IV. 培地作製，継代培養，凍結保存の方法

1. ストック液と培地の作り方

1.1. ストック液

培地は一般に多量栄養素，微量金属，およびビタミン類から成る。これらの諸成分のストック液を作製しておくと，培地作製が簡便になる。これらのうち微量金属やビタミン類のストック液の濃度は非常に低いので，まず，秤量しやすい，より高濃度の液を作製し，それを順次希釈してストック液を作製する必要がある。以下，各々についてストック液の濃度と作り方について述べる。

1.1.1 多量栄養素

各栄養素につき，10 mg/mL の濃度のストック液を別々に作製し，冷蔵庫（5°C）で保管する。

1.1.2. 微量金属

各種のストック液として別々に作製され保管される場合と，いくつかの金属溶液を混合した混液で保管される場合がある。

1.1.2.1. 各種ストック液

1 ～ 10 mg/mL の濃度で各種金属のストック液を作製し，冷蔵庫（5°C）に保管する。

1.1.2.2. 混合ストック液

i) 1 ～ 10 mg/mL の濃度で各種金属液を作製する。
ii) 必要量の 80% の蒸留水をビーカーに加える。
iii) 十分に撹拌しながら各種金属液を必要量添加する。
iv) 蒸留水を加え，最終量に調整し，冷蔵庫（5°C）に保管する。

1.1.3. ビタミン類

ビタミン B_{12}，ビオチン，チアミンの 3 種のビタミンだけで多くの藻類が増殖するので，殆どの培地はこれら 3 種のビタミン類だけが添加されている。しかし，培地によっては，他のビタミン類が添加されている場合もある。

1.1.3.1. ビタミン B_{12}，ビオチン，チアミン

i) ビタミン B_{12} とビオチンについては，各々 100 µg/mL の原液，チアミンについては 10 mg/mL の原液を作製し，それぞれ 1 mL ずつ小分けして –20°C のフリーザーに保管する。
ii) 各ビタミンについて，保存原液の 1 mL を融解し，蒸留水で 1/100 に希釈し，ビタミン B_{12} とビオチンについては 1 µg/mL，チアミンについては 100 µg/mL のストック液を作製し，冷蔵庫に保管しながら使用する。

1.1.3.2. ビタミン類混液

培地によっては，多種のビタミン類が混液の形で添加される場合がある。大量に作製しておくとよい。

i) 各種のビタミンについて 0.1 ～ 1 mg/mL の原液を作製する。
ii) 必要量の 80% の蒸留水をビーカーに加える。
iii) 十分に撹拌しながら各種ビタミンを必要量加える。
iv) 蒸留水で最終量に調整し，10 mL ずつ小分けし，使用する分は冷蔵庫（5°C）に，使用しない分は –20°C のフリーザーに保存する。

1.2. 培地

培地は，合成培地と強化培地に大別される。すべての淡水藻類や一部の海産藻類は合成培地で，ほとんどの海産藻類は強化培地で保存されている。ほとんどの培地は，試験管等に分注した後オートクレーブ滅菌して使用するが，濾過滅菌しなければならない培地もある。

1.2.1. 淡水藻類用合成培地

i) 必要量の 80 ～ 90% の蒸留水をビーカーに加える。
ii) Tris, glycylglycine, HEPES, TAPS, Bicine, MES 等の緩衝剤（必要とされる場合）を必要量天秤で秤量し，十分に撹拌しながら添加する。
iii) 各種栄養塩を各々のストック液から必要量添加する。
iv) 蒸留水で最終量に調整する。
v) 緩衝剤が使用されている場合は，1 mol/L HCl あるいは 1 mol/L NaOH で，使用されていない場合は各々 1/10 の濃度で pH を調整する。
vi) 培地 10 mL ずつを試験管 (18 × 150 mm) に分注し，オートクレーブで滅菌する（121°C，20 min）。

1.2.2. 海産藻類用合成培地

i) 必要量の 80% の蒸留水をビーカーに加える。
ii) 十分に撹拌しながら，緩衝剤（Tris, NTA 等）および多量栄養塩類（NaCl, $MgSO_4 \cdot 7H_2O$, KCl, $CaCl_2 \cdot 2H_2O$）を必要量天秤で秤量し，添加する。
iii) 他の各種栄養塩を各々のストック液から必要量添加する。
iv) 蒸留水で最終量に調整する。
v) 1 mol/L HCl で pH を調整する（通常 8.0）。
vi) 培地 10 mL ずつを試験管に分注し，オートクレーブで滅菌する（121°C，20 min）。

1.2.3. 海産藻類用栄養塩強化培地

i) 汚染のない外洋海水を採取し，ワットマン GF/C フィルターでろ過し，粒子を除く。通常の外洋海水の塩分

は約 35‰である。
ii) 必要量の 80 ～ 90% の海水をビーカーに加える。
iii) 必要量の Tris 等の緩衝剤を天秤で秤量し（必要とされる場合），攪拌しながら溶解する。
iv) 他の栄養塩類を各々のストック液から必要量添加する。
v) 海水で最終量に調整する。
vi) pH を測定する。指示されている場合は 1 mol/L HCl で調整する (通常 8.0)。
vii) 培地 10 mL ずつを試験管に分注し，オートクレーブで滅菌する（121°C，20 min)。

1.2.4. 濾過滅菌

MNK 培地は濾過滅菌をして使用している。オートクレーブ滅菌（121°C, 20 min）したフィルターセット（ミリポアフィルター 0.22 μm）を用いて濾過滅菌する。濾過滅菌された培地は，滅菌シリンジや滅菌した分注器を用いて，あらかじめ滅菌された試験管に 10 mL ずつ分注する。分注は無菌室で行う。

1.2.5. 寒天斜面培地

通常寒天は 1.5% の濃度で滅菌する前に液体培地に加える。
i) 寒天を必要量天秤で秤量し，液体培地に添加し，オートクレーブまたはホットプレートで熱し，溶解する。
ii) 溶解後，速やかに 10 mL ずつ試験管に分注し，オートクレーブで滅菌する（121°C，20 min)。
iii) 滅菌後，試験管上部に直径 1 cm の枕木をして寝かせ，放冷して培地を斜面状に固める。

1.2.6. 原生動物用培地

培地には，餌となるバクテリアを増殖させるための有機物が含まれている。穀類を添加する培地は，予め，小麦や米をシャーレなどに入れ，乾熱滅菌（150°C，30 min）し，冷蔵保存したものを，使用直前に液体培地 10 mL に対して 1 粒添加する。

1.2.7. シャジクモ類用培地

1.2.7.1. 培養土

本施設では，培養土に用いる黒土，川砂，腐葉土，苦土石灰は園芸店で購入しているが，水田や池等の底泥は独自に採取している。土質によって株の生育に多少の差が生じる。使用する培養土の種類は，各保存株データおよび培地リストに示した。

1.2.7.2. 水

培地に加える水は，通常，脱イオン水（または蒸留水）を用いるが，汽水産の株の場合は 1/3 Herbst 人工海水を，脱イオン水でさらに 1/3 ～ 1/2 に希釈して使用する。

1.2.7.3. 培地作製

i) 容器の底から 1/4 ～ 1/5 まで土を入れる。
ii) 脱イオン水（または蒸留水）で土を湿らす。
iii) 容器にゆるく蓋をし，オートクレーブで 2 回滅菌する (121°C, 20 min)。その際，1 回目のオートクレーブ後，一晩放置し再度オートクレーブする。
iv) 冷めたら，オートクレーブ滅菌した脱イオン水（または蒸留水）を静かに加える。単藻株用の培地では，土に水を注ぐ操作をクリーンベンチ内で行う。

