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# STUDIES ON SELECTED CORALLINACEAE (RHODOPHYTA) AND OTHER ALGAE IN A DEFINED MARINE CULTURE MEDIUM

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**Abstract:** A marine culture medium (MCM) has been developed and shown to have the unique ability to support the growth of several coralline algae. The results of experiments designed to determine the effects of varying certain ionic concentrations and buffers are presented for this defined medium. Optima of 5 mM $Ca^{2+}$, 1 mM $SO_4^{2-}$ and 1 $\mu$M $BO_3^{3-}$ (lower than the respective sea-water levels) were found for growth or oxygen evolution in *Corallina*. No organic buffer was needed for growth of Corallinaceae, but growth stimulation was observed for a strain of *Callithamnion* (Ceramiaceae) when Tris–(hydroxymethyl)amino-methane buffer was added. This stimulation could not be duplicated with other similar buffers. Results of growth studies with a diverse selection of marine macrophytes have indicated that MCM generally supports growth better than sea water alone but often not as well as enriched sea water. The best MCM growth results were observed with members of the Rhodophyceae and certain Chlorophyceae.

## INTRODUCTION

Although an almost bewildering array of defined culture media have been developed for marine organisms (see reviews by Provasoli *et al.*, 1957; Droop, 1969; McLachlan, 1973; Kinne, 1976; Ukeles, 1976), many remain untested on macroscopic algae, and apparently none has been used successfully for growing taxa of Corallinaceae (Rhodophyta). Previous culture-based studies of Corallinaceae (e.g., Von Stosch, 1964, 1969; Jones & Moorjani, 1973; Chihara, 1973, 1974; Johansen & Colthart, 1975; Notoya, 1976; Brown *et al.*, 1977; Chamberlain, 1977,1978; Smith & Roth, 1979) all employed undefined, natural sea-water based media. In a number of cases plants became necrotic, developed abnormally, or grew very slowly. The only two published accounts of culture work on coralline life histories (Von Stosch, 1969; Chamberlain, 1977) involved undefined media. West & Hommersand (1981) have stated that before more extensive culture work on coralline life histories can be undertaken, much more needs to be known about the nutritional physiology of these algae and more suitable culture media need to be developed. Thus, as a prelude to work on experimental assessment of variation in morphological–anatomical attributes of potential taxonomic significance and on sexual and apomictic cycles in the Corallinaceae, it seemed highly desirable to develop a

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chemically defined culture medium which would support normal growth, calcification and reproduction of plants raised from spores.

Preliminary examinations of the chemical composition of a variety of extant defined media and comparisons with natural sea water revealed that most of these media possess a number of potentially undesirable characteristics. First, the concentrations of some ions (notably phosphate and a number of micronutrients – see Table IV and McLachlan, 1973) are substantially greater in the defined media than in natural sea water, but the general necessity for such high concentrations apparently has not been demonstrated. Secondly, the concentrations of some other ions (notably calcium, sulfate and borate), while generally similar to those in sea water, appear to be both high compared to concentrations used in freshwater media (Nichols, 1973) and totally unnecessary for ensuring stability of the medium. Thirdly, a number of extant defined media employ organic buffers which are directly or indirectly metabolizable (e.g., glycylglycine – see McLachlan, 1973; Tris – see Hanisak, 1979, p. 321) and/or which have demonstrated negative effects (e.g., Tris – see Smith & Foy, 1974; Van Steveninck, 1975) and/or whose effects on marine macroscopic algae are unknown. Finally some media also contain organic salts (e.g., sodium glycerophosphate – see McLachlan, 1973) which promote bacterial growth, and many media use a single salt (e.g., magnesium sulfate) as the source of two nutrients, thereby making manipulation of individual nutrient levels more difficult for experimental purposes. It was decided, therefore, to design empirically a stable, chemically defined marine culture medium (MCM) and to preliminarily assess its suitability for growing Corallinaceae and other macroscopic marine algae. Design goals included using a separate salt for each nutrient, employing inorganic compounds insofar as possible, determining whether Tris (widely used as a buffer in marine media) or another organic buffer was a necessary ingredient, and determining whether the high levels of certain nutrients found in other marine media and natural sea water are necessary. This report summarizes results of three series of experiments: one designed to determine suitable concentrations for a number of nutrients; one designed to examine the need for and role of Tris and several other buffers; and one designed to test the suitability of MCM relative to Provasoli's enriched sea water (PES – see McLachlan, 1973) and to natural sea water for growing selected taxa of Corallinaceae from spores and for maintaining other macroscopic algae in closed-system cultures. Because available data (see Johansen, 1981) suggest that the growth rates of most coralline algae are slow, clones of *Callithamnion byssoides* which grow comparatively fast (Spencer, unpubl. data), were used to test algal response to specific changes in the medium. The medium most suitable for growth of *Callithamnion* and the Corallinaceae was then tested on a variety of marine macrophytes.

## Materials and Methods

The physical and chemical conditions employed during the various experiments are summarized in Tables I and II respectively. Provasoli's enriched sea water (PES, see

