euglenophyceae
R Rhodophyceae

TABLE 1-2. *pH and concentration of defined media*

		Concentration (total mol/l)		
	pH	Macro	Micro	Macro + micro
Beijerinck	6.8	5×10^{-3}	4×10^{-4}	5×10^{-3}
Bold Basal	6.6	4×10^{-2}	5×10^{-5}	4×10^{-2}
Bozniak Community	8.0	7×10^{-6}	1×10^{-6}	8×10^{-6}
Cg 10	8.0	1×10^{-3}	1×10^{-4}	1×10^{-3}
Chu no. 10	6.5–7.0	9×10^{-4}	—	9×10^{-4}
Rodhe VIII	7.0–7.5	2×10^{-3}	—	2×10^{-3}
Volvox	7.0	5×10^{-2}	3×10^{-3}	5×10^{-2}
Waris	6.0	1×10^{-2}	—	1×10^{-2}
Woods Hole MBL	7.1–7.3	2×10^{-2}	—	2×10^{-2}

b. Stock II – use 40 ml/l

KH_2PO_4	9.07 g/l

c. Stock III – use 60 ml/l

K_2HPO_4	11.61 g/l

d. Micronutrients – use 1 ml/l

H_3BO_3	1.0 g/100 ml
$CuSO_4.5H_2O$	0.15
EDTA	5.0
$ZnSO_4.7H_2O$	2.2
$MnCl_2.4H_2O$	0.5
$FeSO_4.7H_2O$	0.5
$CoCl_2.6H_2O$	0.15
$(NH_4)_6Mo_7O_{24}.4H_2O$	0.10

The micronutrients are dissolved one at a time in 100 ml warm water. Following the addition of each, the pH is adjusted to 5 with KOH pellets. The final solution should have a pH *ca.* 6.5 (iron precipitates at 7.0).

2. *Bold Basal pH 6.6* (Nichols and Bold 1965; also known as Bristol solution). Six individual stock solutions are made of the macronutrients. The three minor constituents are also separate stock solutions as is the micronutrient solution.

a. Macronutrients – use 10 ml each/940 ml

$NaNO_3$	10 g/400 ml
$CaCl_2.2H_2O$	1
$MgSO_4.7H_2O$	3
K_2HPO_4	3
KH_2PO_4	7
NaCl	1

b. EDTA – use 1 ml/l

EDTA	50 g/l
KOH	31

c. Iron – use 1 ml/l

$FeSO_4.7H_2O$	4.98 g/l
H_2SO_4	1.0 ml/l

d. Boron – use 1 ml/l

H_3BO_3	11.42 g/l

e. Micronutrients – use 1 ml/l

$ZnSO_4.7H_2O$	8.82 g/l
$MnCl_2.4H_2O$	1.44

MoO_3	0.71
$CuSO_4.5H_2O$	1.57
$Co(NO_3)_2.6H_2O$	0.49

3. Bozniak Community pH 8.0 (Bozniak 1969). Prior to preparation, all glassware is ashed at 600 °C for 30 min. The autoclave is scrubbed inside with 95 % ethanol to minimize organic contamination; adjust pH of water to 8.0. Separate stock solutions of each macronutrient, the micronutrient solution and the vitamin solution may be prepared and stored frozen. The $Ca(NO_3)_2$ is autoclaved separately and added aseptically when cool to avoid precipitation of calcium phosphate. The ferric citrate–citric acid and vitamin stocks are membrane filtered (0.22 μm GS Millipore Filter, Millipore Corp., Ashby Rd, Bedford, Massachusetts 01730; 55 Montpellier Blvd, Montreal 379, Quebec). These are added after sterilization of the other constituents to the cooled medium.

a. Macronutrients – use 1 ml each/l

$Ca(NO_3)_2.4H_2O$	24.4 g/l
$MgSO_4.7H_2O$	1.0
$NaHCO_3$	16.5
$K_2HPO_4.3H_2O$	0.8
KH_2PO_4	0.8
Na_2SiO_3	5.8
H_3BO_3	0.5
Na_2EDTA	10.0

b. Citrate–citric acid – use 1 ml each/l

Ferric citrate	1.0 g/l
Citric acid	1.0

c. Vitamins – use 1 ml each/l

Thiamine.HCl	1×10^{-6} g/l
Cyanocobalamin	10×10^{-6}

d. Micronutrients

K_2CrO_4	0.0037 g/l
$CoCl_2.6H_2O$	0.020
$MnCl_2.4H_2O$	0.890
$ZnCl_2$	0.0104
$VOSO_4.2H_2O$	0.0039
MoO_3	0.0075
$CuSO_4$	0.00125