1.2.7.4. 二酸化ゲルマニウム溶液の作製法

保存されているシャジクモ類株の多くは単藻化されていない。したがって，本施設では混在する藻類，特に珪藻の増殖を抑えるために以下に示す方法で二酸化ゲルマニウム溶液（濃度 1 mg/L）を作製し，培地に添加している。
i) 1 mol/L の水酸化ナトリウム溶液 200 mL を沸騰させる。
ii) 突沸させないよう気をつけて二酸化ゲルマニウム 0.5 g を添加する。
iii) 溶液を室温まで冷ます。
iv) 1 mol/L の塩酸で pH を調整する（7.8 ～ 8.0)。
v) 蒸留水を加えて 500 mL にする。
vi) オートクレーブで滅菌し（121°C，20 min)，冷えたら冷蔵庫で保存する。

2. 培地リスト

Media list (p. 214 ～ 227）参照。

3. 継代培養の方法

3.1. 微細藻類，原生動物，淡水産紅藻

株は，ねじ口試験管に培養された状態で送付される。株を受け取ったら，キャップを緩め，保存株データに示された培養条件に合った適当な場所に保管する。株を維持するには，以下の方法で植え継ぎ，培養を行う。なお，培地は株を受け取る前に作製しておく。

i) 植え継ぐ前に培地を培養条件と同じ温度にする。
ii) 無菌操作にて，適量の細胞懸濁液を新鮮な培地に植え継ぐ。本施設では，予め滅菌した綿栓ピペットを用いて接種している（口絵プレート 7-1, 2)。細胞が沈殿する株や容器に付着する株ではピペッティングによって懸濁させてから培養液を取り出す。但し，細胞壁を持たない細胞では，ピペッティングによって細胞が壊れるので, 攪拌せず, 細胞の多いところを静かに吸い取る。接種する細胞懸濁液の量は, 藻類の種や株の状態によって異なり，10 mL の培地に，よく増殖した培養では 1, 2 滴と少量でよいが，細胞が大きく細胞密度が低い場合は 4，5 滴と多めに接種する。寒天培地の場合は，ガ

スバーナーで滅菌した白金耳で細胞塊を掻き取り，新鮮な寒天培地の上になすり付ける。

iii) 保存株情報で指定された温度と光条件下で培養し（口絵プレート 7-3, 4），指定された期間毎に，新鮮な培地に植え継ぐ。明暗周期は 12 時間明期 12 時間暗期とし，ねじ口試験管のねじ蓋をゆるくする。

iv) 本施設では，1 週間毎に，目視または顕微鏡で生育状況を確認している。生育が悪い場合は，再度植え継ぎ，培地や培養条件を検討している。

原生動物株では，以下の点に留意する。

i) 植え継ぐ際に，培地に穀類，餌となる生物などを接種する場合がある（口絵プレート 7-5）。または，事前に餌となる生物を接種する場合がある。

ii) 餌として藻類を接種した場合を除き，培養に光を必要としない。

iii) 培養容器に付着する性質をもつ株では，植え継ぎの際にピペッティングする必要がある。

3.2. シャジクモ類

シャジクモ類株は，藻体の一部を切り取った状態で送付される。株を受け取ったら，速やかに以下の方法に従って藻体を新鮮な培地へ植え込む。

i) 培地は株を受け取る前に作製しておく。その際，濃度 1 mg/L の二酸化ゲルマニウム溶液を 1 ～ 2 mL 添加しておく（900 mL マヨネーズ瓶の場合。単藻株では不要。）。

ii) 培養土へ竹串またはピンセットを用いて，藻体をやさしく植え込む（口絵プレート 7-6）。この時，節部が必ず 1 つは培養土の中に埋め込まれた状態とする。なお，シラタマモ属（*Lamprothamnium*）は仮根部の球状体を，ホシツリモ属（*Nitellopsis*）は星状体を土に埋め込む。

iii) 保存株情報に示された温度と光条件下で培養する。株は新鮮な培地へ植え込んでから約 2 週間後に新たに生長を開始する（極端な高温，低温でなければ，実験室などの直射日光の当たらない，明るい窓辺でも培養できる）。

iv) 良好な生長が確認された後に，更に株を継代培養する場合は，下記の方法に従って，保存株情報に示された期間毎に新鮮な培地に移植する必要がある。

a) 生長した藻体の上端から 3 ～ 4 節を，ハサミまたはピンセットを用いて切り取る（口絵プレート 7-7）。

b) 絵筆を用いて，切り取った藻体表面に付着している他の藻類を取り除き（口絵プレート 7-8），よくすすぐ（単藻株では不要）。

c) 二酸化ゲルマニウム溶液を添加した新鮮な培地へ，ii) と同様に植え込む。

4. 凍結保存法

NIES コレクションでの凍結保存は，プログラムフリーザーを用いて徐々に –40°C まで下げた後，液体窒素で –196°C まで急速凍結させる二段階凍結法を用いて行っている。シアノバクテリアの多くの株，緑藻と単細胞性紅藻の一部の株，および大型の淡水産紅藻について，現在本施設で採用している凍結方法の概要を紹介する。また，微細藻類の凍結法については Mori *et al.* (2002) および森 (2007) に詳細な説明がある。

引用文献

Mori, F., Erata, M. & Watanabe, M. M. 2002. Cryopreservation of cyanobacteria and green algae in the NIES-Collection. Microbiol. Cult. Coll. 18: 45-55.

森史 2007. 微細藻類の凍結保存法. 日本微生物資源学会誌 23: 89-93.

4.1. 微細藻類の凍結保存

4.1.1. 準備するもの

i) 細胞懸濁液：対数増殖期終期から定常期初期の細胞。

ii) 培地：通常当該株の培養に用いている，滅菌済みの培地。

iii) 凍結保護剤：シアノバクテリアの凍結保存には，適当な培地で希釈した 6% ジメチルスルホキシド（DMSO）を用意する。緑藻および紅藻には 10%DMSO を用いる。これらは最終濃度の 2 倍の濃度である。DMSO は Millex-LG フィルターで濾過滅菌しておく。

iv) 器具および機器

① クリーンベンチおよび無菌操作に必要な器具類。

② 2 mL クライオチューブ：あらかじめ滅菌済みのものを使用する。チューブには株番号等，必要事項をラベルしておく。

③ プログラムフリーザー：NIES コレクションでは Planer Kryo 320-1.7 を使用している。

④ デュワー瓶：10 L シャトルドラム JIK-S10 を使用している。

⑤ 大きめのピンセット（19 cm），クライオ手袋，クライオエプロン，ゴーグル。

⑥ 箱，ラック，液体窒素槽：NIES コレクションでは Nunc ポリカーボネート製ストレージボックス，8 段ステンレスラック，大陽日酸 DR-245LM を使用している。

⑦ 凍結保存容器：DR-245LM（大陽日酸）の液体窒素槽を使用している。

⑧ 恒温槽：サーマルロボ TR-1 を使用している。

4.1.2. 凍結手順

i) 滅菌した器具を用い，ii) から v) の操作はクリーンベンチで行う。

ii) 滅菌した培地で最終濃度の2倍になるよう希釈した凍結保護剤を，氷上で冷やしておく。
iii) あらかじめ株番号等をラベルした2 mLクライオチューブに細胞懸濁液（対数期終期から定常期初期の細胞）0.5 mLを分注する。
iv) 冷やしてあった凍結保護剤0.5 mLを加え，クライオチューブを振って混合する。
v) 室温に15分間静置する。
vi) プログラムフリーザーにクライオチューブをセットし，毎分–1℃の冷却速度で–40℃まで冷却する（口絵プレート7-9）。
vii) プログラムフリーザー内（–40℃）で15分間保持する。
viii) クライオチューブをプログラムフリーザーから速やかに取り出し，デュワー瓶に入れた液体窒素中に投入する（口絵プレート7-10）。
ix) 1時間後，クライオチューブをストレージボックスに詰めてラックに収納し，液体窒素保存槽（気相）内に保管する（口絵プレート7-11）。