McLachlan, 1973 for recipe) was utilized for treatments involving enriched sea water. MCM was prepared by adding the ingredient stocks listed in Table III sequentially to the NaCl stock. Tris was not used in some experiments and $Na_2SO_4$ and $NaHCO_3$ were added directly as salts rather than stock solutions when producing final concentrations $> 1.0$ mM $Na_2SO_4$ or 5.0 mM $NaHCO_3$. Details of individual stock preparations are as follows. (1) $CaCl_2$ and $MgCl_2$ are difficult to weigh accurately because of deliquescence. See McLachlan (1973, p. 35) for method of preparing stock solutions of high accuracy. (2) Micronutrient mix contains 6.6 mM $Na_2EDTA$, 1 mM $FeCl_3$, 1 mM $MnCl_2$, 1 mM $ZnCl_2$, 1 mM $Na_2MoO_4$, 1 mM $H_3BO_3$, 0.3 mM $CuCl_2$ and 0.2 mM $CoCl_2$. To prepare, dissolve salts sequentially in 800 ml $H_2O$ and bring the final volume to 1 liter. MCM concentrations are 0.001 of stock concentrations. (3) Vitamin mix contains 1.48 mM thiamine, 4.04 $\mu$M biotin and 0.74 $\mu$M cyanocobalamin. Prepare in 3 steps: (a) dissolve 10 mg biotin and 10 mg cyanocobalamin in 80 ml $H_2O$ and bring final volume to 100 ml, (b) separately dissolve 500 mg thiamine–HCl in $\approx$800 ml $H_2O$, (c) add 10 ml of solution A to solution B then bring volume to 1 liter with $H_2O$ and use as a stock solution. Solution should be stored frozen. MCM concentrations are 0.001 of vitamin stock concentrations. (4) $NaHCO_3$ stock solutions should be prepared in ice cold water and used immediately. (5) The Tris stock is 8.0 g of Tris–(hydroxymethyl)-aminomethane in 800 ml $H_2O$; bring volume to 1000 ml. Prepare new stock every 30 days.

Field samples used directly in experiments or employed as a source of spores were transported to the laboratory in plastic bags, stored in an ice-filled chest and then transferred to a 40-l aerated sea-water holding tank at 10–15 °C for 12–48 h prior to use. For certain experiments, portions from one or several *Corallina* plants with a total drip-free wet wt of 950–1000 mg were inoculated into 80 × 100 mm Pyrex deep storage dishes containing 250 ml of medium. For spore-initiated coralline experiments, plant fragments bearing tetrasporangial conceptacles were placed in 20–25 ml of sterilized sea water in 95 × 15 mm Petri dishes and the conceptacles were broken open. Within 30–60 s, the spore tetrads within mature, excised tetrasporangia separate and the

TABLE I

Experimental physical conditions.

| Parameter | Organism | | |
|---|---|---|---|
| | Corallinaceae | *Callithamnion* | Other algae |
| Dish diameter (mm) | 180 90 | 70 | 70 |
| Dish depth (mm) | 80 50 | 50 | 50 |
| Medium volume (ml) | 250 100 | 50 | 100–1000 |
| Photoperiod (L : $\overline{D}$) | 16 : $\overline{8}$ | 16 : $\overline{8}$ | 16 : $\overline{8}$ |
| Photon flux density ($\mu$mol · m$^{-2}$ · s$^{-1}$) | 10–20 | 5–7 | 10–25 |
| Temperature (°C) | 15 | 20 | 15–24 |
| Agitation | none | 115 rpm | none |

individual spores are released. These spores were transferred in capillary pipettes to 35 × 10 mm Petri dishes containing 5 ml of the appropriate medium, and the Petri plates then were placed within 90 × 50 mm crystallization dishes and left undisturbed to allow spore adhesion to the bottom of the Petri plate. After 72–96 h, counts of living (i.e., pigmented) and dead (i.e., non-pigmented) spores were taken and the dishes were then flooded with an additional 95 ml of medium.

For experiments involving *Callithamnion byssoides* Arnott ex Harvey in Hooker (from Georgia, JAW 685) the inoculum was prepared from stock culture plants by chopping filaments with a razor blade, placing the resulting fragments in a funnel fitted with a

TABLE II

Summary of test media used for various experiments during the developmental phase of MCM.

| Compound | Test medium: | Nutrient concentration A | B | C |
|---|---|---|---|---|
| $MgCl_2$ | | 20 mM | 20 mM | 40 mM |
| KCl | | 10 mM | 10 mM | 10 mM |
| $CaCl_2$ | | 10 mM | 10 mM | 2.5 mM |
| $NaNO_3$ | | 1 mM | 1 mM | 1 mM |
| $Na_2SO_4$ | | 20 mM | 20 mM | 1 mM |
| $Na_2HPO_4$ | | 30 μM | 30 μM | 30 μM |
| $FeCl_3$ | | 1 μM | 1 μM | 1 μM |
| $H_3BO_3$ | | 40 μM | 1 μM | 1 μM |
| $Na_2HCO_3$ | | 10 mM | 5 mM | 5 mM |

In addition all formulations contained the following substances in the stated concentrations: 450 mM NaCl, 1 μM $MnCl_2$, 1 μM $ZnCl_2$, 1 μM $Na_2MoO_4$, 0.3 μM $CuCl_2$, 0.2 μM $CoCl_2$, 1.5 μM thiamine HCl, 4 nM biotin, 0.74 nM cyanocobalamin, 1.2 × total micronutrient concentration $Na_2EDTA$.

TABLE III

Preparation of MCM.

| Ingredient | Concentration in stock | Amount of stock per l (ml) | Final concentration |
|---|---|---|---|
| NaCl | 600 mM | 750 | 450 mM |
| $MgCl_2$ | 4 M | 10 | 40 mM |
| KCl | 1 M | 10 | 10 mM |
| $CaCl_2$ | 500 mM | 10 | 5 mM |
| $NaNO_3$ | 100 mM | 10 | 1 mM |
| $Na_2SO_4$ | 100 mM | 10 | 1 mM |
| $Na_2HPO_4$ | 30 mM | 1 | 30 μM |
| Micronutrients | – | 1 | – |
| Vitamins | – | 1 | – |
| $NaHCO_3$ | 50 mM | 50 | 2.5 mM |
| Tris | 660 mM | 1 | 0.66 mM |
| $H_2O$ | – | 147 | – |

For details of individual stock preparations see p. 63.

