PROC. N.S. INST. SCI (1989)
Volume 38, pp. 145-168

PURIFYING ALGAL CULTURES - A REVIEW OF CHEMICAL METHODS*

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Chemical methods that have been used to obtain axenic algal cultures are reviewed. The methods include the use of antibiotics, hypochlorite, iodine, hormones, phenol and detergents singly or in combination. Data are given on the use of antifungal agents such as caffeine, griseofulvin and the polyene macrolides e.g. nystatin. When appropriate, the use of physical methods e.g. gel adsorption, sonication, filtration, and osmosis, is described where these techniques are used in conjugation with antifungal/antibacterial compounds.

On a examiné les méthodes chimiques en usage pour la production de cultures axéniques d'algues. Ces méthodes comprennent l'usage d'antibiotiques, de l'hypochlorite, de l'iode, des hormones, du phénol et de détersifs, employés séparément ou en combinaison. Des données sont présentées quant à l'usage de substances antifongiques telles que le caféine, le griséofulvin, et les macrolides de polyene, e.g. le nystatine. L'usage de méthodes physiques, e.g. l'adsorption sur gèle, la sonication, la filtration, et l'osmose, est décrite où on se sert de ces méthodes en conjonction de composés antifongigues ou antibactériennes.

Introduction

Antibiotics and other chemical agents have been used in conjunction with physical methods for many years to obtain axenic cultures of algae. These procedures are widely dispersed in the literature and no systematic attempt has been made to summarize them. The present report draws together this information into a form accessible for scientists and students wishing to purify algae from natural sources. Procedures for isolating algae have been compiled c.f. Stein (1973). Data concerning the source, formula, physical appearance, activity, mode of action and solubility of some of the commonly used antibiotics employed to free algal tissues from bacteria and fungi, can be found in CRC (1980a-e). The taxonomic classification follows that of Bold and Wynne (1978).

Antibacterial Methods Used to Obtain Axenic Cultures

Cyanochloronta - The elimination of bacteria from blue-green algae has proved to be a difficult task. Tchan and Gould (1961), without mentioning the species tested or the concentrations of antibiotics used, reported that none of the following antibiotics eradicated bacteria from *Cyanochloronta;* streptomycin, neomycin, bacitracin, penicillin, chloramphenicol, tetracycline, erythromycin, nystatin, novobiocin, oleandomycin, polymyxin. Combinations of tetracycline and chlortetracycline; penicillin, erythromycin, and aureomycin; and polymyxin, penicillin, erythromycin, and tetracycline were also ineffective. They found that bacteria tested alone were sensitive to some of these antibiotics, but not when associated with the blue-greens, suggesting a protective effect of the algae. Vance (1966) exposed *Microcystis aeruginosa* to several antibiotics in the concentration range 0.01 - 1000 μg mL^{-1}. Only neomycin and dihydrostreptomycin inhibited the growth of bacteria under conditions that allowed algal growth. Four bacteria were isolated; *Xanthomonas oryzae, Brevibacterium insectiphilum, B. linens,* and a small unidentified gram negative rod, and these were sensitive to both antibiotics. A subsequent test employing 1 μg mL^{-1} of neomycin and dihydrostreptomycin singly and together established that only the combination

*NRCC No. 30854

produced axenic cultures. However, over the following 6-9 weeks all but two of the axenic cultures on agar slants died. It was discovered that the two survivors were contaminated with a small unidentified gram negative rod, whereas the dead cultures were uncontaminated, suggesting that bacteria may play a role in blue-green metabolism.

Jones *et al.* (1973) used a two-fold dilution series containing a mixture of benzylpenicillin (7-1000 ng mL^{-1}), chloramphenicol (0.3-40 μg mL^{-1}), and neomycin (0.1-16μg mL^{-1}) in an attempt to free *Oscillatoria* sp. from bacteria. With the exception of green-fluorescent pseudomonads, all bacteria were killed. The authors speculated that polymyxin B or one of the newer penicillins e.g. carbenicillin (developed for human *Pseudomonas* infections), kanamycin or colistin might eliminate the pseudomonads.

The use of antibiotics to selectively inhibit contaminants in blue-green algal cultures is not an effective approach because the latter are often as sensitive as the former. This is not surprising because the blue-green algae are prokaryotic (Benson 1985).

Chlorophycophyta - A number of antibiotics used singly and in combination has been applied to green algae to obtain axenic cultures. Galloway and Krauss (1959) tested the potential selective toxicity of terramycin, streptomycin, polymyxin-B-sulfate, tetracycline, penicillin G, bacitracin, chloromycetin, and gramicidin used singly on three species of algae (*Scenedesmus obliquus, Chlorella pyrenoidosa* and *Chlorella vulgaris*), three genera of bacteria (*Flavobacterium sp., Achromobacter sp.* and *Pseudomonas sp.*), and one fungus (*Aspergillus sydowii*). The point at which growth was completely arrested over a period of 2-5 days at 25°C was determined for concentrations ranging from 0.1 to 1000 μg mL^{-1}. Only polymyxin B showed selective toxicity. In an organic medium, >1 mg mL^{-1} was required to halt the growth of *S. obliquus* and *C. vulgaris* whereas $\leq$ 10 μg mL^{-1} arrested the growth of the fungus as well as the bacteria. However, *C. pyrenoidosa* was inhibited at 5 μg mL^{-1}, illustrating the point made by Droop (1967) that even though algae may be closely related, they can exhibit widely different tolerances to antibiotics. Further experiments revealed that 640 μg mL^{-1} of polymyxin B suppressed photosynthesis of *C. pyrenoidosa*, whereas *S. obliquus* and *C. vulgaris* were unaffected. A combination of penicillin and streptomycin applied over several days enabled Berglund (1969) to sterilize multicellular green algae. *Enteromorpha sp.* and *Cladophora gracilis* exposed to 1 mg mL^{-1} and 0.7 mg mL^{-1} for 16 and 17 days, respectively, were freed of all contaminants. Similarly, *Enteromorpha linza* was purified after 8-12 days in 1.2 mg mL^{-1} penicillin and 0.7 mg mL^{-1} streptomycin. However, *Bryopsis plumosa* remained contaminated despite exposure to penicillin, streptomycin, griseofulvin, undecyclenic acid and "Jodopax" alone and in combination. Millner *et al.* (1979) added 35 μg mL^{-1} each of penicillin G and streptomycin sulfate to the enzyme medium they were using to prepare protoplasts from *Enteromorpha intestinalis*, but no mention was made of the sterility of the culture. Gibor and Izawa (1963) sterilized *Acetabularia mediterranea* by washing it thoroughly in sterile seawater and then in a solution containing (per mL) 1 mg streptomycin and penicillin, 200 μg chloramphenicol and neomycin, and 34 μg mycostatin. Later, Green *et al.* (1967) purified *A. mediterranea* with a combination of the antibiotics (per mL) 1 mg penicillin, 200 μg neomycin, and 34 μg mycostatin.

Sterile mixtures of several antibiotics were employed by Jones *et al.* (1973) in four experiments as indicated in Table I, to purify cultures of *Pediastrum boryanum* and *Chlorella vulgaris*. In the first experiment, Series A, a six-tube dilution series was prepared such that each concentration was only half of the previous one. The initial mixture contained (μg mL^{-1}): 2000 benzylpenicillin, 500 tetracycline, 60 chloramphenicol, 50 aureomycin, 400 ceporin, 10 neomycin-SO_4 and 10 streptomycin-SO_4.

Table I Concentrations of antibiotics ($\mu g\ mL^{-1}$) in algal cultures of *Chlorella vulgaris* and *Pediastrum boryanum* (adapted from Jones et al. 1973).

Antibiotic	Series A[a] Dilution Tube a	b	c	d	e	f
Benzylpenicillin	2000	1000	500	250	125	62.5
Tetracycline	500	250	125	62.5	31.25	15.6
Chloramphenicol	60	30	15	7.5	3.75	1.9
Aureomycin	50	25	12.5	6.25	3.12	1.55
Ceporin	400	200	100	50	25	12.5
Neomycin-SO_4	10	5	2.5	1.25	0.6	0.3
Streptomycin-SO_4	10	5	2.5	1.25	0.6	0.3

[a] Series B = 2X conc. of Series A minus streptomycin.
Series C = 2X conc. of Series A minus (streptomycin + neomycin).
Series D = 2X conc. of Series A minus (streptomycin + neomycin + ceporin).