4.1.3. 解凍手順

i) 恒温槽を40℃に設定し，準備しておく。
ii) 恒温槽中にて，クライオチューブ内の氷晶が完全に消えるまで手でよく振り，融解する（口絵プレート7-12）。
iii) クリーンベンチで，解凍した細胞懸濁液を新しい液体培地の入った試験管に移してよく攪拌し，通常培養より暗めの光条件で数日間培養し（株によって異なる），その後通常の培養条件に移す。

4.2. 淡水産紅藻の凍結保存

4.2.1. 準備するもの

i) 細胞培養液：植え替え後2週間以上経った藻体。但し藻体が大きい場合は，ピンセットおよびハサミを使って細かく裁断し，2週間以上培養する。
ii) 培地：Bold 3N培地。
iii) 凍結保護剤：チスジノリ（*Thorea okadae*），フトチスジノリ（*T. hispida*）およびオキチモズク（*Nemalionopsis tortuosa*）には40% DMSOを用い，オキチモズクには30%メタノールも使用する。これらは最終濃度の2倍の濃度である。DMSOおよびメタノールはMillex-LGフィルターで濾過滅菌し，滅菌したBold 3Nで希釈してある。
iv) 器具および機器：微細藻類の場合と同様。

4.2.2. 凍結手順

i) 滅菌した器具を用い，ii)からiv)の操作はクリーンベンチで行う。
ii) Bold 3N培地でDMSOを40%，メタノールを30%に希釈し，氷上で冷やしておく。
iii) 2 mLクライオチューブに細胞培養液0.8 mLを分注する。
iv) iii)へ40%DMSOまたは30%メタノールを0.8 mLずつ加え，クライオチューブを振って混合する。DMSOを加えた場合は，室温に15分間静置する。
v) 4.1.2. vi)からix)と同じ手順で凍結する。

4.2.3. 解凍手順

i) 恒温槽を40℃に設定し，培地を氷水で冷やしておく。
ii) クライオチューブを速やかに恒温槽へ入れ，手でよくチューブを振る（口絵プレート7-12）。クライオチューブ内の氷晶が完全に消える寸前に氷水へ移す。
iii) クリーンベンチで，直ちにチューブ内の細胞懸濁液を50 mL遠心管に移し，氷水で冷やした新しい培地40 mLを加え，静置する。
iv) 上澄み液をピペットで完全に取り除く。
v) 再び新しい培地を40 mL加え，静置し，上澄み液をピペットで完全に取り除く。
vi) 藻体を新しい液体培地の入った三角フラスコに移し，通常の培養条件で培養する。

IV. MEDIA PREPARATION, SUBCULTURE AND CRYOPRESERVATION

1. How to prepare stock solutions and media

1.1. Stock solutions

Media are generally composed of three types of components; macronutrients, trace metals, and vitamins. For convenience we recommend to prepare stock solutions of these components. Stock solutions of trace metals and vitamins are prepared at extremely low concentrations, and therefore required dilution steps. The following methods are currently used at the NIES-Collection.

1.1.1. Macronutrients

Prepare stock solutions of individual macronutrients separately at a concentration of 10 mg/mL, and store them in a refrigerator (5°C).

1.1.2. Trace metals

These elements are prepared as either separate stock solutions or mixed stock solutions.

1.1.2.1. Separate stock solutions

Prepare stock solutions of individual metals separately at concentrations of 1–10 mg/mL, and store in a refrigerator (5°C).

1.1.2.2. Mixed stock solution

i) Prepare each metal solution as for the separate stock solutions shown in 1.1.2.1
ii) Add approximately 80% of the final volume of distilled water in a beaker.
iii) First, dissolve the required amount of Na_2EDTA, while stirring, if applicable.
iv) Add the required volume of each trace metal solution one at a time, while stirring.
v) Adjust to the final volume by adding distilled water, and store in a refrigerator (5°C).

1.1.3. Vitamins

Vitamins requirement is in majority fulfilled with three vitamins; vitamin B_{12}, biotin, and thiamine HCl. Therefore, most of the media contain only these three vitamins. However, several media contain additional vitamins.

1.1.3.1. Vitamin B_{12}, biotin, and thiamine HCl

i) Prepare 0.1 mg/mL solutions of vitamin B_{12} and biotin and a 10 mg/mL solution of thiamine HCl. Disperse 1 mL of each solution into a separate micro-tube, and store in a freezer at –20°C.
ii) Thaw and dilute the vitamin solution to 1/100 to prepare stock solutions of 1 μg/mL vitamin B_{12} or biotin, and a stock solution of 100 μg/mL thiamine HCl. Store in a refrigerator (5°C).

1.1.3.2. Other vitamins

Additional vitamins are added to some media as a mixture. We recommend to prepare a large volume of mixed stock solutions at once.

i) Prepare each vitamin solution at concentrations of 0.1–1.0 mg/mL. (Store these original solutions in a freezer at –20°C, if needed.)
ii) Add approximately 80% of the required volume of distilled water in a beaker.
iii) Add the required volume of each vitamin solution one at a time, while stirring.
iv) Adjust to the final volume by adding distilled water.
v) Dispense 10 mL of the vitamin mixture into several vessels, and store in a refrigerator (5°C) for use or in a freezer (–20°C) for storage.

1.2. Media preparation

Two categories of media are usually used; synthetic and enriched. The former is used for maintenance of all freshwater algal cultures and some marine ones and the latter for most marine ones. Most of the media are dispensed to test tubes and autoclaved before use, whereas some media should be filter sterilized.

1.2.1. Synthetic medium for freshwater algae

i) Add approximately 80–90% of the required volume of distilled water to a beaker.
ii) Dissolve appropriate quantities of buffers such as Tris (hydroxymethyl) aminomethane (known as Tris), glycylglycine, HEPES, TAPS, Bicine, or MES (if required), while stirring.
iii) Add the appropriate nutrients from previously prepared stock solutions, while stirring.
iv) Adjust to the final volume by adding distilled water.
v) Check and adjust pH as specified in the media list with either 1 mol/L HCl or 1 mol/L NaOH (if buffers are used) or with either 0.1 mol/L HCl or 0.1 mol/L NaOH (if no buffers are used).
vi) Dispense 10 mL of medium into each test tube (18

× 150mm) and sterilize by autoclaving (121°C, 20 min).

1.2.2. Synthetic medium for marine algae

i) Add approximately 80% of the required volume of distilled water to a beaker.
ii) Dissolve appropriate quantities of Tris, nitrilotriacetic acid (known as NTA) and major salts such as NaCl, $MgSO_4 \cdot 7H_2O$, KCl and $CaCl_2 \cdot 2H_2O$, while stirring.
iii) Add the other nutrients from previously prepared stock solutions.
iv) Adjust to the final volume by adding distilled water.
v) Check and adjust pH with 1mol/L HCl, if pH is specified in the media list. (usually pH 8.0)
vi) Dispense 10 mL of medium into each test tube and sterilize by autoclaving (121°C, 20 min).

1.2.3. Enriched seawater medium

i) Collect offshore seawater free from pollution, and remove particulate matter by filtering through Whatman GF/C filters.
ii) Check salinity. (Usually salinity of offshore seawater is 35‰)
iii) Add approximately 80–90% of the required volume of seawater to a beaker.
iv) Dissolve appropriate quantities of Tris (if required).
v) Add the appropriate nutrients from previously prepared stock solutions.
vi) Adjust to the final volume by adding the filtered seawater.
vii) Check and adjust the pH to 8.0 with 1 mol/L HCl if required.
viii) Dispense 10 mL of medium into each test tube and sterilize by autoclaving (121°C, 20 min).

1.2.4. Filter sterilization

MNK medium should be filter sterilized by using a filter apparatus with a filter (Millipore 0.22 μm), which is previously autoclaved (121°C, 20 min). Then, the medium is dispensed into previously sterilized test tubes by using a sterilized syringe or dispenser under aseptic conditions.