20 μm Nitex screen and rinsing with culture medium. The rinsed material was finally resuspended in ≈20 ml of culture medium. Uniformly suspended inoculum was then transferred into dishes using a wide-mouth pipette. The inocula for other algae consisted of visually equal amounts of vegetative material obtained form pre-existing cultures (California) or from field-collected samples (Australia).

TABLE IV

Comparison of MCM, PES, sea water, and ASP-6.

| Component | MCM | PES[a] | Sea water[b] | ASP-6[c] |
|---|---|---|---|---|
| Salinity | 32‰ | 30.7‰ | 34.3‰ | – |
| $Cl^-$ | 505 mM | 489 mM | 548 mM | 424 mM |
| $Na^+$ | 457 mM | 471 mM | 470 mM | 415 mM |
| $Mg^{2+}$ | 40 mM | 48 mM | 54 mM | 33 mM |
| $K^+$ | 10 mM | 8.9 mM | 10 mM | 9.4 mM |
| $Ca^{2+}$ | 5 mM | 9.2 mM | 10 mM | 10 mM |
| $HCO_3^-$ | 2.5 mM[d] | 2.3 mM[d] | 2.3 mM[d] | – |
| $NO_3^-$ | 1 mM | 660–709 μM | 0.7–50 μM | 3.5 mM |
| $SO_4^-$ | 1 mM | 25 mM | 28 mM | 33 mM |
| $PO_4^{3-}$ | 30 μM | 26–59 μM | 0.03–33 μM | 317 μM |
| $Fe^{3+}$ | 1 μM | 10.2–15.4 μM | 0.02–5.2 μM | 35.8 μM |
| $Mn^{2+}$ | 1 μM | 2.8–3.0 μM | 0.02–0.18 μM | 18 μM |
| $Zn^{2+}$ | 1 μM | 0.38–51 μM | 0.08–0.21 μM | 7.7 μM |
| $MoO_4^{2-}$ | 1 μM | 0.003–17 μM | 0.003–0.17 μM | 5.2 μM |
| $BO_3^{3-}$ | 1 μM | 457 μM | 430 μM | 185 μM |
| $Cu^{2+}$ | 0.3 μM | 0.16–0.38 μM | 0.16–0.38 μM | 0.32 μM |
| $Co^{2+}$ | 0.2 μM | 0.070–0.076 μM | 0.002–0.008 μM | 0.17 μM |
| Thiamine | 1.48 μM | 0.237 μM | 0–0.000059 μM | 5.9 μM |
| Biotin | 4.0 nM | 3.3 nM | – | 20.5 nM |
| Cyanocobalamin | 0.74 nM | 1.18 nM | 0–0.0036 nM | 0.69 nM |
| Tris | 660 μM | 506 μM | 0 | 8.25 mM |
| EDTA | 6.6 μM | 10 μM | 0 | 0 |

[a] Calculated estimates using data from Barnes (1954), Collier (1970), and McLachlan (1973).
[b] Determined from data given by Collier (1970, Tables 1–5, 1–6, and 1–7).
[c] Values based on data given by McLachlan (1973).
[d] Varies depending on pH.

Rates of oxygen evolution were determined from polarographic data generated using a Clark type $O_2$ electrode (see Delieu & Walker, 1972). Samples of *Corallina* were finely chopped with a razor blade prior to introduction into the electrode chamber. Each sample subsequently was recovered from the chamber, placed in 90% acetone and pulverized; chlorophyll extraction was allowed to take place for 24 h in the dark at 2 °C and subsequent determinations were made using formula 1 of Jensen (1978, p. 62). For the other algae, plants were removed from culture vessels by filtration through a funnel fitted with 20 μm Nitex (Tetco, Inc.) screening (0.45-μm Millipore filters in the case of *Prymnesium*), washed briefly with 10 mM phosphate buffer (pH 7.0), and then frozen overnight. Thawed plants were extracted with 90% acetone in an ice bath for 1 h and

chlorophyll *a* determinations were made using the formula in Jensen (1978).

Kjeldahl digestion (according to Steyermark, 1961) was followed by ammonia nitrogen determination using the indanetrione hydrate (ninhydrin) spectrophotometric method of Jacobs (1960). Interference of cations in the assays was avoided by the use of EDTA (Meyer & Riklis, 1953). Digestion was carried out on the complete sample following chlorophyll determinations. Samples were heated in 18 × 150 mm test tubes in a 70 °C water bath for 1 h to evaporate the acetone. Five hundred microliters of concentrated $H_2SO_4$ were then added followed by 320 mg $K_2SO_4$ and 8 mg HgO. Capped with a glass vial, the tubes were heated for 4 h in a sand bath on a hot plate so that gently refuxing of $H_2SO_4$ went 3 cm up the tube after all water was gone. Some of the acid in the cooled tubes was neutralized by the addition of ≈9 ml of 2 M NaOH in 0.4 M Na-acetate. The volume was brought to 10 or 20 ml before the assay.

For the spectrophotometric nitrogen assay 200 to 500 μl of diluted digest were added to a test tube along with enough acetate buffer (0.2 M acetate pH 5.3 with 0.2% EDTA) to make a final volume of 2.0 ml. Indanetrione hydrate reagent was prepared by mixing equal volumes of a 4% stock of indanetrione hydrate in ethylene glycol monomethyl ether (Fisher certified) and the same pH 5.3 acetate buffer. This working stock was made 0.4% with respect to sodium hydrosulfite (reducing agent) immediately before use. After addition of 2.0 ml of this reagent the sample tubes were immediately placed in a boiling water bath for 20 min. Upon cooling the volume of each was brought to 10 ml with 50% ethanol and the $A_{570}$ was determined. Standards of $NH_4Cl$ were run with each determination; accurate results were obtained when the assay tube contained 1 to 7 μg N.

All experiments involving Australian algae were undertaken at La Trobe University; all other work was done in the Department of Botany, the University of California, Berkeley.