Subsequent experiments were carried out at twice the concentrations used in Series A, although with reduced numbers of antibiotics. For example, all of the initial concentrations of antibiotics in Series B were doubled except for streptomycin-SO_4 which was omitted. Similarly, streptomycin and neomycin were excluded from Series C while streptomycin, neomycin and ceporin were dropped from Series D. Two-week-old cultures were incubated for 24 h with antibiotics, whereupon an aliquot was removed and added to Bold's basal medium and grown for two weeks prior to testing for sterility. Sterility was achieved for *P. boryanum* in certain dilution tubes in all four experiments (Series A - tube b; Series B - tubes c and d; Series C - tubes a, b, c, d; Series D - tubes b, c, d). Since sterile cultures were obtained in Series D where only four antibiotics were used, it appears that streptomycin, neomycin and ceporin were of little value in deriving axenic cultures. If streptomycin is largely ineffective in purifying green algal cultures, it should be omitted as Provasoli (1966) reported that it may induce chlorophyll bleaching. It should also be noted that only one tube became bacteria-free in Series A even though all seven antibiotics were present. This may be attributed to the fact that the concentrations in this series were only half of those in Series B, C and D, but it is also possible that there was antagonism occurring between the antibiotics. For example, Speck *et al.* (1951) reported that aureomycin and terramycin antagonized the action of penicillin. With regard to *C. vulgaris*, only tubes b, c, d, e, and f of Series B were rendered sterile. Series A was contaminated with bacteria and yeasts while Series C and D were overrun with yeasts. Apparently the concentrations in Series A were too low to destroy the contaminants, whereas in Series C and D the elimination of bacteria allowed the opportunistic yeasts to proliferate. Provasoli *et al.* (1951) described the same phenomenon where yeasts predominate in the absence of previously established bacteria and he noted that a fungicide would be required to obtain sterile cultures (see below). However, it should be pointed out that neomycin was absent from Series C and D. It was hypothesized that it had played a role in eradicating yeasts from Series B.

A different approach was tried by Provasoli (1958), who placed pieces of *Ulva* sp. thallus on the surface of an agar medium containing a mixture of antibiotics. He found that 120 μg of penicillin together with 1 μg each of chloramphenicol, neomycin, and polymyxin B per mL of agar were sufficient to kill all bacteria and 66% of the diatoms. The bacteria-free algal cultures developed into atypical, short filamentous germlings. Provasoli (1958) postulated that this effect was due to the absence of bacteria and their beneficial effect on the gross morphology of the *Ulva*. Antia and

Kalmakoff (1965) employed a combination of antibiotics together with a non-ionic surfactant, Tween-80 (Brown and Bischoff 1962), to purify *Tetraselmis maculata*. Penicillin (100 μg mL^{-1}), streptomycin (50 μg mL^{-1}), chloramphenicol (25 μg mL^{-1}), and Tween-80 (20-40 μL mL^{-1}) proved to be successful.

Euglenophycophyta - Pappas and Hoffman (1952) tried to free *Euglena gracilis* of bacteria using penicillin (120 μg mL^{-1}), dihydrostreptomycin (1 mg mL^{-1}), and aureomycin (400 μg mL^{-1}) singly, in pairs, and in combination over a 10 day period at 23°C. Success was achieved only when all three antibiotics were combined. Later, Ebringer (1964) found in 14-day exposures that the following concentrations of antibiotics (μg mL^{-1}) tested individually could produce permanent bleaching of the chloroplasts: erythromycin (200); streptomycin (200); kanamycin (500); viomycin (500); carbomycin (500); and spiramycin (2000).

Chrysophycophyta - Chrysophyceae - Pintner and Provasoli (1968) derived axenic cultures of three species of *Chrysochromulina* using two different antibiotic combinations. *C. strobilus* was rendered axenic following treatment for an unspecified period with a solution of AM9 antibiotics in the concentration range 0.06 - 0.1 mL mL^{-1}. AM9 contained (μg mL^{-1}): 20 polymyxin B, 6600 dihydrostreptomycin, 260 tetracycline, 560 chloramphenicol, 3000 penicillin G, and 400 neomycin. The media of the remaining species, *C. kappa* and *C. brevefilum*, were treated with kanamycin (100 μg mL^{-1}) and pencillin (3-6 mg mL^{-1}). Samples were transferred daily to media without antibiotics until bacteria-free cultures were obtained.

Bacillariophyceae - One of the first published studies on purifying diatom cultures involved the use of penicillin and streptomycin singly and in combination. Spencer (1952) exposed *Nitzschia closterium* forma *minutissima* to concentrations of penicillin ranging from 0.03-6 mg mL^{-1} for 16 days and found that the highest concentration in which the diatom survived (0.6 mg mL^{-1}) also permitted bacterial growth. Streptomycin inhibited diatom growth at 0.7 mg mL^{-1} but failed to rid the culture of bacteria. Combinations of both penicillin (0.03, 0.06 and 0.03 mg mL^{-1}) and streptomycin (0.07, 0.13 and 0.7 mg mL^{-1}) over 16 days also were unsuccessful.

A different approach was tried by Berland and co-workers in which bacteria were isolated from planktonic algal cultures and identified. Their susceptibility to a number of antibiotics tested singly was evaluated and compared to that of five species of diatoms. Berland *et al.* (1969) isolated 25 species of bacteria from cultures of *Asterionella japonica, Chaetoceros lauderi, Lauderia borealis, Phaeodactylum tricornutum, Leptocylindricus danicus,* and *Stichochrysis immobilis.* Berland and Maestrini (1969a) tested susceptibility of the diatoms to aureomycin, terramycin, penicillin, kanamycin, neomycin, streptomycin, dihydrostreptomycin, chloramphenicol, and polymyxin B while incubated for one week at room temperature. The effectiveness of each antibiotic was judged relative to its toxicity to the diatoms tested: *A. japonica, C. lauderi, L. danicus, P. tricornutum* and *Rhizoselenia fragillissima.* Aureomycin, terramycin, chloramphenicol, and polymyxin B were not selectively toxic (Table II), because their minimum inhibitory concentrations (MIC) towards the diatoms after a 6-day exposure at 18°C were in the ranges (μg mL^{-1}) 35-100, 5-20, 6->80 and 0.75-55, respectively (Berland and Maestrini 1969b). The MICs (μg mL^{-1}) of kanamycin (250->1000), neomycin (100->1500) and streptomycin (550->1000) to the diatoms tested suggest that, in most cases, the antibiotics are more toxic to bacteria, with the exception of *Flavobacterium* sp. and *Xanthomonas* sp., than to diatoms. By contrast, penicillin with MICs of 700->1000 μg mL^{-1} was more effective against *Flavobacterium* sp. and *Xanthomonas,* but less effective against pseudomonads. No attempt was made to sterilize the diatoms with dihydrostreptomycin so its selective toxicity could not be evaluated.

Table II Minimum inhibiting concentrations ($\mu g\ mL^{-1}$) of antibiotics for bacteria isolated from planktonic algal cultures (from Gerland and Maestrini 1969a).