1.2.5. Agar slants

Agar is usually added at a concentration of 1.5% after liquid medium has been prepared, and before autoclaving.

i) Add the appropriate quantities of agar to the liquid medium and heat by autoclaving or on a hot plate.
ii) After melting, quickly dispense 10 mL of agar medium into each test tube and sterilize by autoclaving (121°C, 20 min).
iii) After sterilization, lay the test tubes down with the upper part of the tubes elevated on a rod (1 cm ϕ), and cool to form agar slants.

1.2.6. Medium for protozoa

These media contain organic matter to encourage multiplication of bacteria as a food source for protozoa. For media containing wheat or rice grains, these cereals should be sterilized by dry heat (150°C, 30 min) in advance, and kept in a cool place. For use, one grain of cereal is added to 10 mL of medium.

1.2.7. Medium for Charales

1.2.7.1. Soils

Black soil, river sand, leaf mould, and garden lime used in the NIES-Collection are purchased from garden centers, whereas bottom mud from paddy fields, reservoirs, and ponds is collected by us. Soil quality influences the growth of Charales to a greater or lesser degree. Please refer to the media list and individual strain data for soil composition.

1.2.7.2. Water

Freshwater strains: Deionized water (or distilled water).

Brackish water strains: one-third to one-half diluted 1/3 Herbst ASW, i.e. the original medium is diluted to one-third to one-half with deionized water (or distilled water).

1.2.7.3. Soil water medium

i) Put appropriate soil into a glass vessel up to one-quarter to one-fifth.
ii) Dampen the soil with deionized water (or distilled water).
iii) Cover the glass vessel with a plastic cap or aluminum foil, and autoclave it twice (121°C, 20 min, overnight cooling down, and again 121°C, 20 min).
iv) After the vessel has cooled down to room temperature, pour sterilized water (see 1.2.7.2 Water) into the glass vessel carefully (do not disturb the soil). When you make media for unialgal strains, use a clean bench (or a clean room) for this process.

1.2.7.4. Germanium dioxide solution

Germanium dioxide solution especially discourages the growth of diatoms. To suppress the growth of undesired diatoms, add germanium dioxide solution (1 mg/L GeO_2) to the media.

i) Boil 200 mL NaOH solution (1 mol/L).
ii) Add 0.5 g GeO_2 to the boiling NaOH solution very

carefully.

iii) Cool down to room temperature.

iv) Check the pH and adjust to 7.8–8.0 with 1 mol/L HCl.

v) Adjust to 500 mL by adding deionized water (or distilled water).

vi) Autoclave (121°C, 20 min).

2. Media list (培地リスト)

2.1. Media for freshwater, terrestrial, hot spring and salt water algae

(淡水産, 陸生, 温泉産, 塩水産藻類用培地)

Media indicated with asterisks (*) are available for distribution. Reference numbers are shown in parentheses after medium names.

(* 分譲可能な培地を示す。培地に関する文献番号を培地名の後のカッコ内に示した。)

1. AAF-6*

Prepare as for AF-6[1] medium but adjust to pH 5.5–5.8.

1) See AF-6

2. Acid-CSi/5*

Dilute CSi medium with distilled water to one-fifth. Adjust to pH 3 with sulfuric acid.

3. AF-6* (338)

$NaNO_3$	14	mg
NH_4NO_3	2.2	mg
$MgSO_4 \cdot 7H_2O$	3	mg
KH_2PO_4	1	mg
K_2HPO_4	0.5	mg
$CaCl_2 \cdot 2H_2O$	1	mg
$CaCO_3$[2]	1	mg
Fe-citrate	0.2	mg
Citric acid	0.2	mg
Biotin	0.2	μg
Thiamine HCl	1	μg
Vitamin B_6	0.1	μg
Vitamin B_{12}	0.1	μg
Trace metals[1]	0.5	mL
Distilled water	99.5	mL
pH 6.6[2]		

1) In the NIES-Collection, $CaCO_3$ is removed and PIV metals are used instead of Trace metals.

2) In the NIES-Collection, 40 mg MES is added and pH is adjusted to 6.6.

4. AF-6/2*

AF-6[1] medium is diluted with distilled water to one-half.

1) See AF-6

5. AFAC*

To 100 mL AF-6[1] medium add 20 mg sodium acetate.

1) See AF-6

6. Allen* (11, 883)

$(NH_4)_2SO_4$	132	mg
KH_2PO_4	27.2	mg
$MgSO_4 \cdot 7H_2O$	24.6	mg
$CaCl_2 \cdot 2H_2O$	7.4	mg
Allen metals[1]	0.01	mL
Distilled water	99.9	mL
pH 2.5[2]		

1) See Allen metals

2) pH is adjusted to 2.5 with 0.5 mol/L H_2SO_4.

7. BBM (33)

$NaNO_3$	25	mg
KH_2PO_4	17.5	mg
K_2HPO_4	10	mg
$MgSO_4 \cdot 7H_2O$	7.5	mg
$CaCl_2 \cdot 2H_2O$	2.5	mg
NaCl	2.5	mg
KOH	3.1	mg
$FeSO_4 \cdot 7H_2O$	0.498	mg
H_3BO_3	1.142	mg
$ZnSO_4 \cdot 7H_2O$	0.882	mg
$MnCl_2 \cdot 7H_2O$	0.144	mg
MoO_3	0.071	mg
$CuSO_4 \cdot 5H_2O$	0.157	mg
$Co(NO_3)_2 \cdot 6H_2O$	0.049	mg
Na_2EDTA	5	mg
Distilled water	100	mL

8. BG-11*

$NaNO_3$	150	mg
$K_2HPO_4 \cdot 3H_2O$	4	mg
$MnSO_4 \cdot 7H_2O$	4	mg
$CaCl_2 \cdot 2H_2O$	7.5	mg
Citric acid	3.6	mg

Ferric ammonium citrate	0.6	mg
Na_2EDTA-Mg	0.1	mg
Na_2CO_3	2	mg
Trace metal mix A_5 + Co[1)]	0.1	mg
Agar	1.5	g
Distilled water	99.9	mL

pH 7.4

1) See Trace metal mix A_5 + Co

9. C* (208)

$Ca(NO_3)_2 \cdot 4H_2O$	15	mg
KNO_3	10	mg
β-Na_2glycerophosphate $\cdot 5H_2O$	5	mg
$MgSO_4 \cdot 7H_2O$	4	mg
Vitamin B_{12}	0.01	μg
Biotin	0.01	μg
Thiamine HCl	1	μg
PIV metals[1)]	0.3	mL
Tris (hydroxymethyl) aminomathane	50	mg
Distilled water	99.7	mL

pH 7.5

Add 1.5 g agar to 100 mL of medium to give a solid medium.

1) See PIV metals

10. C + 10% Seawater (N. Tezuka, unpubl.)

C[1)] medium with 10% filtered seawater.

1) See C

11. C/6G

Mix 1 volume of C[1)] medium and 5 volumes of Lake Nojiri water (sterilized through GF/F filter, and store at 5°C).

1) See C

12. CA* (221)

$Ca(NO_3)_2 \cdot 4H_2O$	2	mg
KNO_3	10	mg
NH_4NO_3	5	mg
β-Na_2glycerophosphate $\cdot 5H_2O$	3	mg
$MgSO_4 \cdot 7H_2O$	2	mg
Vitamin B_{12}	0.01	μg
Biotin	0.01	μg
Thiamine HCl	1	μg
PIV metals[1)]	0.1	mL
Fe (as EDTA; 1:1 molar)[2)]	0.1	mg
HEPES	40	mg
Distilled water	99.9	mL

pH 7.2

1) See PIV metals
2) See Fe (as EDTA; 1:1 molar)

13. CAM*

CA[1)] medium with pH adjusted to 6.5 by buffering with MES instead of HEPES.