## RESULTS

### EXPERIMENTS ASSOCIATED WITH MCM DEVELOPMENT

The MCM formulation (Table III) used in later experiments was derived in part from data obtained during an initial sequence of tests designed to elucidate whether particular concentrations of various nutrients were suited for maintaining coralline red algae in culture. The results, based on experiments involving vegetative branches from field collected plants of *Corallina officinalis* L. (Australia) and *C. vancouveriensis* Yendo (California) as well as on spores and germlings of the latter species subjected to various test media (Table II), indicated that the concentrations of $Ca^{2+}$, $Mg^{2+}$, $SO_4^{2-}$, and $BO_3^{3-}$ could be reduced relative to those of sea water without adverse effects. Thus results from one series of experiments showed that the rates of oxygen evolution (Table V) from vegetative tissues of *C. officianalis* incubated for 7, 11, or 14 days (in a series of trials using sea water and variations of test medium A) were consistently higher

in treatments with 1 $\mu$M $BO_3^{3-}$ than in treatments with 10 $\mu$M or 40 $\mu$M $BO_3^{3-}$ or in sea water controls containing $\approx$430 $\mu$M $BO_3^{3-}$.

TABLE V

Oxygen evolution rates ($\mu$mol $O_2$ · mg Chl $a^{-1}$ · $h^{-1}$) in plants of *Corallina officinalis* grown in test medium A with various concentrations of $BO_3^{3-}$: means and SD are based on three trials.

| Treatment | $BO_3^{3-}$ conc. | Incubation time (days) | | |
|---|---|---|---|---|
| | | 7 | 11 | 14 |
| Sea water control | $\approx$430 $\mu$M | 240 ± 5 | 147 ± 8 | 149 ± 6 |
| 1 | 40 $\mu$M | 217 ± 4 | 159 ± 10 | 137 ± 9 |
| 2 | 10 $\mu$M | 175 ± 6 | 175 ± 7 | 120 ± 6 |
| 3 | 1 $\mu$M | 283 ± 8 | 225 ± 5 | 198 ± 5 |
| 4 | 0 | 265 ± 5 | 145 ± 14 | 180 ± 7 |

TABLE VI

Rates of oxygen evolution ($\mu$mol $O_2$ · mg Chl $a^{-1}$ · $h^{-1}$) in vegetative branches of *Corallina vancouveriensis* grown in various media for 4 days and for 7 days: means and SD are based on five trials.

| Medium | Treatment | Rates of oxygen evolution after | |
|---|---|---|---|
| | | 4 days | 7 days |
| Sea water | – | 268 ± 6 | 243 ± 4 |
| Test medium B | – | 215 ± 2 | 188 ± 9 |
| Test medium B | 2 mM $NO_3$ | 248 ± 5 | 223 ± 7 |
| Test medium B | 0.5 mM $NO_3^-$ | lost | 233 ± 7 |
| Test medium B | 20 mM $K^+$ | 234 ± 9 | 145 ± 6 |
| Test medium B | 5 mM $K^+$ | 278 ± 11 | 147 ± 9 |
| Test medium B | 60 $\mu$M $PO_4^{3-}$ | 200 ± 7 | 190 ± 8 |
| Test medium B | 15 $\mu$M $PO_4^{3-}$ | 258 ± 7 | 186 ± 7 |
| Test medium B | 40 mM $SO_4^{2-}$ | 238 ± 4 | 100 ± 6 |
| Test medium B | 10 mM $SO_4^{2-}$ | 305 ± 6 | 184 ± 9 |
| Test medium B | 40 mM $Mg^{2+}$ | 337 ± 8 | 254 ± 4 |
| Test medium B | 10 mM $Mg^{2+}$ | 219 ± 7 | 220 ± 6 |
| Test medium B | 5 mM $Ca^{2+}$ | 421 ± 8 | 196 ± 10 |
| Test medium B | 2.5 mM $Ca^{2+}$ | 473 ± 6 | 320 ± 8 |

In another series of treatments, vegetative tissues of *C. vancouveriensis* were placed in natural sea water or in test medium B or in variations of test medium B in which the concentrations of six macronutrients were independently doubled and/or halved and/or quartered, and rates of oxygen evolution were compared after incubation periods of 4 and 7 days (Table VI). Responses in media containing 2.5 or 5 mM $Ca^{2+}$ or 10 mM $SO_4^{2-}$ were markedly better than responses in test medium B (10 mM $Ca^{2+}$; 20 mM $SO_4^{2-}$) or in natural sea water ($\approx$ 10 mM $Ca^{2+}$; $\approx$28 mM $SO_4^{2-}$). Responses in 40 mM

$Mg^{2+}$ were better than in 10 mM $Mg^{2+}$ or in test medium B (20 mM $Mg^{2+}$) or in sea water ($\approx$ 54 mM $Mg^{2+}$). These data led to the conclusions that reduced concentrations of $Ca^{2+}$, $Mg^{2+}$, and $SO_4^{2-}$ relative to sea water probably do not adversely affect the species tested. This contrasts with results obtained in the trials involving $K^+$, where after 7 days, plants grown in 20 mM or in 5 mM $K^+$ showed markedly lower oxygen evolution rates than in test medium B or in natural sea water, both containing 10 mM $K^+$. Responses to the concentrations of $NO_3^-$ and $PO_4^{3-}$ tested were similar to those for test medium B and for sea water, suggesting that levels in these ranges probably are not critical.