Genera and Species	Aureomycin	Terramycin	Penicillin	Kanamycin	Neomycin	Streptomycin	Dihydro-streptomycin	Chloram-phenicol	Polymyxin B
Pseudomonas									
P. aestumarina	300	200	4000	200	20	>10000	>5000	100	>900
P. cruciviae	60	50	1	30	30	30	25	2	4
P. marinoglutinosa	400	1000	250	400	100	400	1000	150	15
P. (marinoglutinosa)	300	1500	1500	40	40	30	20	25	10
P. riboflavina	50	20	50	20	20	20	75	25	20
P. stereotropis	80	175	0.25	200	50	150	25	2	25
P. sp. 1	200	700	2000	50	60	30	25	15	50
P. sp. 2	200	750	750	70	50	200	200	7.5	10
P. sp. 3	40	100	12	200	30	100	>1000	1.5	>900
Vibrio									
V. algosus	400	150	1750	400	200	300	200	10	5
V. phytoplanktis	300	175	15	400	175	80	150	1.5	10
Agarbacterium									
A. mesentericus	200	1500	3000	100	30	20	60	5	50
Xanthomonas									
X. sp. 1	100	100	30	100	1500	>10000	>5000	1	500
Achromobacter									
A. parvulus	80	150	1	200	125	>10000	200	2	500
A. stationis	15	50	0.25	7	10	15	15	1.5	50
A. stenohalis	50	20	50	60	15	5	10	1.5	2
A. sp. 1	200	750	750	50	30	40	25	2	7.5
Flavobacterium									
F. aquatile	200	125	50	>5000	>1000	>10000	>5000	30	>1000
F. lutescens	300	75	100	>5000	>2100	10000	9500	75	>1000
F. peregrinum	30	100	2	30	10	7.5	5	5	150
F. sp. 1	30	40	0.05	80	30	>100000	125	0.75	2
F. sp. 2	300	100	750	>5000	>1000	>100000	>5000	100	>1000
F. sp. 3	400	150	400	60	>2100	100000	9500	200	600
Micrococcus									
M. sp. 1	50	250	15	80	400	25	100	2	150
Staphylococcus									
S. aureus	40	150	7	9	30	20	20	5	400
S. aureus peni (-)[a]	20		150						
S. aureus peni. (+)			>1000						

[a] *S. aureus* penicillin (-) and *S. aureus* penicillin (+) are collection strains from the "Faculté de Pharmacie de Marseille", France.

This type of study points out the large differences in susceptibility to the same antibiotic which can occur between species of the same genus. For example, the MIC of penicillin for *Pseudomonas aestumarina* was 4 mg mL^{-1} whereas for *P. stereotropis* is was only 0.25 μg mL^{-1}. Because of these variations and those of the diatom species themselves, treatment with a single antibiotic would be unlikely to render a culture axenic.

Droop (1967) reported that a short exposure (24 h) to a high concentration of a combination of antibiotics would eliminate bacteria. He tested two separate mixtures for diatoms as indicated in Table III. The original concentration (Table III) was decreased by a factor of two through each of six successive subcultures before deleting the antibiotics. In a "typical" experiment an intermediate concentration was usually successful in eliminating the bacteria while yielding a viable alga. Jones *et al.* (1973) tried Droop's mixture 1 (Table III) on *Nitzschia capitellata* and *Amphora but a 24 h exposure bleached the chloroplasts. The mixture was then modified (2 mg* mL^{-1} penicillin, 1.6 mg mL^{-1} ceporin, and 200 μg mL^{-1} aureomycin) and, after a "short exposure", the cultures were rendered axenic. No alteration in growth, pigmentation or cytology in either species occurred. However, using the same treatment as for green algae described above, Jones *et al.* (1973) were unable to sterilize *Tribonema viride* Pasch., due to a heavy growth of *Pencillium* spp. The addition of a fungicide such as nystatin might overcome this problem (see below).

Table III Concentrations of antibiotics used in mixtures to sterilize diatom cultures (adapted from Droop 1967).

Antibiotic	Mixture 1 (μg mL^{-1})	Mixture 2 (μg mL^{-1})
Benzylpenicillin	8000	8000
Streptomycin-SO_4	1600	1600
Chloramphenicol	200	80
Neomycin	-	400

Pyrrhophycophyta - Divan and Schnoes (1982) exposed four "red tide" organisms *Gonyaulax catenella, G. excavata, G. tamarensis* subsp. *excavata,* and *G. tamarensis* Plymouth to a number of antibiotics in attempts to produce an axenic culture. Actively growing cultures were inoculated into seawater medium together with amphotericin B, chloramphenicol, dihydrostreptomycin sulfate, neomycin sulfate, polymyxin B sulfate, potassium penicillin G, and streptomycin sulfate singly and in combination. After 16-17 days of growth at 15°C, the cells were centrifuged and resuspended in medium prior to being examined microscopically for changes in size, morphology and motility. In general, *G. catenella* and *G. excavata* tolerated dihydrostreptomycin, neomycin, penicillin, and streptomycin applied singly over a concentration range of 10-1000 μg mL^{-1}. The other algal species were susceptible to all the antibiotics tested. Amphotericin B and chloramphenicol inhibited dinoflagellate growth in all four species. For example, 25 μg mL^{-1} of amphotericin B inhibited the growth of both *G. catenella* and *G. excavata* while 10 μg mL^{-1} completely arrested the growth of *G. tamarensis* subsp. *excavata* and *G. tamarensis* Plymouth. Chloramphenicol at concentrations >50 μg mL^{-1} caused cell lysis in all species. Five antibiotics with the lowest toxicity to the dinoflagellates (dihydrostreptomycin, penicillin G, polymyxin B, streptomycin, and neomycin) were then evaluated for bactericidal activity. No single antibiotic was completely effective. The group that yielded axenic cultures of *G. catenella* and *G. excavata* consisted of dihydrostreptomycin, neomycin, penicillin G, and amphotericin B at concentrations of 250, 250, 500 and 5 μg mL^{-1},

respectively. This combination did not reduce growth, motility or toxin production in the dinoflagellates. Cultures of *G. tamarensis* subsp. *excavata* and *G. tamarensis* Plymouth could not be rendered axenic as concentrations of antibiotics necessary to eradicate the bacteria were toxic to the dinoflagellates themselves.

Phaeophycophyta - Saga and Sakai (1982) described a method for deriving pure cultures of the macroscopic alga *Dictyosiphon foeniculaceus,* which had been treated with an antibiotic mixture consisting of (mg mL^{-1}): penicillin G (125), streptomycin sulphate (50), and chloramphenicol (20). The alga was wiped with gauze, rinsed in sterile seawater and cut into one cm lengths, whereupon ten sections were introduced into the antibiotic solution and maintained at 5°C for two days. After rinsing in sterile medium, zoospores were collected and spread over the surface of an agar plate. One month later, 0.5 to 1.0 mm masses of filamentous thalli had grown. Those which appeared sterile were transferred to test tubes containing ASP 12-NTA medium (Provasoli 1963) and monitored for cloudiness after growth for an additional month. Using this method, more than 90% of the sections were reported axenic as were some strains of other algae that were tested (e.g. *Pogotrichum yegoense, Ulva pertusa* and *Laminaria angustata).*

Saga and Sakai (1983) also developed axenic cultures of *L. angustata* using a procedure without antibiotics. Sporophytes were cut into 5 cm lengths, wiped with gauze and rinsed several times in sterile seawater, whereupon both ends were dipped in absolute ethanol, burned and cut off with a sterile knife. The remaining tissue was punched axially using a sterile cork borer and the medullary tissue was sectioned approximately 2 mm thick, prior to being inoculated on 50 mL ASP 12-NTA medium solidifed with 1.0% agar. A sterility test using ST3 medium (Provasoli et al. 1957) revealed that all tissues were axenic.

Rhodophycophyta - *Goniotrichum elegans* was freed from bacterial contaminants by treating small pieces of tissue for 12-14 days with penicillin (0.6 mg mL^{-1}), and streptomycin (0.7 mg mL) (Fries 1963). Similarly, *Asterocytis ramosa* was rendered axenic by a three-day treatment in streptomycin (0.7 mg mL^{-1}) followed by eight days in pencillin (4.8 mg mL^{-1}). However, other unidentified species were not freed of contaminants using only these antibiotics. In addition, the combination of antibiotics used by Provasoli (1958) to obtain axenic cultures of *Ulva* was tested on *Nemalion multifidum, Antithamnion plumula,* and *Ceramium strictum* without success. Similarly, Cheney *et al.* (1986), using a modified AM9 combination were unable to free *Gracilaria lemaneiformis* from bacteria after a 24-48 h exposure. Bradley et al. (1988), however, did report some success with small pieces of tissue exposed to discs impregnated with antibiotics on agarose plates. Unialgal cultures of *Agardhiella subulata, G. lemaneiformis,* and *Porphyra yezoensis* were separately and aseptically chopped into 1 to 3 mm pieces and spread evenly across agarose solidified media in petri dishes. A variety of antibiotic discs was then placed on the agar surface. Following incubation at 22°C for three to five days, healthy pieces of tissue were removed from clear zones, where the bacteria were inhibited, and placed on other agarose plates to test for sterility. After a further five to seven days at 22°C, the pieces that still appeared "clean" were transferred to new medium and regenerated into whole plants. In a typical experiment, six of twenty-four antibiotic discs would produce clear zones. Of the ten pieces of tissue from the polymyxin B and erythromycin zones, only one of each proved to be axenic and grew into mature plants. This method also yielded axenic cultures of *Porphyra yezoensis, Enteromorpha linza* and *Laminaria japonica.* However, *A. subulata* did not survive longer than five days unless plant growth regulators were added. A concentration of α-naphthaleneacetic acid (0.01 mg L^{-1}) and zeatin (0.01 mg L^{-1}) ensured survival for ten days. In one experiment four axenic pieces were obtained from the rifampicin zone while one was recovered from the erythromycin zone.