4. Cg 10 pH 8.0 (Van Baalen 1967). Major nutrients are added as salts, or can be dissolved in stock solutions in appropriate concentrations. Aliquots of these stock solutions are then added to the final medium before bringing the medium up to volume. The micronutrient solution ('A5') is made as a single stock solution, and adjusted to pH 8.5.

a. Macronutrients

$MgSO_4.7H_2O$	0.25 g/l
K_2HPO_4	1.0
$Ca(NO_3)_2.4H_2O$	0.025
KNO_3	1.0
Na_2.EDTA	0.010
$Fe_2(SO_4)_3.6H_2O$	0.004
Glycylglycine	1.0

b. Micronutrient 'A5' – use 1 ml/l

H_3BO_3	2.86 g/l
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
MoO_3 (85%)	0.018
$CuSO_4.5H_2O$	0.079
$CoCl_2.6H_2O$	0.010

5. Chu no. 10 pH 6.5–7.0 (Chu 1942). Prior mixing of stock solutions is suggested to avoid repetitive weighing.

$Ca(NO_3)_2$	0.04 g/l
K_2HPO_4	0.01
$MgSO_4.7H_2O$	0.025
Na_2CO_3	0.02
Na_2SiO_3	0.025
$FeCl_3$	0.8 mg/l

6. Rodhe VIII pH 7.0–7.5 (Rodhe 1948). Prior mixing of stock solutions is suggested to avoid repetitive weighing.

$Ca(NO_3)_2$	60 mg/l
$MgSO_4$	5
Na_2SiO_3	20
K_2HPO_4	5
Ferric citrate	1.0
Citric acid	1.0
$MnSO_4$	0.03

7. Volvox pH 7.0 (Darden 1966; Starr 1969). Stock solutions of the macronutrients are prepared separately. The micronutrient solution is prepared separately. Glycylglycine, glycerophosphate and the vitamins are considered as macronutrients. Starr (1969) suggests increasing the vitamins (biotin, 2.5 increase; cyanocobalamin, 0.5 increase).

a. Macronutrients

$Ca(NO_3)_2.4H_2O$	0.118 g/l	use 10 ml/l
$MgSO_4.7H_2O$	0.04	use 10 ml/l
Na_2.glycerophosphate.$5H_2O$	0.05	use 10 ml/l
KCl	0.05	use 10 ml/l
Glycylglycine	0.5	use 10 ml/l
Biotin	1.0 μg	use 1 ml/l
Cyanocobalamin	1.0 μg	use 1 ml/l

b. Micronutrients – use 3 ml/l

$FeCl_3.6H_2O$	0.097 g/l
$McCl_2.4H_2O$	0.041
$ZnCl_2$	0.005
$CoCl_2.6H_2O$	0.002
Na_2MoO_4	0.004
Na_2.EDTA	0.750

Adjust pH to 7.0 with 1N NaOH

8. Waris pH 6.0 (Waris 1953). Separate stocks of each macronutrient and the iron sequestrine solution may be prepared.

a. Macronutrients

KNO_3	0.1 g/l
$MgSO_4.7H_2O$	0.02
$(NH_4)_2HPO_4$	0.02
$CaSO_4$	0.05

b. Iron sequestrine

EDTA	1.30 g/l
$FeSO_4.7H_2O$	1.25
1N KOH	13.5 ml/l

pH is adjusted to 6.0

FeNaEDTA (ferric sequestrine) may be substituted for the solution (use 2.49 g FeNaEDTA/liter)

9. Woods Hole MBL pH 7.2 (R. R. L. Guillard, personal communication). Stock solutions of each macronutrient are prepared at 1000-fold concentrations thus 1 ml of each stock is added to 1 l medium. Stock solutions of each