TABLE VII

Germination responses of *Corallina vancouveriensis* spores after 38 days to test medium B, with varying levels of indicated nutrients: each culture was inoculated with 20–25 spores; results represent range values for triplicate cultures in each treatment.

| Treatment | Ion concentration | | | | % Spore germination and development |
|---|---|---|---|---|---|
| | $Ca^{2+}$ (mM) | $Mg^{2+}$ (mM) | $PO_4^{3-}$ ($\mu$M) | $NO_3^-$ (mM) | |
| 1 | 10 | 40 | 30 | 1.0 | 0 |
| 2 | 10 | 40 | 30 | 0.5 | 0 |
| 3 | 10 | 40 | 15 | 1.0 | 0–8 |
| 4 | 10 | 40 | 15 | 0.5 | 0 |
| 5 | 10 | 20 | 30 | 1.0 | 0 |
| 6 | 10 | 20 | 30 | 0.5 | 0–14 |
| 7 | 10 | 20 | 15 | 1.0 | 0 |
| 8 | 10 | 20 | 15 | 0.5 | 0 |
| 9 | 2.5 | 40 | 30 | 1.0 | 100 |
| 10 | 2.5 | 40 | 30 | 0.5 | 100[a] |
| 11 | 2.5 | 40 | 15 | 1.0 | 100[a] |
| 12 | 2.5 | 40 | 15 | 0.5 | 83–100[a] |
| 13 | 2.5 | 20 | 30 | 1.0 | 5–36 |
| 14 | 2.5 | 20 | 30 | 0.5 | 63–92 |
| 15 | 2.5 | 20 | 15 | 1.0 | 68–100 |
| 16 | 2.5 | 20 | 15 | 0.5 | 0 |
| Sea water | – | – | – | – | 0 |

[a] Geniculae present on several crusts.

Spore viability and germination responses in relation to different nutrient regimes also were examined in two series of treatments. In one set of trials responses were examined in a 38-day experiment using test medium B with all possible combinations of 2.5 and 10 mM $Ca^{2+}$, 20 and 40 mM $Mg^{2+}$, 15 and 30 $\mu$M $PO_4^{3-}$ and 0.5 and 1.0 mM $NO_3^-$. Complete survival of inoculated spores of *C. vancouveriensis* occurred only in media containing both 2.5 mM $Ca^{2+}$ and 40 mM $Mg^{2+}$ (Table VII). Very low survival rates or total death occurred in all cultures containing 10 mM $Ca^{2+}$. Distinct trends with respect to $NO_3^-$ and $PO_4^{3-}$ concentrations were not evident. These data were similar to those obtained with vegetative tissues; the data also suggested that $Ca^{2+}$ concen-

tration may be critical for the survival and development of spores and sporelings. Consequently a further experiment using test medium C was conducted to determine spore survival and germination rates of *C. vancouveriensis* in relation to $Ca^{2+}$ concentration. The results (Table VIII) indicated clearly that 5 mM $Ca^{2+}$ is optimal for spore

TABLE VIII

Germination responses of *Corallina vancouveriensis* to test medium C, with different concentrations of $Ca^{2+}$: data are presented as percent germination of inoculum; results represent range values for triplicate cultures inoculated with 100–250 spores; ungerminated but viable spores were not included.

| No. | Treatment | $Ca^{2+}$ (mM) | Bodega population (days) | | | Cypress Pt. population (days) | | |
|---|---|---|---|---|---|---|---|---|
| | | | 4 | 7 | 14 | 4 | 7 | 14 |
| 1 | Sea water | ≈10 | 40–73 | 34–61 | 26–46 | 22–58 | 16–58 | 12–48 |
| 2 | PES | ≈10 | 35–95 | 35–86 | 35–86 | 28–58 | 41–63 | 40–59 |
| 3 | Test med. C | 0.001 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | Test med. C | 0.01 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | Test med. C | 0.1 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | Test med. C | 1.0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | Test med. C | 2.5 | 12–45 | 6–41 | 0–27 | 20–45 | 9–36 | 9–19 |
| 8 | Test med. C | 5.0 | 63–87 | 54–74 | 33–65 | 74–88 | 72–91 | 53–79 |
| 9 | Test med. C | 10 | 0–2 | 0 | 0 | 0–6 | 0 | 0 |
| 10 | Test med. C | 25 | 2–13 | 0 | 0 | 0–2 | 0 | 0 |

development. No spores survived when $Ca^{2+}$ levels were increased to 10 mM or more or were reduced to 1 mM or less. Results from this experiment also suggest that spores would germinate and develop successfully in a 5 mM $Ca^{2+}$ medium containing 40 mM $Mg^{2+}$ and 1 mM $SO_4^{2-}$ (compare test media B and C, Table II). Data from separate experiments (see below) provide evidence that a spectrum of other algae also can survive in a medium containing 1 mM $SO_4^{2-}$ (which is much lower than that of natural sea water or other media – see Table IV).

Based on results from the above series of experiments and discussions between the first author and Prof. G. C. Gerloff (University of Wisconsin, Madison) on general algal nutrition, the MCM formulation was derived from modifications to test media B and C and then used in subsequent experiments.

## BUFFERING

Because of their more rapid growth, cultures of *Callithamnion byssoides* were used to examine buffer effects on growth. In a preliminary experiment the growth responses of *C. byssoides* in sea water, PES and MCM were compared. Responses in both PES and MCM were substantially better than in natural sea water (Table IX) and, based on doubling time, *C. byssoides* grew nearly as well in MCM as in PES. These data suggested that the MCM formulation derived from experiments on Corallinaceae potentially were acceptable for growing non-corallinaceous algae.