The use of antibiotics can have adverse sublethal effects on the physiology of algae. Sheath (1975) reported that chloramphenicol and cycloheximide (see below) at concentrations of 100 μg mL^{-1} reduced the rate of photosynthesis in *Porphyridium purpureum* by about 50% and 75%, respectively. In addition, starch accumulation was prevented by rifampicin (an inhibitor of RNA polymerase) when added to *P. purpureum* after transfer of the alga from the dark to the light.

Antibiotics Used in Conjunction with Osmotic Methods

Chlorophycophyta - Brown (1982) developed a method of freeing algal cultures of bacteria based on the principle that bacterial cells treated with antibiotics at a high salinity will accumulate high concentrations of osmotic solutes and have fragile cell walls which, when abruptly changed to a hypotonic medium, would lyse, leaving the more sturdy eucaryotic algae to survive and become axenic. Artificial seawater (primary salts - 478 mM NaCl, 25.6 mM $MgCl_2$, 28.9 mM $MgSO_4$, 10.5 mM $CaCl_2$, 10.4 mM KCl, 2.4 mM $NaHCO_3$ and 97 μM H_3BO_3) with the addition of f/2 nutrients less silicate (Guillard and Ryther 1962) was used to grow marine unicells consisting of two strains of *Nanochloris* (Univ. Washington isolate 20-2-2 and Utex 2055), *N. oculata* (Utex 2164) and two freshwater species, *Mesotaenium caldariorum* (Utex 41) and *Chlorella sp.* (Univ. W. Ont. isolate 56) at 20°. The two *Nanochloris* spp. were grown in 200% primary salts until the exponential stage of growth, when an antibiotic mixture consisting of penicillin G. (2 mg mL^{-1}), ampicillin (2 mg mL^{-1}), carbenicillin (1 mg mL^{-1}), cycloserine (100 μg mL^{-1}), and vancomycin (300 μg mL^{-1}) was added and the culture was incubated for 72 h. The cells were then centrifuged, resuspended in 7% seawater and subjected to 2 min of ultrasonic treatment. The others were treated in a similar manner except that initially they were grown to the exponential phase in 100% seawater (*Chlorella sp.* and *N. oculata*) or 7% seawater (*M. caldariorum*), whereupon they were centrifuged and resuspended in sterile 200% seawater with antibiotics. Both controls and antibiotic-treated cells were washed, centrifuged and resuspended in 7% seawater. The resulting algal cultures were tested for contamination by inoculation into three types of medium as well as by light and TEM microscopy. All of the algae tested, with the exception of *M. caldariorum*, survived the treatment. However, only two cultures became axenic; both were *Nanochloris sp.* In one case, 75% of the cultures were axenic, whereas, in the other only 33% were free of bacteria.

Brown (1982) pointed out that all of the antibiotics used were bacterial cell wall inhibitors designed to work together with osmotic shock and sonication to lyse bacterial cells. He discouraged the use of commonly recommended bacteriostatic agents such as streptomycin or chloramphenicol in combination with cell wall inhibitors as they render the inhibitors ineffective (Tipper and Wright 1979).

Antibiotics Used With Hypochlorite Treatments

Phaeophycophyta - Druehl and Hsiao (1969) exposed four species of brown algae, *Nereocystis luetkeana, Laminaria saccharina, Costaria costata* and *Alaria marginata* to a mixture of antibiotics consisting of penicillin G (620 μg mL^{-1}), streptomycin sulfate (250 μg mL^{-1}), chloramphenicol (100 μg mL^{-1}), and/or a 1% solution of sodium hypochlorite in order to obtain axenic cultures. Mature sori were cut from the sporophyte, wiped off and rinsed several times in sterile seawater whereupon they were immersed in the antibiotic mixture for 3 h, followed by 20-30 min in NaOCl. The sori were aseptically trimmed, rinsed 5 times in sterile ASP 2M medium (Druehl and Hsiao 1969) and placed in the medium. Meiospores, released usually within 2 h, were transferred to culture tubes containing sterility test medium and examined over a 10 week period for the presence of bacteria. All species tested became axenic except *C. costata*. Under axenic conditions, the percentage germination of the meiospores and the development of the gametophytes were the same as those of the controls.

However, the sporophyte development was delayed in *A. marginata* and absent altogether in the other species. No explanation was given for this phenomenon, except that the contaminants may have produced a factor necessary for sporophyte development (see below).

Antibiotics Used With Agar Cleaning

Rhodophycophyta - Several antibiotic mixtures consisting of various combinations of chloramphenicol, neomycin, polymyxin, tetramycin, novobiocin, erythromycin, penicillin, streptomycin, and magnamycin were tried unsuccessfully by Tatewaki and Provasoli (1964) to treat three species of *Antithamnion*. They then turned to the "dip and drag" technique of pulling a small thallus piece through 20 mL of agar to which 0.5-1.5 mL of antibiotic mixture had been added. Some success was achieved by washing the thallus 15 times in agar followed by 6 washings in liquid medium and antibiotics. The antibiotic mixture contained ($\mu g\ mL^{-1}$): penicillin (600), dihydrostreptomycin (100), chloramphenicol (50), neomycin (50), polymyxin (6), and tetracycline (21).

Iwasaki (1965) adapted the Tatewaki and Provasoli (1964) method in efforts to isolate axenic tissues of *Porphyra tenera*. An antibiotic mixture containing 24 mg of penicillin and 20 mg of streptomycin was added to 20 mL of agarized enriched seawater and young buds 1 mm long were placed on the surface before being dragged inside the agar. Following each washing, the thallus was brought to the surface and the adhering agar was removed. This procedure was repeated seven times, whereupon the tissue was left overnight in the agar and then washed an additional five times before being transferred to sterile liquid medium. After one month the tissue was transferred to new medium and kept for two months prior to being examined microscopically for bacteria. Of the ten original buds, four were axenic, but only one survived and grew. Recently, Cheney *et al.* (1986) rendered *Gracilaria tikvahiae* axenic using a modification of this method.

Chlorophycophyta - Several species of *Oedogonium* were freed of bacteria by the agar "drag" method of Machlis (1962). Vegetative filaments were washed with distilled water and sterile medium and then dragged through 0.7% agar, followed by three successive plates of 0.7% agar containing 1.5 mg mL^{-1} of penicillin. The filaments were left embedded for 24 h and then transferred to culture tubes of medium without agar. Roughly 40-50% of the filaments became axenic using this technique.

Antibiotics Used With Filter Washing

Pyrrhophycophyta - Soli (1964) devised a system whereby *Peridinium trochoideum* was caught on a filter disc and washed with sterile seawater to remove loosely adhering bacteria. This was followed by a 5 h exposure to a combination of penicillin, streptomycin, polymyxin B, and neomycin, each at a concentration of 30 $\mu g\ mL^{-1}$. Then the sterile seawater was again flushed through the system, leaving the dinoflagellate axenic.

Antibiotics Used with Iodine

Rhodophycophyta - Fries (1963) reported that *Nemalion multifidum* was freed of bacteria by initial treatment with penicillin and streptomycin followed by immersion in a 0.125% molecular iodine solution for an unspecified length of time. As there were still small "unknown particles" adhering, the tissue pieces were inoculated onto agar plates and the plates inverted. Small axenic tips were excised as the alga grew through the agar towards the light.