1) See CA

14. Carefoot* (41)

$NaNO_3$	24.7	mg
$CaCl_2 \cdot 2H_2O$	1.1	mg
$MgSO_4 \cdot 7H_2O$	4.7	mg
K_2HPO_4	0.9	mg
KH_2PO_4	2.3	mg
NaCl	1.5	mg
PIV metals[1)]	0.5	mL
Distilled water	99.5	mL

pH 7.5

In the NIES-Collection, 0.02 μg vitamin B_{12}, 0.02 μg biotin and 2 μg thiamine HCl are added to this medium.

1) See PIV metals

15. CB*

To C[1)] medium add Bicine instead of Tris (hydroxymethyl) aminomethane, and adjust pH to 9.0.

1) See C

16. CB-V*

Make B-V[1)] medium with C[2)] medium.

1) See B-V
2) See C

17. CC (215)

C[1)] medium with pH adjusted to 3.0 by buffering with 1,2,3,4-cyclopentane tetracarboxylic acid instead of Tris (hydroxymethyl) aminomethane.

1) See C

18. CSi*

C[1)] medium with pH adjusted to 7.0 by buffering with

50 mg HEPES instead of Tris (hydroxymethyl) aminomethane. Thereafter, 10 mg $Na_2SiO_3 \cdot 9H_2O$ is added.

1) See C

19. CSi + Cu

To 100 mL CSi[1)] medium add 0.25 mg $CuSO_4 \cdot 5H_2O$ and 100 mg agar.

1) See CSi

20. CSi/5*

Dilute CSi[1)] medium with distilled water to 1/5.

1) See CSi

21. CT* (1039)

C[1)] medium with pH adjusted to 8.2 by buffering with 40 mg TAPS instead of Tris (hydroxymethyl) aminomethane.

1) See C

22. CYT*

To 100 mL C[1)] medium add 100 mg yeast extract and 200 mg tryptone.

1) See C

23. DH + Fe (I.I. Brown, unpubl.)

D stock medium[1)]	5	mL
HEPES	0.12	mg
$FeCl_3 \cdot 6H_2O$	11.4	mg
Distilled water	95	mL
pH 8.24 – 8.26		

After autoclaving, keep in room temperature overnight. Next day, adjust pH to 7.5–7.6 and add 1.5 g agar.

1) See D stock medium

24. DY-V*

MES	20	mg
$MgSO_4 \cdot 7H_2O$	5	mg
KCl	0.3	mg
NH_4Cl	0.27	mg
$NaNO_3$	2	mg
β-Na_2glycerophosphate $\cdot 5H_2O$	0.22	mg
H_3BO_3	0.08	mg
$Na_2EDTA \cdot 2H_2O$	0.8	mg
$Na_2SiO_3 \cdot 9H_2O$	1.4	mg
$FeCl_3 \cdot 6H_2O$	0.1	mg
$CaCl_2 \cdot 2H_2O$	7.5	mg
Vitamin B_{12}	0.05	µg
Biotin	0.05	µg
Thiamine HCl	10	µg
DY trace metal solution[1)]	0.1	mL
Distilled water	99.9	mL
pH 6.8		

1) See DY trace metal solution

25. HUT* (207)

KH_2PO_4	2	mg
$MgSO_4 \cdot 7H_2O$	2.5	mg
Sodium acetate	40	mg
Potassium citrate	4	mg
Polypeptone	60	mg
Yeast extract	40	mg
Vitamin B_{12}	0.05	µg
Thiamine HCl	0.04	mg
Distilled water	100	mL
pH 6.4		

Add 150 mg agar to 100 mL of medium to give a semi-solid medium.

26. M-11* (120, 1087)

$NaNO_3$	10	mg
K_2HPO_4	1	mg
$MgSO_4 \cdot 7H_2O$	7.5	mg
$CaCl_2 \cdot 2H_2O$	4	mg
Na_2CO_3	3	mg
$FeSO_4 \cdot 7H_2O$	0.1	mg
$Na_2EDTA \cdot 2H_2O$	0.1	mg
Distilled water	100	mL
pH 8.0		

27. MA* (211)

$Ca(NO_3)_2 \cdot 4H_2O$	5	mg
KNO_3	10	mg
$NaNO_3$	5	mg
Na_2SO_4	4	mg
$MgCl_2 \cdot 6H_2O$	5	mg
β-Na_2glycerophosphate $\cdot 5H_2O$	10	mg
$Na_2EDTA \cdot 2H_2O$	0.5	mg
$FeCl_3 \cdot 6H_2O$	0.05	mg
$MnCl_2 \cdot 4H_2O$	0.5	mg

$ZnCl_2$	0.05	mg
$CoCl_2 \cdot 6H_2O$	0.5	mg
$Na_2MoO_4 \cdot 2H_2O$	0.08	mg
H_3BO_3	2	mg
Bicine	50	mg
Distilled water	100	mL

pH 8.6

28. MAF-6*

To 100 mL of AF-6[1] medium add 10 mg glucose and 10 mg sodium acetate.

1) See AF-6

29. M-Allen*

$(NH_4)SO_4$	262	mg
KH_2PO_4	54	mg
$MgSO_4 \cdot 7H_2O$	50	mg
$CaCl_2 \cdot 2H_2O$	14	mg
A2 trace elements stock solution[1]	0.2	mL
Distilled water	99.4	mL

pH 2.5[2]

After autoclaving, add 0.4 mL of A2 Fe stock solution[3] (filter-sterilized).

1) See A2 trace elements stock solution
2) pH is adjusted to 2.5 with 0.5 mol/L H_2SO_4.
3) See A2 Fe stock solution

Indicated as "MA" medium in reference.

30. M-Allen (+ U)*

To 100 mL M-Allen[1] medium add 50 mg uracil.

1) See M-Allen

31. MBM* (215)

KNO_3	25	mg
$MgSO_4 \cdot 7H_2O$	7.5	mg
K_2HPO_4	7.5	mg
KH_2PO_4	17.5	mg
NaCl	2.5	mg
$CaCl_2 \cdot 2H_2O$	1	mg
Fe solution[1]	0.1	mL
A_5 solution[2]	0.1	mL
Agar	1.5	g
Distilled water	99.8	mL

pH 6.0

1) See Fe solution.
2) See A_5 solution.

Indicated as "Modified Bristol medium" in reference.

32. M Chu No. 10* (47)

$Ca(NO_3)_2 \cdot 4H_2O$	2	mg
KH_2PO_4	0.62	mg
$MgSO_4 \cdot 7H_2O$	2.5	mg
Na_2CO_3	2	mg
$Na_2SiO_3 \cdot 9H_2O$	2.5	mg
HCl (1 mol/L)[1]	0.025	mL
$Na_2EDTA \cdot 2H_2O$	0.2	mg
$FeCl_3 \cdot 6H_2O$	0.1	mg
H_3BO_3	0.248	mg
$MnCl_2 \cdot 4H_2O$	0.139	mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.1	mg
Vitamin B_{12}	1	μg
Thiamine HCl	0.1	μg
Biotin	0.1	μg
Distilled water	100	mL

1) In the NIES-Collection, pH is adjusted to 7.6 with 1 mol/L HCl.

33. MDM* (1013)

KNO_3	100	mg
$MgSO_4 \cdot 7H_2O$	25	mg
K_2HPO_4	25	mg
NaCl	10	mg
$CaCl_2 \cdot 2H_2O$	1	mg
Fe solution[1]	0.1	mL
A_5 solution[2]	0.1	mL
Agar	1.5	g
Distilled water	99.8	mL

pH 8.0

1) See Fe solution
2) See A_5 solution

34. MG* (210)

$Ca(NO_3)_2 \cdot 4H_2O$	2	mg
KNO_3	10	mg
β-Na_2glycerophosphate $\cdot 5H_2O$	3	mg
$MgSO_4 \cdot 7H_2O$	2	mg
Vitamin B_{12}	0.01	μg
Biotin	0.01	μg
Thiamine HCl	1	μg
PIV metals[1]	0.1	mL

Fe (as EDTA; 1:1 molar)[2)]	0.1	mL
HEPES	40	mg
Distilled water	99.8	mL

pH 7.2

1) See PIV metals
2) See Fe (as EDTA; 1:1 molar)

35. MGM

MG[1)] medium with pH adjusted to 6.5 by buffering with MES instead of HEPES.