Results form experiment A (Table X) confirm that growth in MCM is nearly as rapid as in PES (compare treatments 1 and 2; also see treatments 6 and 8) and demonstrate that when both $HCO_3^-$ and Tris are omitted, growth in MCM is reduced markedly (compare treatments 2 and 3). The addition of only bicarbonate has considerable effect in increasing the growth rate (compare treatments 3 and 4) while the addition of only Tris increases growth even more (compare treatments 3, 4 and 5). The addition of bicarbonate (treatment 4) or Tris (treatment 5) appear to have a similar effect on the pH. The presence of both results in a higher pH but does not result in greater growth

TABLE IX

Growth responses of *Callithamnion byssoides* to selected nutrient regimes: MCM is from Table III chlorophyll *a* and total organic nitrogen were determined after 28 days; the inoculum contained 9.4 μg N; doubling times were determined from nitrogen determinations; data are from three replicates.

| No. | Medium | pH[a] | μg Chl *a* | μg N | Average doubling time (days) |
|---|---|---|---|---|---|
| 1 | Sea water | 8.15 | 0.1 ± 0.09 | 27 ± 2.8 | 18 |
| 2 | PES | 8.28 | 18.5 ± 2.4 | 430 ± 28 | 5.1 |
| 3 | MCM | 8.63 | 8.3 ± 1.7 | 251 ± 21 | 5.9 |

[a] pH measured at the midpoint of the light cycle after 7 days.

TABLE X

The effect of added bicarbonate and added buffers on the growth of *Callithamnion byssoides*: the procedure is the same as in Table IX except for inoculum size; the organic buffers are Tris–(hydroxymethyl)amino-methane, *N*-Tris(hydroxymethyl)methyl glycine (tricine) and 4-(2-hydroxyethyl)-1-piperazine-propane sulfonic acid (HEPPS).

| Treatment | Medium | Variation | pH[a] | μg Chl *a* | μg N | Doubling time (days) |
|---|---|---|---|---|---|---|
| Experiment A (inoculum contained 14.7 μg total N) | | | | | | |
| 1 | PES | none | 8.20 | 42 ± 5.0 | 520 ± 65 | 5.4 |
| 2 | MCM[c] | none | 8.40 | 38 ± 11 | 410 ± 65 | 5.8 |
| 3 | MCM[b] | no $HCO_3^-$, no Tris | 6.78 | 0.60 ± 0.25 | 32 ± 14 | 25 |
| 4 | MCM | no Tris | 8.29 | 16 ± 3.0 | 170 ± 13 | 7.9 |
| 5 | MCM[b,c] | no $HCO_3^-$ | 8.22 | 45 ± 4.2 | 450 ± 14 | 5.7 |
| Experiment B (inoculum contained 17.3 μg total N) | | | | | | |
| 6 | PES | none | 8.21 | 22 ± 4.3 | 338 ± 125 | 6.5 |
| 7 | PES | no Tris | 8.20 | 9.3 ± 3.6 | 173 ± 76 | 8.4 |
| 8 | MCM[b] | no Tris | 8.26 | 7.2 ± 1.4 | 105 ± 15 | 11 |
| 9 | MCM | none | 8.36 | 16 ± 5.5 | 334 ± 180 | 6.6 |
| 10 | MCM[b] | 2.0 mM tricine | 7.89 | 5.6 ± 2.7 | 147 ± 36 | 9.1 |
| 11 | MCM[b] | 2.0 mM HEPPS | 7.88 | 9.1 ± 4.9 | 104 ± 2 | 11 |

[a] pH determined at the midpoint of the light cycle just before harvest in A and in the final light cycle in B.

[b] Titrated to pH 7.7 with NaOH initially.

[c] Addition of Tris caused a flocculent precipitate during growth.

relative to medium containing only Tris (compare treatments 2 and 5). Thus, it would appear that Tris not only acts as a buffer, but also stimulates growth.

TABLE XI

The effect of varying Tris concentration on the growth of *C. byssoides*: procedures are as in Table IX; data are final total μg of chlorophyll; initial pH adjusted to 7.7.

| Tris conc. (mM) | MCM | PES |
|---|---|---|
| 0.66 | 43 ± 8.1 | 14 ± 14.7 |
| 0.2 | 35 ± 7.3 | 25 ± 3.4 |
| 0.1 | 31 ± 11 | 22 ± 6.6 |
| 0.01 | 33 ± 8.7 | 21 ± 4.1 |
| 0 | 35 ± 8.6 | 22 ± 3.7 |

Results from Treatment 3 (Table X) confirm that Tris acts as a growth stimulator. Omission of Tris from either PES (compare treatments 6 and 7) or MCM (compare treatments 8 and 9) results in reduced growth. Reduced growth in MCM also occurs when other organic buffers with comparable buffering ranges are substituted for Tris (compare treatment 8 with treatments 10 and 11). These results suggest that stimulation of growth by Tris is not due to its buffering effect alone. Table XI shows that the level of Tris in PES (0.66 mM) is required for the stimulation and may be suboptimal.

As indicated in Table XIII, stimulation by Tris was not observed with all algae tested and often does not account for the improved growth in PES. Preliminary results with the Corallinaceae indicate no effect of Tris addition to MCM upon their growth.

## THE GROWTH OF DIVERSE ALGAE ON MCM

Results from studies of germination and development responses (Table VIII) of spores of *Corallina vancouveriensis* to natural sea water, PES and MCM (without Tris) suggest that survival and germination in MCM (treatment 8, Table VIII) is similar to or better than in PES and that both MCM and PES are better than sea water. After 40 days, many of the sporelings growing in MCM had produced upright branches with one to four intergeniculae whereas sporelings in PES and sea water had produced basal crusts only. Preliminary work (Bramwell & Woelkerling, unpubl. data) on several species of the *Heteroderma–Fosliella* complex (Corallinaceae, Rhodophyta) indicates that in culture tetraspores of these nongeniculate taxa germinate and produce crusts in MCM and PES but not in natural sea water. Further studies are now in progress. Data on growth responses of *Callithamnion byssoides* (Tables IX, X) also indicate that growth in MCM is nearly as rapid as in PES and far better than in natural sea water.