Antibiotics Used with Iodine and Sonication

Rhodophycophyta - Gibor et al. (1981) have outlined a three-step procedure for obtaining axenic cultures: 1) ultrasonic treatment up to several minutes to eliminate

most epiphytes, 2) immersion in 1% w/v betadine (iodinated polyvinyl pyrrolidone): seawater up to five minutes, and 3) immersion in a solution of antibiotics in unspecified enriched seawater for one week. Using this procedure sterile tissues of *Porphyra lanceolata, Eucheuma uncinatum, Antithamnion* sp., *Gelidium nudifrons,* and *Gracilaria* sp. were established. Polne *et al.* (1980) found that ultrasound was effective in removing epiphytes from several species of algae including *Gelidium robustum, G. nudifrons, Gastroclonium coulteri,* and *E. uncinatum.* The algae, encrusted with diatoms, filamentous red and brown algae and bacteria, were immersed in seawater, placed in the ultrasonic bath for two minutes, removed and resuspended in sterile seawater. This procedure, repeated twice, removed most of the epiphytes except where they were well established. Gibor *et al.* (1981) noted that if some epiphytes remained, they might also survive the betadine and antibiotic procedures, thereby resulting in bacteria-free cultures that were not unialgal. in a later study, Polne-Fuller and Gibor (1984) cut discs of tissue from *Porphyra perforata,* sonicated them in sterile freshwater for 30 s, rinsed them in sterile seawater and repeated the procedure twice using sterile seawater. The discs were then immersed in a 1% betadine solution for 7 min and rinsed in sterile seawater. After treatment in a 1:10 dilution of the following antibiotic mixture (1 g penicillin G, 2 g streptomycin, 1 g kanamycin, 25 mg nystatin, 200 mg neomycin in 100 mL distilled water) the tissues were described as axenic.

Antibiotics with Hormones and Tissue Excision

Rhodophycophyta - Using medullary tissue, Chen and Taylor (1978) were able to render *Chondrus crispus* axenic. Apical fragments taken from fronds with a diameter >2 mm were dragged through filtered seawater solidified with 0.8% agar and containing (μg mL^{-1}): penicillin G (300), streptomycin sulfate (100), neomycin (50), and kanamycin (200). They were then incubated for two days in the same antibiotic mixture in SWMD-1 medium (Chen and Taylor 1978), whereupon the procedure was repeated. To promote growth, the tissue was placed in medium containing kinetin ($10^{-6}M$) and α-napthaleneacetic acid (10^{-6} *M*) for one week. A segment was then excised near the apex and the cortical tissue was removed under aseptic conditions. Tissue cubes (2 mm on a side) were cut from unpigmented segments of subcortical and medullary tissue and were inoculated for four weeks in TC-1 medium (Chen and Taylor 1978) on a gyratory shaker. Those segments with the characteristic reddish-brown colour were found to be axenic after checking under oil immersion and SEM for bacteria.

Hypochlorite Treatments

Phaeophycophyta - Fries (1977) found that frond tips of *Fucus spiralis* and *Fucus vesiculosus* remained contaminated after antibiotic treatments and decided that a stronger sterilization method was needed. The tips of *F. spiralis* fronds were cut and immersed in a saturated calcium hypochlorite solution for 2-5 minutes and were then washed in sterile seawater prior to being placed on ASP6 F2 medium (Fries 1977) which had been solidified with 5 g agar per litre. Those pieces which appeared to be free of bacteria after one week were transferred to new plates and then to ASP6 F2 medium one week later. The segments had lost their colour except for a few cells in the apical pit region, some spots on the mid-rib or around the cryptostomata. Some cells began to divide within one month and differentiated into several shoots, while others remained dormant for several months. This method was not very efficient in producing axenic tissues for only four tips were determined to be bacteria-free out of "many hundred treated tips". Nevertheless, Fries (1984) was able to induce the growth of plantlets from the axenic culture of *F. spiralis.* Those tissues which were axenic grew much more slowly than plants supporting bacteria unless growth prom-

oting substances such as phenylacetic acid or p-hydroxyphenylacetic acid were added (Fries 1977).

Essentially the same procedure was used by Fries (1980) for sporophytes of *Laminaria digitata* and *Laminaria hyperborea,* except that pieces of tissue 5 x 5 mm from the thallus where the stipe broadens into a blade were dipped in saturated calcium hypochlorite for 30-60 s. Sterility was tested according to the methods of Fries (1970) but the success rate was not reported.

Potassium Tellurite

Potassium tellurite is a bacteriostatic agent which has been used in agar media to allow the algae to "outgrow" bacteria. Caution should be exercised in its use, as it is now labelled a teratogen.

Ducker and Willoughby (1964) washed apices of *Falkenbergia rufolanosa* in sterile seawater, dragged them through 1% agar containing 0.01% potassium tellurite and incubated them in light at 16°C. The alga was able to outgrow the bacteria in the agar and produce "clean" filaments; however, no axenicity test was conducted.

Rosowski and Hoshaw (1970) used a combination of techniques to isolate *Zygnema* sp. into axenic culture. Filaments were chopped with five razor blades spaced 1.5 mm apart and the resulting cells were placed in sterile distilled water and sonicated for 5 s. They were then centrifuged, washed and sonicated for an additional 5 s prior to being recentrifuged and washed 5 times in sterile distilled water. The suspension was then sprayed onto a 1.5% agar medium containing 10 mg L^{-1} potassium tellurite and incubated at room temperature. Bacteria-free filaments, that is those that did not give rise to bacterial colonies when streaked on nutrient agar, were then transferred to agar slants.

Detergents and Phenol

Contaminated cultures of *Chlorella pyrenoidosa, Anacystis nidulans* and *Chlamydomonas* sp. were apparently freed of bacteria by centrifuging the cells with a detergent and phenol. McDaniel *et al.* (1962) added 2 drops of laboratory detergent to a 50 mL culture containing 0.3-0.8 mL of centrifuge-packed cells and shook it rigorously, prior to mixing it with a 1% phenol solution for 20 s, allowing it to stand for 15 min and centrifuging for 5 min. The supernatant was discarded and distilled water added before centrifuging again for 5 min. The cells from the pellet were then plated out on Knop's medium with 0.5% glucose and 2% agar. The authors reported a 75% success rate, although the axenicity was only tested by examining gram-stained smears.

Antifungal Agents

Polyene Macrolide Antibiotics - Polyene macrolides contain a polyene chromophore in the lactone ring and show antifungal but no antibacterial behaviour (Dekker 1969). They are especially active against yeasts and filamentous fungi. Ghosh and Ghosh (1963a) found that the growth of *Candida albicans* was completely arrested by 1 μg mL^{-1} of amphotericin B and 5 μg mL^{-1} of nystatin. Similarly, the growth of *Neurospora crassa* was reduced 50% by concentrations of 0.054, 0.20 and 0.27 μg mL^{-1} of amphotericin B, filipin, and nystatin, respectively (Kinsky 1961). Divan and Schnoes (1982) used 5.0 μg mL^{-1} amphotericin B to rid a dinoflagellate culture of a filamentous fungus. Both Kinsky (1961) and Ghosh and Ghosh (1963b) observed that cytoplasmic constituents were lost following exposure to antibiotics. This latter finding led to the hypothesis that polyene antibiotics bind to sterols in the cell membrane causing the loss of selective permeability (Kinsky 1961, 1962; Dekker 1969).

Polyene antibiotics have a broad range of antifungal activity. Using an agar dilution method, Frank *et al.* (1958-59) showed that almost all phytopathogenic fungi tested

were sensitive to nystatin, with inhibitory concentrations ranging from ≤ 0.3 - 18 μg mL^{-1} agar over a 48-120 h period. However, some species within the Phycomycetes were resistant. *Phytophthora infestans* and *Pythium debaryanum* tolerated 35 and 1420 μg mL^{-1} agar, respectively. Similarly, Vaartaja (1960) reported that both *P. debaryanum* and *Pythopthora cactorum* were resistant to individual concentrations of 500 μg mL^{-1} of pimaricin, rimocidin and nystatin. Somewhat surprisingly, these antibiotics were not very effective against fungal plant diseases in the field. The reason is apparently related to their instability after application as a result of their sensitivity to UV light. For instance, the antifungal activity is lost within a few days in aqueous solution in daylight (Dekker 1969).