1) See MG

36. Modified acetate medium (mAC) (672)

To 100 mL AF-6[1)] medium, add 40 mg glucose, yeast extract, tryptone, and sodium acetate.

1) See AF-6

37. Modified M-1 (mM-1) (194)

$CaCl_2 \cdot 2H_2O$	0.5	mg
$NaNO_3$	2.5	mg
NH_4Cl	0.5	mg
$CaSO_4 \cdot 2H_2O$	0.4	mg
$MgSO_4 \cdot 7H_2O$	0.5	mg
$Na_2SiO_3 \cdot 9H_2O$	0.2	mg
Fe (as EDTA; 1:1 molar)[1)]	25	mL
mM-1 Trace elements[2)]	0.1	mL
K_2HPO_4	6.96	mg
KH_2PO_4	266.5	mg
Distilled water	74.9	mL

pH 5.1 – 5.3

1) See Fe (as EDTA; 1:1 molar)
2) See mM-1 Trace elements

38. MW* (794)

Urea	0.85	mg
$NaNO_3$	0.17	mg
NH_4Cl	0.042	mg
$Ca(NO_3)_2 \cdot 4H_2O$	10	mg
$CaCO_3$	1	mg
$CaCl_2 \cdot 2H_2O$	1.4	mg
KNO_3	1	mg
$KHCO_3$	0.9	mg
β-Na_2glycerophosphate $\cdot 5H_2O$	2	mg
$MgSO_4 \cdot 7H_2O$	1.5	mg
PIV metals[1)]	0.05	mL
Vitamin B_{12}	0.02	μg
Thiamine HCl	2	μg
Biotin	0.02	μg
Glycylglycine	10	mg
Distilled water	100	mL

pH 7.2

1) See PIV metals

39. MW/5*

MW[1)] medium is diluted with distilled water to 1/5.

1) See MW

40. N-Free*

NaCl	7	mg
$MgSO_4 \cdot 7H_2O$	38	mg
$CaCl_2 \cdot 2H_2O$	10.6	mg
K_2HPO_4	60	mg
$Fe_2(SO_4)_3 \cdot 6H_2O$	1	mg
$Na_2EDTA \cdot 2H_2O$	2.7	mg
H_3BO_3	0.3	mg
$MnSO_4 \cdot 4H_2O$[1)]	0.2	mg
$Na_2Mo_4 \cdot 2H_2O$	0.8	mg
$ZnSO_4 \cdot 7H_2O$	0.03	mg
$CuSO_4 \cdot 5H_2O$	8	μg
$CoCl_2 \cdot 6H_2O$	3.7	μg
Agar	1.5	g
Distilled water	100	mL

pH 7.5

1) In the NIES-Colletion, 0.2 mg $MnSO_4 \cdot 4H_2O$ is replaced by 0.22 mg $MnSO_4 \cdot 5H_2O$.

41. O* (215, 880)

Glucose	100	mg
Tryptone	100	mg
Yeast extract	100	mg
Beef extract[1)]	50	mg
Agar	150	mg
Distilled water	100	mL

1) In the NIES-Collection, beef extract is removed. Indicated as "*Ochromonas* medium" in reference.

42. P 35* (211)

NH_4NO_3	10	mg
$MgSO_4 \cdot 7H_2O$	4	mg
KCl	5	mg

$CaCl_2 \cdot 2H_2O$	7.4	mg
β-Na_2glycerophosphate · $5H_2O$	5	mg
Sodium acetate	100	mg
Vitamin B_{12}	0.01	µg
Biotin	0.01	µg
Thiamine HCl	1	µg
PIV metals[1)]	0.3	mL
Tris (hydroxymethyl) aminomethane	50	mg
Distilled water	99.7	mL

pH 8.0

1) See PIVmetals

43. Pro* (215, 880)

To 100 mL MBM[1)] medium add 100 mg proteose peptone.

1) See MBM

Indicated as "Proteose medium" in reference.

44. SOT* (684)

$NaHCO_3$	1.68	g
K_2HPO_4	50	mg
$NaNO_3$	250	mg
K_2SO_4	100	mg
NaCl	100	mg
$MgSO_4 \cdot 7H_2O$	20	mg
$CaCl_2 \cdot 2H_2O$	4	mg
$FeSO_4 \cdot 7H_2O$	1	mg
$Na_2EDTA \cdot 2H_2O$	8	mg
A_5 solution[1)]	0.1	mL
Distilled water	99.9	mL

1) See A_5 solution

45. SW (768)

Put a small amount of dried soil into a test tube and add 20 mL distilled water.

46. TAP

NH_4Cl	40	mg
$CaCl_2 \cdot 2H_2O$	5.1	mg
$MgSO_4 \cdot 7H_2O$	10	mg
K_2HPO_4	11.9	mg
KH_2PO_4	6.03	mg
Hutner's trace elements[1)]	0.1	mL
Acetic acid	0.1	mL
Tris (hydroxymethyl) aminomethane	242	mg
Agar	1.5	g
Distilled water	99.8	mL

1) See Hutner's trace elements

47. Tre* (215, 880)

To 100 mL MBM[1)] medium add 1 g proteose peptone and 2 g glucose.

1) See MBM

Indicated as "Trebouxia medium" in reference.

48. URO* (364, 558)

NH_4NO_3	0.5	mg
β-Na_2glycerophosphate · $5H_2O$	0.4	mg
$MgSO_4 \cdot 7H_2O$	1	mg
$CaCl_2 \cdot 2H_2O$	1	mg
KCl	0.1	mg
Thiamine HCl	1	µg
Vitamin B_{12}	0.01	µg
Biotin	0.01	µg
Fe-EDTA	0.05	mg
PIV metals[1)]	0.1	mL
Distilled water	99.9	mL

pH 7.5[2)]

1) See PIV metals
2) pH is adjusted to 7.5 with 0.1 mol/L HCl.

49. URO-H*

To 100 mL URO[1)] medium add 40 mg HEPES.

1) See URO

50. URO-T*

To 100 mL URO[1)] medium add 50 mg Tris (hydroxymethyl) aminomathane.

1) See URO

51. VT* (772, 882)

$Ca(NO_3)_2 \cdot 4H_2O$	11.78	mg
β-Na_2glycerophosphate · $5H_2O$	5	mg
$MgSO_4 \cdot 7H_2O$	4	mg
KCl	5	mg
Vitamin B_{12}	0.01	µg
Biotin	0.01	µg
Thiamine HCl	1	µg
PIV metals[1)]	0.3	mL
Glycylglycine	50	mg

Distilled water	99.7	mL

pH 7.5

1) See PIV metals

52. VTAC* (655)

To 100 mL VT[1] medium add 20 mg sodium acetate.

1) See VT

53. VTYT (215)

To 100 mL VT[1] medium add 10 mg yeast extract and 20 mg tryptone.

1) See VT

54. W (1036)

$Ca(NO_3)_2 \cdot 4H_2O$	10	mg
KNO_3	1	mg
$MgSO_4 \cdot 7H_2O$	1.5	mg
β-Na_2glycerophosphate $\cdot 5H_2O$	2	mg
Urea	1.7	mg
Thiamine HCl	0.2	μg
Vitamin B_{12}	0.002	μg
Biotin	0.002	μg
PIV metals[1]	0.05	mL
Glycylglycine	10	mg
Distilled water	99.95	mL

pH 7.5

1) See PIV metals

2.2. Media for marine and brackish water microalgae

(海産および汽水産藻類用培地)

Media indicated with asterisks (*) are available for distribution. Reference numbers are shown in parentheses after medium names.