The suitability of MCM as a general culture medium was also examined in three additional experiments. In the first, growth of a variety of marine algae in MCM, PES, and natural sea water was compared. The results (Table XII) indicate that while the

TABLE XII

Growth of diverse algae in MCM, PES and natural sea water (from three replicates).

| Alga and source | Days in culture | μg Chl *a*[a] | | |
|---|---|---|---|---|
| | | PES | MCM | Sea water |
| Rhodophyta | | | | |
| *Audouinella saviana* (Meneghini) Woelk., JAW 1553, Tunisia | 44 | 28 ± 4.7 | 17 ± 6.0 | 0.7 ± 0.4 |
| *Branchioglossum woodii* (J. Ag.) Kylin, JAW 653, Mexico | 47 | 22 ± 9.4 | 2.5 ± 1.0 | <0.05 |
| *Caloglossa leprieurii* (Mont.) J. Ag., JAW 686, Georgia | 66 | 2.3 ± 0.5 | 4.2 ± 1.8 | <0.05 |
| *Champia parvula* (C. Ag.) Harv., JAW 2310, Rhode Island | 47 | 37 ± 22 | 9.1 ± 3.6 | <0.05 |
| *Erythrocladia* sp., JAW 645, Mexico | 44 | 16 ± 3.6 | 17 ± 3.4 | <0.05 |
| *Spyridia filamentosa* (Wulf.) Harv., JAW 677, Hawaii | 49 | 22 ± 4.5 | 12 ± 1.5 | 1.6 ± 0.7 |
| Phaeophyta | | | | |
| *Dictyota dichotoma* (Huds.) Lam., UTEX 1676-1677 | 59 | 22 ± 3.4 | 12 ± 1.4 | 5.0 ± 1.6 |
| *Giffordia oviger* (Harv.) Hollenberg & Abbott, JAW 640, Calif. | 35 | 20 ± 5.8 | <0.05 | 0.13 ± 0.12 |
| *Sphacelaria furcigera* Kütz., JAW 2192, Mexico | 47 | 153 ± 45 | 0.3 ± 0.2 | 2.6 ± 1.2 |
| Chlorophyta | | | | |
| *Cladophora* sp., JAW 2237, Philippines | 66 | 75 ± 33 | 6.6 ± 1.3 | 0.7 ± 0.12 |
| *Microdictyon boergesenii* Setchell, JAW 1555, Bahamas | 59 | 106 ± 45 | 2.3 ± 0.8 | 0.7 ± 0.24 |
| *Percursaria percursa* (C. Ag.) J. Ag., UTEX 1423 | 42 | 359 ± 57 | 9.2 ± 5.1 | 6.0 ± 2.3 |
| *Rhizoclonium riparium* (Roth) Harv., JAW 474, Wash. | 42 | 156 | 48 ± 6.1 | 7.3 ± 2.3 |
| *Valonia ventricosa* J. Ag., JAW 1569, Florida | 59 | 41 ± 24 | 62 ± 18 | 3.1 ± 3.7 |
| Chrysophyta | | | | |
| *Prymnesium parvum* H. Carter, UTEX 995 | 35 | 155 ± 44 | 56 ± 10 | 8.8 ± 1.5 |

[a] Values not corrected for spectral overlap when other chlorophylls present.

TABLE XIII

Growth of various algae in sea water (SW), MCM without Tris, MCM with Tris and PES: data are presented as average chlorophyll *a* of three cultures; isolates are the same as in Table XII.

| Alga | Chlorophyll *a* (μg) | | | |
|---|---|---|---|---|
| | SW | MCM | MCM + Tris | PES |
| *Branchioglossum* | 4.2 ± 0.7 | 77 ± 2.0 | 81 ± 2.4 | 18.5 ± 5.6 |
| *Audouinella* | 3.3 ± 0.5 | 19 ± 14 | 44 ± 20 | 11.9 ± 5.4 |
| *Dictyota* | 5.7 ± 0.4 | 10.3 ± 4.6 | 24.6 ± 23 | 65 ± 48 |
| *Sphacelaria* | 1.9 ± 1.4 | 18.7 ± 4.0 | 18.8 ± 3.1 | 71 ± 6 |
| *Microdictyon* | 3.9 ± 0.2 | 3.9 ± 1.4 | 12 ± 4.1 | 49.5 ± 12 |
| *Percursaria* | 5.4 ± 1.1 | 12 ± 5.1 | 13 ± 6 | 122 ± 13 |

TABLE XIV

Representative taxa from Australia which have grown successfully in MCM for periods of at least 5 months at 15 °C, 16 h light–8 h dark cycle.

Chlorophyta
- *Bryopsis gemellipara* J. Agardh
- *Caulerpa cactoides* (Turner) C. Agardh
- *C. flexilis* Lamouroux
- *C. geminata* Harvey
- *C. hedleyi* Weber van Bosse
- *C. longifolia* C. Agardh
- *C. scalpelliformis* (R. Brown in Turner) C. Agardh
- *C. simpliciuscula* (Turner) C. Agardh
- *C. trifaria* Harvey
- *Chaetomorpha aerea* (Dillwyn) Kützing
- *C. darwinii* (Hooker) Kützing
- *Codium australicum* Silva
- *C. pomoides* J. Agardh
- *Dictyosphaerea sericia* Harvey

Rhodophyta
- *Audouinella daviesii* (Dillwyn) Woelkerling
- *Audouinella* sp.
- *Bostrychia radicans* (Montagne) Montagne
- *Botryocladia obovata* (Sonder) Kylin
- *Caloglossa leprieurii* (Montagne) J. Agardh
- *Ceramium* sp.
- *Epymenia wilsonis* Sonder
- *Griffithsia monilis* Harvey
- *Heteroderma* sp.
- *Jania* sp.
- *Lomentaria* sp.
- *Peyssonnelia* sp.
- *Polysiphonia* sp.

growth of all Chlorophyta, Chrysophyta and Rhodophyta tested were substantially greater in both MCM and PES than in natural sea water, most taxa grew better in PES than in MCM. The three Phaeophyta tested grew poorly both in MCM and sea water compared to PES. In a related experiment the growth of six taxa was compared in sea water, PES, MCM with Tris and MCM without Tris. The results (Table XIII) show that with the exception of *Sphacelaria*, growth in MCM with Tris was greater than growth in MCM without Tris. In all cases the poorest growth occurred in natural sea water.