Algae and protozoa both contain sterols in the protoplast membranes and may be sensitive to polyene antibiotics. Lampen and Arnow (1961) exposed eight species of algae representing four divisions (Cyanochloronta, Chlorophycophyta, Euglenophycophyta, and Chrysophycophyta) to nystatin for 72 h and found that the growth of all species except *Navicula pelliculosa* was inhibited by concentrations ranging from 1 to 30 μg mL^{-1} (Table IV). Nystatin appeared to act by disrupting the ability of the algae to maintain elevated internal K^+ concentrations. The polyene macrolide antibiotics appear to be effective against most species of fungi and yeasts tested. Nevertheless, their selective toxicities towards algae should be evaluated before employing them as fungicides in algal cultures (see below).

Caffeine - Bowne (1964) reported that caffeine at concentrations of 0.01-0.03 *M* would kill fungi and non-photosynthetic protozoa and "if anything, stimulate the algae". *Euglena* and *Chlamydomonas* cultures were freed from species of *Aspergillus, Penicillium, Saccharomyces* and *Paramecium* by caffeine in various media including pond water, pea infusion, modified Cramer-Myers salt medium and Beijerinck's mineral medium with acetate.

Griseofulvin - The sensitivity of fungi to griseofulvin varies with the species tested (Brian 1949). For example, the growth of *Microsporum gypseum* and four species of *Trichophyton* was completely arrested at 10 μg mL^{-1} griseofulvin (El-Nakeeb *et al.* 1965). The growth of *Alternaria tenuis, Cercospora melonis* and *Glomerella cingulata* was inhibited 33-65% at 10 μg mL^{-1} while *Aspergillus niger, Fusarium nivale* and *Neurospora crassa* were only marginally affected (9-20% inhibition). Yeasts and bacteria were resistant to griseofulvin (Roth *et al.* 1959; Shockman and Lampen 1962; El-Nakeeb *et al.* 1965). Data on the effects of griseofulvin on algae are few. Mizukami

Table IV Sensitivity of algal growth to nystatin (adapted from Lampen and Arnow 1961).

Division	Organism	MIC[a] of nystatin (72 h) (μg mL^{-1})
Euglenophycophyta	*Euglena gracilis* no. 752	30
	E. gracilis no. 753	30
Chlorophycophyta	*Chlorella vulgaris*	3
	Scenedesmus obliquus	1
	Chlamydomonas reinhardti	1
	Prototheca zopfii	1
Chrysophycophyta	*Ochromonas malhamensis*	1
	Navicula pelliculosa	>60[b]
Cyanochloronta	*Plectonema boryanum*	1[b]

[a] Minimum inhibiting concentrations.
[b] Incubated for 5 days.

and Wada (1983) found that apical growth in *Bryopsis plumosa* exposed to 10 μg mL^{-1} griseofulvin for two days ceased. Densely packed protoplasm at the tip disappeared and new growth points were not induced. They suggested that the morphological abnormalities were the result of microtubule inhibition. Kiermayer and Meindl (1979)

Table V Concentrations of cycloheximide completely inhibiting the growth of species representative of the Cyanochloronta, Chlorophycophyta, Xanthophyceae, Bacillariophyceae, and Euglenophycophyta after a three-week exposure.

Group	*Species*	Cycloheximide (μg mL^{-1})
Cyanochloronta	*Microcystis aeruginosa*	NI[a,e]
		NI[b,f]
		NI[c,g]
	Anabaena cylindrica	NI[a,e]
		NI[d,e]
	Aphanocapsa sp.	NI[a,e]
	Gloeothece rupestris	NI[a,e]
	Coelosphaerium kuetzingianum	NI[a,e]
	Oscillatoria tenuis	NI[a,e]
	Lyngbya sp.	NI[a,e]
	Tolypothrix tenuis	NI[a,e]
	Tolypothrix sp.	NI[a,e]
	Gloeotrichia echinulata	NI[a,e]
	Phormidium foveolarum	NI[a,e]
	Phormidium sp.	NI[d,e]
	Cylindrospermum licheniforme	NI[c,g]
Chlorophycophyta	*Ankistrodesmus falcatus*	1[a]
	Haematococcus lacustris	1[a]
		1[d]
	Scenedesmus obliquus	2[c]
		1[a]
	Raphidonema longiseta	1[a]
	Hormidium subtile	20[a]
	Chlorella variegata	NI[c,g]
	Chlorella pyrenoidosa	20[a]
	Chlamydomonas agloeformis	50[a]
		50[d]
Chrysophycophyta		
Xanthophyceae	*Tribonema aequale*	1[a]
	Polydriella helvetica	50[d]
Bacillariophyceae	*Navicula minima*	20[a]
	Navicula pelliculosa	2[d]
	Gomphonema parvulum	2[c]
	Nitzschia palea	2[c]
Euglenophycophyta	*Euglena gracilis*	100[d]

[a] Zehnder and Hughes (1958).
[b] Vance (1966)-length of exposure of *M. aeruginosa* not given.
[c] Palmer and Maloney (1955).
[d] Hunter and McVeigh (1961).
[e] Not inhibited by the maximum concentration tested (200 μg mL^{-1}).
[f] Not inhibited by the maximum concentration tested (1 μg mL^{-1}).
[g] Not inhibited by the maximum concentration tested (2 μg mL^{-1}).

did observe microtubule inhibition in *Micrasterias denticulata* exposed to 0.01-0.001% griseofulvin. Because griseofulvin is effective against a limited number of fungi and may be toxic to algae, it cannot be regarded as a general fungicide for cleaning algal cultures.

Selectivity Toxic Agents Allowing Differentiation Between Algae

Agents Used to Purify Cyanochloronta Cultures - Cycloheximide - Representative species of Cyanochloronta, Chlorophycophyta, Chrysophycophyta and Euglenophycophyta have been subjected singly to cycloheximide concentrations ranging from 1 to 1000 μg mL^{-1} over a 3-week period to determine whether selective toxicity existed (Table V). In general, the blue-green algae were unaffected by cycloheximide whereas members of the other groups had their growth rates inhibited at ≤ 100 μg mL^{-1}. The green together with the yellow-green alga and diatoms suffered arrested growth at ≤ 50 μg mL^{-1}. Berland and Maestrini (1969b) also found cycloheximide to be toxic to diatoms as five species exposed to 5 μg mL^{-1} died. *Euglena gracilis* was the only species where 100 μg mL^{-1} was necessary to inhibit growth. The growth of *Chlorella variegata* was not affected by 2 μg mL^{-1} cycloheximide, the only concentration which was tested, but *C. pyrenoidosa* was inhibited at 20 μg mL^{-1}.

Zehnder and Hughes (1958) observed the growth of *Gloeothece rupestris* over 5 weeks (Table V) and reported that at 100 and 200 μg mL^{-1} cycloheximide growth was "less vigorous" than the controls (50 μg mL^{-1} was ineffective). In another experiment, a mixed culture of eight species of blue-green and nine species of green algae was grown in cycloheximide at levels from 0-100 μg mL^{-1}. After 27 days at the highest concentration, only blue-green algae were present. Zehnder and Hughes (1958) then took samples of algae from the natural environment (e.g. waterbloom, moist soil, wet

Table VI Concentrations of nystatin completely inhibiting the growth of species representative of Cyanochloronta, Chlorophycophyta, Xanthophyceae, Bacillariophyceae, and Euglenophycophyta after a three-week exposure (adapted from Hunted and McVeigh 1961).

Group	*Species*	Nystatin (μg mL^{-1})
Cyanochloronta	*Anabaena cylindrica*	NI[a]
	Fremiyella diplosiphon	NI
	Nostoc sp.	NI
	Phormidium sp.	200
Chlorophycophyta	*Hormidium* sp.	1
	Chlorococcum minutum	1
	Ankistrodesmus falcatus	20
	Chlamydomonas agloeoformis	20
	Chlorella pyrenoidosa	50
	Stichococcus bacillaris	100
	Haematococcus lacustris	200
	Scenedesmus obliquus	NI[a]
	Goccomyxa elongata	NI
Chrysophycophyta		
Xanthophyceae	*Polydriella helvetica*	200
Bacillariophyceae	*Navicula pelliculosa*	100
Euglenophycophyta	*Euglena gracilis*	50

[a] No inhibition at highest concentration tested (200 μg mL^{-1}).

moss), and discovered that 50 μg mL^{-1} cycloheximide suppressed all of the green algae and diatoms and allowed the cyanophytes to flourish. Hunter and McVeigh (1961) took algae which had been completely inhibited by cycloheximide, but which still had apparently viable cells, and cultured them for one week after the test. No growth occurred. Therefore cycloheximide can be useful in isolating cyanobacterial cultures from algal contaminants and fungi, but not from bacteria (see above).