(* 分譲可能な培地を示す。培地に関する文献番号を培地名の後のカッコ内に示した。)

55. BESM*

Make diluted seawater by mixing 27.5 mL seawater and 70 mL distilled water. Make ESM[1] medium by using this diluted seawater instead of original seawater.

1) See ESM

56. BESM 2*

Make diluted seawater by mixing 47.5 mL seawater and 50 mL distilled water. Make ESM[1] medium by using this diluted seawater instead of original seawater.

1) See ESM

57. ESM* (721)

$NaNO_3$	12	mg
K_2HPO_4	0.5	mg
Vitamin B_{12}	0.1	μg
Biotin	0.1	μg
Thiamine HCl	10	μg
Fe-EDTA	25.9	μg
Mn-EDTA	33.2	μg
Tris (hydroxymethyl) aminomethane	100	mg
Soil extract[1]	2.5	mL
Seawater	97.5	mL

pH 8.0

The amount of Soil extract depends on the quality of the soil. In the NIES-Collection, Soil extract was reduced from 5 mL to 2.5 mL after 2002.

Add 1.5 g agar to 100 mL of medium to give a solid medium.

1) See Soil extract

58. ESM2*

Prepare as for 100 mL ESM[1] medium with 95.5 mL instead of 97.5 mL seawater and with 5 mL instead of 2.5 mL Soil extract[2].

1) See ESM
2) See Soil extract

59. f/2* (119)

$NaNO_3$	7.5	mg
$NaH_2PO_4 \cdot 2H_2O$	0.6	mg
Vitamin B_{12}	0.05	μg
Biotin	0.05	μg
Thiamine HCl	10	μg
$Na_2SiO_3 \cdot 9H_2O$	1	mg
f/2 metals[1]	0.1	mL
Seawater	99.9	mL

1) See f/2 metals

60. f/2 + NH_4Cl*

To 100 mL f/2[1] medium add 2.67 mg NH_4Cl.

1) See f/2

61. IMK

Into 100 mL seawater dissolve 21 mg powder medium of Daigo IMK (Nihon Pharmaceutical Co., Ltd.).
In the NIES-Collection, IMK medium is used after autoclaving (121°C, 20 min).

62. M-ASP7 (1058)

NTA	7	mg
NaCl	2.5	g
$MgSO_4 \cdot 7H_2O$	900	mg
KCl	70	mg
$CaCl_2 \cdot 2H_2O$	30	mg
$NaNO_3$	5	mg
$NaH_2PO_4 \cdot 2H_2O$	2	mg
Vitamin B_{12}	0.1	µg
Vitamin mix S_3[1]	1	mL
$Na_2SiO_3 \cdot 9H_2O$	1	mg
P_N metals[2]	3	mL
Tris (hydroxymethyl) aminomethane	100	mg
Distilled water	96	mL

pH 8.0

1) See Vitamin mix S_3
2) See P_N metals

63. MF

f/2[1] medium with $Na_2SiO_3 \cdot 9H_2O$ replaced by 1 mL Soil extract[2] and adjusted to pH 8.0 by buffering with 100 mg Tris (hydroxymethyl) aminomethane.

1) See f/2
2) See Soil extract

64. mIMR* (modified IMR) (759)

KNO_3	1.26	mg
K_2HPO_4	0.22	mg
Na_2SiO_3[1]	0.61	mg
mIMR trace metals[2]	0.1	mL
Thiamine	20	µg
Vitamin B_{12}	1	µg
Biotin	0.1	µg
Seawater	100	mL

1) In the NIES-Collection, Na_2SiO_3 is replaced by $Na_2SiO_3 \cdot 9H_2O$.
2) See mIMR trace metals

65. MKM (1013)

KNO_3	75	mg
KH_2PO_4	2.5	mg
$MgSO_4 \cdot 7H_2O$	2	mg
Fe-citrate	250	µg
Agar	1.5	g
Seawater	50	mL
Distilled water	50	mL

66. MNK* (617)

$NaNO_3$	2	mg
K_2HPO_4	0.1	mg
$Na_2HPO_4 \cdot 12H_2O$	0.028	mg
Vitamin B_{12}	0.015	µg
Biotin	0.015	µg
Thiamine HCl	2	µg
$CoSO_4 \cdot 7H_2O$	0.12	µg
$ZnSO_4 \cdot 7H_2O$	0.24	µg
$MnCl_2 \cdot 4H_2O$	0.9	µg
$CuSO_4 \cdot 5H_2O$	0.006	µg
Na_2SeO_3	0.003	µg
$Na_2MoO_4 \cdot 2H_2O$	0.07	µg
$Na_2EDTA \cdot 2H_2O$	0.37	µg
Fe-EDTA	2.6	µg
Mn-EDTA	3.3	µg
Seawater	100	mL

Vitamins should be added at the end of the preparation. This medium should not be autoclaved but filter-sterilized.

67. PRO-99*

$NaH_2PO_4 \cdot 2H_2O$	3	mg
NH_4Cl	4.28	mg
PRO-99 trace metals[1]	0.01	mL
Seawater	100	mL

1) See PRO-99 trace metals

68. WESM*

Make diluted seawater by mixing 87.5 mL seawater and 10 mL distilled water. Make ESM[1] medium by using this diluted seawater instead of seawater.

1) See ESM

2.3. Bacteria-free check media for freshwater algae
(淡水産藻類用無菌検査培地)

Reference numbers are shown in parentheses after medium names.
(培地に関する文献番号を培地名の後のカッコ内に示した。)

69. B-I (222)

Appropriate medium	100	mL
Proteose peptone	100	mg

70. B-II (222)

Appropriate medium	100	mL
Yeast extract	500	mg

71. B-III (222)

Appropriate medium	100	mL
Peptone	500	mg
Beef extract	300	mg

72. B-IV (222)

Appropriate medium	100	mL
Glucose	100	mg
Peptone	100	mg

73. B-V (222)

Appropriate medium	100	mL
Sodium acetate	50	mg
Glucose	50	mg
Tryptone	50	mg
Yeast extract	30	mg

74. YT (215)

Appropriate medium	100	mL
Yeast extract	100	mg
Tryptone	200	mg

2.4. Bacteria-free check media for marine algae
(海産藻類用無菌検査培地)

Reference numbers are shown in parentheses after medium names.
(培地に関する文献番号を培地名の後のカッコ内に示した。)

75. Bf/2 (1129)

ASP 7[1)]	100	mL
Trypticase	50	mg
Yeast extract	5	mg

1) In the NIES-Collection, ASP 7 is replaced by f/2 medium. See f/2.

76. MM 23 (M. Tatewaki, pers. comm.)

NaCl	1.8	g
$MgSO_4 \cdot 7H_2O$	500	mg
KCl	60	mg
$NaNO_3$	100	mg
$CaCl_2 \cdot 2H_2O$	36.7	mg
K_2HPO_4	6	mg
Sucrose	400	mg
PII metals[1)]	2	mL
$FeCl_3 \cdot 6H_2O$	48	μg
Thiamine HCl	10	μg
Biotin	0.1	μg
Vitamin B_{12}	0.2	μg
C-Source Mix II[2)]	1	mL
Tris (hydroxymethyl) aminomethane	100	mg
Distilled water	97	mL

pH 8.0

1) See PII metals
2) See C-Source Mix II

77. STP* (771)

$NaNO_3$	20	mg
K_2HPO_4	1	mg
Sodium glutamate	50	mg
Glucose	20	mg
Glycine	10	mg
D,L-Alanine	10	mg
Vitamin mix 8[1)]	0.1	mL
Trypticase	20	mg
Yeast autolysate[2)]	20	mg
Sucrose	100	mg
Soil extract[3)]	5	mL
Sea water	80	mL
Distilled water	15	mL

pH 7.5

1) In the NIES-Collection, Vitamin mix 8 is replaced by Vitamin mix S_3. See Vitamin mix S_3.
2) In the NIES-Collection, yeast autolysate is replaced by yeast extract.
3) See Soil extract

2.5. Trace metals, vitamin mixtures and soil extracts (微量金属，ビタミン混液，土壌浸出液)

Media indicated with asterisks (*) are available for distribution. Reference numbers are shown in parentheses after medium names.
(* 分譲可能な培地を示す。培地に関する文献番号を培地名の後のカッコ内に示した。)

78. A2 Fe stock solution*

EDTA · 2Na	700	mg
$FeCl_3 \cdot 6H_2O$	400	mg
Distilled water	100	mL

Sterilize by passing through a Millipore filter (0.22 μm).