Results from both of the above experiments suggest that a variety of red and green algae can be maintained in MCM although most tend to grow more slowly than in PES. In a third experiment, vegetative fragments or spores of a series of red and green algae (Table XIV) were inoculated into MCM without Tris and grown for more extended periods of time. After 5 months, these taxa retained normal morphology, and all produced new tissue. Although comparative growth rate data were not obtained, species of *Caulerpa* appear to grow particularly well in MCM, possibly because of the low concentration of sulfate. O'Kelley (1974) notes that some coenocytic green algae may exclude sulfate, and this suggests that sulfate levels in sea water may not be optimal for such algae. Attempts, however, to grow *Ulva*, *Plocamium*, and seedlings of the seagrass *Amphibolis* in MCM failed; after several weeks the plants became necrotic and eventually they died. In additional experiments tetrasporophytes of *Cumagloia andersonii* (Farlow) Setchell and Gardner were successfully cultured in MCM (DeCew & Zupan, unpubl. data). Tetrasporangia developed and released viable spores in MCM but not in other media (PES and sea water).

## Discussion

West & Hommersand (1981) have indicated that two prerequisites to undertaking more extensive laboratory-based experimental studies involving the Corallinaceae were the development of more suitable culture media and a better understanding of the nutritional physiology of these algae. Results from the present study hopefully contribute to the attainment of both goals. MCM ostensibly is the first chemically defined marine culture medium in which taxa of Corallinaceae have been grown from spores and which has been optimized specifically for culturing these plants. Based on results from studies of *Corallina*, it appears that calcium concentrations can influence markedly the viability rate of spores (Tables VII, VIII) and that 5 mM $Ca^{2+}$ is nearly optimal. This is approximately half the concentration of $Ca^{2+}$ found in PES, sea water, or some other media (Table IV). It also appears that taxa of Corallinaceae may grow better under chemically defined conditions when the concentration of $BO_3^{3-}$ is reduced by a factor of 430 (Table V) and when the concentration of $SO_4^{2-}$ is reduced by a factor of 28 (Tables IV, VI) as compared (Table IV) to sea water. In contrast, 10 mM $K^+$ (the level found in sea water – see Table IV) appears more optimal than other levels tested (Table VI), based on 7-day trials.

MCM has been designed so that each nutrient is supplied independently as a sodium or chloride salt and so that, except for vitamins, only inorganic compounds are used as nutrient sources. This contrasts with most other defined media (see McLachlan, 1973) where multinutrient and/or organic compounds are employed. Tris has been included as an optional ingredient because of its apparent effect as a growth stimulator (Tables X, XI, XIII). Tris does not appear to be essential for the survival of *Corallina* or of other algae tested (Table XIII) since the results reported in Tables VI–VIII were based on media devoid of Tris. In some previous sea-water media preparations Tris has been shown to be detrimental to algal growth (Harrison *et al.*, 1980) or to calcification (Smith & Roth, 1979). Results from this study (Table X) also show that Tris is not essential for buffering purposes and that the addition of 2.5 mM bicarbonate serves as an effective buffer in MCM as it does in sea water (see Moberg *et al.*, 1934).

The reason for the stimulatory effect of Tris upon *Callithamnion* growth is unknown. The phenomenon could be related to the precipitation that occurs during growth of cells in the presence of Tris. Specific ion concentrations could be affected. Additional possible side effects include alteration of membrane permeability to certain ions (Van Steveninck *et al.*, 1973) or an enhancement of bacterial growth. Since the precipitate resulting from Tris is laden with bacteria and the level of growth stimulation is variable, an indirect bacterial effect may be the most likely explanation. Ogata (1966) explored the stimulatory effect of Tris upon *Porphyra* photosynthesis and concluded that its buffering increased the effective $CO_2$ concentration. While such an effect is not precluded by the results of the present study, the inability of other organic buffers to similarly stimulate *Callithamnion* growth makes an additional explanation necessary.

MCM appears to have potential as a defined culture medium for Corallinaceae and also for at least some other macroscopic algae, but additional studies are needed to test MCM further. Results presented here have involved only four taxa of Corallinaceae – two species of *Corallina* and one species each of *Jania* and *Heteroderma*. To date only plants of the last taxon have been grown from spores to reproductive maturity (Bramwell & Woelkerling, unpubl. data), and additional coralline taxa need to be tested. Limited attempts to grow several other coralline taxa from spores have failed, but this may be due to the nonviability of spores during certain seasons of the year rather than to any shortcomings of MCM. Ducker (pers. comm.) also has found that spores of certain Corallinaceae appear to be viable only at certain times of the year.

For most other algae tested (Tables XII, XIII) MCM does not support growth as well as PES, but MCM has the advantage of being chemically defined whereas PES is not. MCM appears to be most suited for various red algae and, in its present form, largely unsuited for brown algae. Among green algae, coenocytic forms such as species of *Caulerpa* and *Valonia* grow exceptionally well whereas mixed results were obtained for multicellular taxa. The suitability for planktonic algae remains generally untested, but some modifications (e.g., addition of a silicate salt) would probably be necessary. Reasons for the varying success and failure of MCM for non-coralline algae have not been examined in this study, but results published by Fries (1966) and Pedersen (1969)

suggest that investigating the potential effects of added bromide and/or iodide to MCM could prove productive.

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