Nystatin - Hunter and McVeigh (1961) exposed representatives of the Cyanochloronta, Chlorophycophyta, Chrysophycophyta and Euglenophycophyta to concentrations of nystatin ranging from 1-200 μg mL^{-1} (Table VI) and found that, in general, blue-green algae were tolerant while others were not. Of the four species of Cyanochloronta tested, only *Phormidium* sp. was inhibited at 200 μg mL^{-1}. The growth of the remaining species in Table VI was completely arrested at $\leq$ 200 μg mL^{-1}, except in the cases of *Scenedesmus obliquus* and *Goccomyxa elongata*.

It would appear from a comparison of Tables V and VI that nystatin is not as effective as cycloheximide. However, in most cases its mycocidal effect would be useful to decrease the fungal contamination of blue-green algae.

Agents used to eliminate Cyanochloronta - Blue-green algae are more sensitive to streptomycin than green algae or diatoms (Foter *et al.* 1953; Palmer and Maloney 1955; Jacob and Talpasayi 1977; Harrass *et al.* 1985). Table VII shows that the minimum inhibitory concentration (MIC) for blue-greens lies in the range 0.09-2.0 μg mL^{-1}. Considering that Palmer and Maloney (1955) used only a single concentration of 2.0 μg mL^{-1} to test different algal species including *Microcystis aeruginosa*, and that Harrass et al. (1985) determined an MIC for *M. aeruginosa* of only 0.28 μg mL^{-1} (Table VII), it is likely that the range is actually smaller (0.09-0.86 μg mL^{-1}), indicating a relatively uniform sensitivity to streptomycin.

With the exception of *Chlamydomonas reinhardtii* (MIC = 0.66 μg mL^{-1}), all of the green algae tested were at least 2-3 times more resistant to streptomycin than the blue-greens, although the range of sensitivity was greater (2.0-66.0 μg mL^{-1}), indicating that no single species would be representative of the group. Similarly, the diatoms also displayed a greater, but more uniform tolerance, with MIC's from 2 to 9 μg mL^{-1} (Table VII).

The data from the different sources in Table VII agree well, with the exception of those for *Scenedesmus obliquus*. Harrass *et al.* (1985) reported an MIC of 21.0 μg mL^{-1} whereas Palmer and Maloney (1955) observed a complete inhibition of growth at 2.0 μg mL^{-1}. This difference remains unexplained. To ascertain whether streptomycin was selectively toxic, Harrass et al. (1985) set up a paired culture containing *Anabaena cylindrica* (MIC = 0.28 μg mL^{-1}) and *S. obliquus* (MIC = 21.0 μg mL^{-1}) in media containing 6.6 μg mL^{-1} streptomycin. Within 4 days growth of *A. cylindrica* had stopped, leading to a unialgal culture of *S. obliquus*.

Bacitracin was also more toxic to blue-green algae than to other algae (Hunter and McVeigh 1961). *A. cylindrica* and *Phormidium* sp. were completely inhibited by 2 μg mL^{-1} bacitracin while 50 μg mL^{-1} was necessary to arrest the growth of *Navicula pelliculosa*. Several species: *Polydriella helvetica, H. lacustris, C. agloeoformis* and *E. gracilis,* were resistant to the highest concentration tested, 200 μg mL^{-1}.

Blue-green algae have also been reported to be sensitive to neomycin (Foter et al. 1953; Jacob and Talpasayi 1977), although whether neomycin is selectively toxic is an open question. From the limited information available, blue-green algae showed the same sensitivity (6 μg mL^{-1}) as *Chlamydomonas* sp., *Gomphonema* sp., and *Nitzschia* sp. *Scenedesmus* exhibited a greater resistance as it was inhibited at 25-49 μg mL^{-1} (Foter et al. 1953). Jacob and Talpasayi (1977) provided no data, but mentioned that erythromycin was equally inhibitory, but not lethal, to 14 blue-green algae tested, and that penicillin had little effect.

Table VII Minimum inhibitory concentrations (MIC) of streptomycin that arrested the growth of representative species of Cyanochloronta, Chlorophycophyta and Chrysophycophyta.

Group	Species	MIC ($\mu g\ mL^{-1}$)	Exposure Time (days)
Cyanochloronta	*Microcystis aeruginosa*	2.0[a,d]	21
	Microcystis aeruginosa	0.28[b]	35
	Lyngbya sp.	0.09[b]	35
	Oscillatoria tenuis	0.28[b]	35
	Anabaena cylindrica	0.28[b]	35
	Anabaena flos-aquae	0.28[b]	35
	Aphanizomenon flos-aquae	0.86[b]	35
	Nostoc sp.	<2.0[c]	30
	Phormidium sp.	<2.0[c]	30
	Cylindrospermum licheniforme	2.0[a]	21
Chlorophycophyta	*Chlamydomonas reinhardtii*	0.66[b]	35
	Chlamydomonas sp.	9-18[c]	30
	Pediastrum sp.	2.1[b]	35
	Selenastrum capricornutum	2.1[b]	35
	Ankistrodesmus sp.	6.6[b]	35
	Stigeoclonium sp.	6.6[b]	35
	Scenedesmus obliquus	2.0[a]	21
	Scenedesmus obliquus	21.0[b]	35
	Scenedesmus sp.	18-36[c]	30
	Ulothrix sp.	21.0[b]	35
	Chlorella vulgaris	66.0[b]	35
	Chlorella variegata	NI[a,e]	21
Chrysophycophyta	*Navicula* sp.	6.6[b]	35
	Gomphonema parvulum	NI[a,e]	21
	Gomphonema sp.	4-9[c]	30
	Nitzschia sp.	2-4[c]	30
	Nitzschia palea	2.0[a]	21

[a] Palmer and Maloney (1955).
[b] Harrass et al. (1985).
[c] Foter et al. (1953)
[d] 2.0 $\mu g\ mL^{-1}$ only concentration tested.
[e] Not inhibited by 2.0 $\mu g\ mL^{-1}$.

It appears that streptomycin and bacitracin would be the antibiotics of choice to eliminate blue-green contaminants from other algal cultures.

Agents Used to Eliminate Bacilliarophyceae

Germanium Dioxide - Lewin (1966) examined the selective toxicity of germanium compounds against a mixed culture containing blue-green, green, brown, and red algae. These were inoculated into artificial seawater with concentrations of germanium dioxide (GeO_2) ranging from 0-100 mg L^{-1}. After one week the suspensions were streaked onto agar plates. No diatoms were found from cultures containing $\geq$3.0 mg L^{-1} GeO_2. In a further experiment with pure cultures of diatoms (2 species of *Amphipora* and *Nitzschia*, 4 strains of *Cylindrotheca closterium*, 2 strains of *Phaeodactylum tricornutum, Navicula incerta, Skeletonema costatum, Cyclotella cryptica*) exposed to 1, 3, and 10 mg L^{-1} GeO_2 for 12 days, the average growth rate was inhibited 42, 67, and 83%, respectively. Lewin (1966) postulated that GeO_2 could take part in reactions similar to those of SiO_2, with germanium replacing silicon in the final

mineralized shell. She recommended concentrations of 1-10 mg L^{-1} GeO_2 to rid algal cultures of diatoms.

Borden and Stein (1969) used 6-10 mg L^{-1} GeO_2 to control diatoms in cultures of the green alga, *Codium fragile*. Similarly, Chen *et al.* (1969; 1970) used GeO_2 to eliminate diatoms from cultures of red algae. However, problems with selective toxicity of GeO_2 towards brown algae emerged. McLachlan *et al.* (1971) noted that with 4 species of *Fucus*, an exposure of several days to 5-10 mg L^{-1} GeO_2 resulted in apical necrosis of embryos and mature thalli even though the diatoms were not suppressed. Tatewaki and Mizuno (1979) reported that 2.5-5.0 mg L^{-1} GeO_2 inhibited the growth of all brown algae examined but had no effect on that of the green and red algae tested.

Markham and Hagmeier (1982), after observing that a *Laminaria saccharina* culture stopped growing suddenly after a routine addition of GeO_2 to control diatoms, proceeded to test diatoms and macroalgae for their susceptibility to GeO_2. Diatoms were subjected to 0.009-1.79 mg L^{-1} GeO_2 for 4-9 days at 10 and 18°C. *Chaetoceros didymus* and *Rhizosolenia delicatula* were strongly inhibited by 0.045 and 0.134 mg L^{-1} GeO_2, respectively. *Thalassiosira rotula* displayed a 50% growth inhibition at 0.18 mg L^{-1} GeO_2. *Coscinodiscus concinnus* was unaffected. The effects on growth were independent of temperature in the range studied.

In a 6-day test, GeO_2 at concentrations ranging from 0.22-8.95 mg L^{-1} had no adverse effects on the red algae: *Polysiphonia urceolata, Porphyra umbilicalis,* and *Chondrus crispus.* The only green species tested, *Ulva lactuca,* exhibited a decline in % diameter increase d^{-1} (7.4% compared with 9.0% for the controls) at the maximum concentration used. With regard to brown algae, the specific growth rate (% length increase d^{-1}) of *Fucus spiralis* decreased from 4.1 to 3.2% at 8.95 mg L^{-1} GeO_2, but the most significant effect was observed for *Laminaria saccharina* where GeO_2 at 3.5 and 8.95 mg L^{-1} resulted in a growth inhibition of 50 and 67%, respectively. Although the latter species appeared healthy, the fronds were brittle, as they broke into pieces when handled. Germanium is reported to interfere with silicon metabolism, blocking the initial uptake of silicate and inhibiting the metabolism of that already accumulated, thereby preventing cell division by arresting wall formation (Markham and Hagmeier 1982). Parker (1969) noted that silicon was present in some brown algae, thus the lack of silicon may have led to the observed brittleness of the *Laminaria* thallus. Darley and Volcani (1969) reported that the molar ratio of Ge to Si is important in the mode of action of GeO_2. Using the test organism *Cylindrotheca fusiformis,* they showed that wall formation was inhibited at Ge/Si ratios of 0.1 and 0.2. However, at a Ge/Si ratio of 0.5, DNA synthesis and growth of the diatom were inhibited. Markham and Hagmeier (1982) suggested that lower levels of GeO_2 be used with brown algae, in the range of 0.045-0.179 mg L^{-1} as this appears to be "safe" for them, while still eliminating most diatoms.

Algal Metabolites Affecting Other Algae and Microrganisms

Many algae excrete substances which affect other algal species as well as bacteria and fungi. Juttner (1979) found that an excretion product of *Cyanidium caldarium,* geranylacetone, at a concentration of 20 mg mL^{-1} almost totally inhibited the carotenoid synthesis of *Synechococcus* 6911. Jorgensen (1956) reported that algal metabolites from one species could influence the growth of another. For example, *Chlorella pyrenoidosa* cultured separately in 21-day filtrates of *Scenedesmus quadricauda* and *Nitzschia palea* exhibited an inhibition of growth with the former and an acceleration of growth in the latter. *S. quadricauda* grew more quickly in 21-day filtrates of both *C. pyrenoidosa* and *N. palea.*

Some of the antimicrobial and antifungal algal metabolites are listed in Table VIII. Unfortunately, most of the test organisms employed to ascertain their activity are not

Table VIII The antimicrobial effects of algal metabolites.

Group	*Species*	Metabolite	Organism Inhibited
Cyanochloronta	*Lyngbya majuscula*	δ-lactone	*Mycobacterium smegmatis*[a]
		δ-lactone	*Streptococcus pyogenes*[a]
		-	*Candida albicans*[b]
		-	*Penicillium* sp.[b]
		-	*Rhizopus oryzae*[b]
		-	*Cryptococcus neoformans*[b]
Chlorophycophyta	*Caulerpa racemosa*	-	*Candida albicans*[b]
			Penicillium sp.[b]
			Rhizopus oryzae[b]
			Cryptococcus neoformans[b]
	Halimeda sp.	trialdehyde	marine bacteria & fungi[a]
		halimedatrial	marine bacteria & fungi[a]
Phaeophycophyta	*Dictyota indica*	-	*Candida albicans*[b]
			Penicillium sp.[b]
			Rhizopus oryzae[b]
			Cryptococcus neoformans[b]
	Dictyopteris zonaroides	zonarol, isozonarol	*Phytophthora cinnamomi*[b]
			Rhizoctonia solani[c]
			Sclerotinia scleratiorum[c]
			Sclerotium rolfii[c]
	Dictyopteris undulata	farnesylhydroquinone	antimicrobial[a]
		yakuzanol	
	Sargassum kjellmanianum	kjellmanianone	antibacterial[a]

Rhodophycophyta	*Laurencia obtusa*	-	*Candida albicans*[b] *Penicillium* sp.[b] *Rhizopus oryzae*[b] *Cryptococcus neoformans*[b]
	Laurencia yamada	chondriol	antiviral & antimicrobial[c]
	Laurencia brongniaratii	brominated indoles	antimicrobial[a]
	Laurencia sp.	laurinterol debromolaurinterol	*Staphlococcus aureus*[c] *Mycobacterium smegmatis*[c] *Candida albicans*[c]
	Chondria oppositiclada	cycloeudesmol	*Staphlococcus aureus*[c] *Mycobacterium smegmatis*[c] *Candida albicans*[c]
	Chondria californica	sulphone	*Vibrio anguilarum*[c] *Proteus mirabilis*[c] *Salmonella typhimurium*[c] *Escherichia coli*[c]

[a] Faulkner 1984.
[b] Accorinti 1983.
[c] Faulkner 1978.

normally found as algal epiphytes. The blue-green alga, *Lyngbya majuscula*, produces substances effective against both bacteria (*Mycobacterium smegmatis, Streptococcus pyogenes*) and fungi (*Candida albicans, Penicillium* sp., *Rhizopus oryzae*, and *Cryptococcus neoformans*). It also secretes inflammatory toxins lyngbyatoxin A and debromoaplysiatoxin which cause contact dermatitis known as "swimmers itch" (Moore 1981).

The antifungal metabolites are widespread as they are found in green (*Caulerpa racemosa, Halimeda* sp.), brown (*Dictyota indica, Dictyopteris zonaroides*) and red algae (*Laurencia* sp., *L. obtusa, Chondria oppositiclada*) (Table VIII). Accorinti (1983) speculated that the inhibitory effects on fungi could be due to polyene antibiotics (see above). The antimicrobial activity of many marine algae appears to be due to the production of brominated phenols and sesquiterpene phenols (Faulkner 1978). For example, laurinterol and debromolaurinterol isolated from several species of *Laurencia* and cycloeudesmol from *Chondria oppositiclada* inhibited *Staphlococcus aureus, M. smegmatis* and *C. albicans* (Table VIII). Sulfur-containing compounds such as sulphone from *Chondria oppositiclada* have also shown antimicrobial activity (Table VIII). These are some of the algal metabolites exhibiting antimicrobial and antifungal behaviour. As more natural products are isolated, it is probable that more compounds active against bacteria and fungi will be discovered.

The techniques reviewed here to purify algal cultures show that there is no single method applicable for every species. It does not appear to date that antimicrobial and antifungal products from the algae themselves have been used to rid cultures of epiphytic contamination. Different algae will in all probability be contaminated with a wide spectrum of bacteria and, to a lesser extent, fungi and other algae, each of which will differ in sensitivity to the various chemical agents used to eliminate them. A mixture of chemicals, rather than a single agent, has been necessary to prepare axenic cultures. In addition, closely related species of algae may exhibit quite different responses to the same chemical. It will therefore be necessary to experiment with different types and concentrations of chemicals, keeping in mind that they must be selectively toxic. The techniques gathered here provide the reader with background information and may suggest useful approaches for the isolation of algae into pure culture.

Acknowledgements

I thank Drs. J.S. Craigie and J. van der Meer for reviewing the manuscript and offering helpful suggestions.

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(Received 19 May 1989).