79. A2 trace element stock solution*

H_3BO_3	285	mg
$MnCl_2 \cdot 4H_2O$	180	mg
$ZnCl_2$	10.5	mg
$Na_2MoO_4 \cdot 2H_2O$	39	mg
$CoCl_2 \cdot 6H_2O$	4	mg
$CuCl_2 \cdot 2H_2O$	4.3	mg
Distilled water	100	mL

80. A_5 solution* (195)

H_3BO_3	286	mg
$MnSO_4 \cdot 7H_2O$[1)]	250	mg
$ZnSO_4 \cdot 7H_2O$	22.2	mg
$CuSO_4 \cdot 5H_2O$	7.9	mg
$Na_2MoO_4 \cdot 2H_2O$	2.1	mg
Distilled water	100	mL

1) In the NIES-Collection, 250 mg $MnSO_4 \cdot 7H_2O$ is replaced by 217 mg $MnSO_4 \cdot 5H_2O$.

81. Allen metals* (11)

Fe–EDTA	30.16	g
$MnCl_2 \cdot 4H_2O$	1.79	g
H_3BO_3	2.86	g
$ZnSO_4 \cdot 7H_2O$	220	mg
$CuSO_4 \cdot 5H_2O$	79	mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	130	mg
NH_4VO_3	23	mg
Distilled water	100	mL

In the NIES-Collection, Allen metals are used after dilution with distilled water to 1/1000.

82. C-Source Mix II* (M. Tatewaki, pers. comm.)

Glycine	100	mg
D,L-Alanine	100	mg
L-Aspargine	100	mg
Sodium acetate · $3H_2O$[1)]	200	mg
Glucose	200	mg
L-Glutamic acid	200	mg
Distilled water	100	mL

1) In the NIES-Collection, 200 mg sodium acetate · $3H_2O$ is replaced by 120 mg sodium acetate, anhydrous.

83. D stock medium (42)

NTA	0.2	g
D trace mix[1)]	1	mL
$FeCl_3 \cdot 6H_2O$	0.58	mg
$CaSO_4 \cdot 2H_2O$	120	mg
$MgSO_4 \cdot 7H_2O$	200	mg
NaCl	16	mg
KNO_3	200	mg
$NaNO_3$	1.4	g
Na_2HPO_4[2)]	220	mg
Distilled water	99	mL

1) See D trace mix
2) In the NIES-Collection, 220 mg Na_2HPO_4 is replacedy by 550 mg $Na_2HPO_4 \cdot 12H_2O$.

84. D trace mix (42)

Conc H_2SO_4	0.05	mL
$MnSO_4 \cdot H_2O$[1)]	228	mg
$ZnSO_4 \cdot 7H_2O$	50	mg
H_3BO_3	50	mg
$CuSO_4 \cdot 5H_2O$	2.5	mg
$Na_2MoO_4 \cdot 2H_2O$	2.5	mg
$CoCl_2 \cdot 6H_2O$	4.5	mg
Distilled water	100	mL

1) In the NIES-Collection, 228 mg $MnSO_4 \cdot H_2O$ is replaced by 349 mg $MnSO_4 \cdot 5H_2O$.

85. DY trace metal solution*

$MnCl_2 \cdot 4H_2O$	20	mg
$ZnSO_4 \cdot 7H_2O$	4	mg
$CoCl_2 \cdot 6H_2O$	0.8	mg
$Na_2MoO_4 \cdot 6H_2O$[1)]	2	mg
Na_3VO_4	0.2	mg
H_2SeO_3	0.2	mg

Distilled water	100	mL

1) In the NIES-Collection, $Na_2MoO_4 \cdot 6H_2O$ is replaced by $Na_2MoO_4 \cdot 2H_2O$.

86. Fe (as EDTA; 1:1 molar)* (770)

$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	70.2	mg
$Na_2EDTA \cdot 2H_2O$	66	mg
Distilled water	100	mL

1 mL of this solution contains 0.1 mg Fe.

87. Fe solution (215)

$FeSO_4 \cdot 7H_2O$	200	mg
Distilled water	100	mL
Conc · H_2SO_4[1)]	0.026	mL[1)]

1) 2 drops/500 mL (Ref. 215)

88. f/2 metals* (119)

$Na_2EDTA \cdot 2H_2O$	440	mg
$FeCl_3 \cdot 6H_2O$	316	mg
$CoSO_4 \cdot 7H_2O$	1.2	mg
$ZnSO_4 \cdot 7H_2O$	2.1	mg
$MnCl_2 \cdot 4H_2O$	18	mg
$CuSO_4 \cdot 5H_2O$	0.7	mg
$Na_2MoO_4 \cdot 2H_2O$	0.7	mg
Distilled water	100	mL

89. Hutner's trace elements

$Na_2EDTA \cdot 2H_2O$	5	g
$ZnSO_4 \cdot 7H_2O$	2.2	g
H_3BO_3	1.14	g
$MnCl_2 \cdot 4H_2O$	506	mg
$FeSO_4 \cdot 7H_2O$	499	mg
$CoCl_2 \cdot 6H_2O$	161	mg
$CuSO_4 \cdot 5H_2O$	157	mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	110	mg
Distilled water	100	mL

Adjust the pH to 6.5–6.8 with KOH (~1.6 g). Store the solution in a refrigerator (5°C). The solution should have turned to violet color before use. This process takes a while and is necessary.

90. mIMR trace metals*

$FeCl_3 \cdot 6H_2O$	100	mg
$MnSO_4 \cdot 4H_2O$	85.5	mg
$ZnSO_4 \cdot 7H_2O$	25	mg
$Na_2MoO_4 \cdot 2H_2O$	13	mg
$CoCl_2 \cdot 6H_2O$	0.4	mg
$CuSO_4 \cdot 5H_2O$	0.4	mg
Na_2SeO_3	0.173	mg
Na_2EDTA[1)]	600	mg
Distilled water	100	mL

1) In the NIES-Collection, Na_2EDTA is replaced by $Na_2EDTA \cdot 2H_2O$.

91. mM-1 Trace elements (194)

$CuSO_4 \cdot 5H_2O$	10	mg
$MnCl_2 \cdot 7H_2O$	10	mg
Br (1 mol/L solution)	0.01	mL
$ZnSO_4 \cdot 7H_2O$	10	mg
$CoCl_2 \cdot 6H_2O$	5	mg
$BaCl_2 \cdot 2H_2O$	1	mg
H_3BO_3	10	mg
$FeCl_3 \cdot 6H_2O$	10	mg
$Na_2MoO_4 \cdot 2H_2O$	5	mg
Distilled water	100	mL

Indicated as "Trace elements" in reference.

92. mTYGM-9*

Pre-solution

K_2HPO_4	0.28	mg
KH_2PO_4	40	mg
Casein Digest	0.2	g
Yeast extract	0.1	g
NaCl	0.75	g
Mucin, gastric	0.2	g
Distilled water	97	mL

Sterilize Pre-solution by autoclaving (121°C, 15 min), add aseptically 3 mL horse serum and 50 µL 10% Tween 80 dissolved in absolute ethanol (both filter-sterilized). Keep in a cool place.

93. P II metals* (769)

$Na_2EDTA \cdot 2H_2O$	100	mg
H_3BO_3	114	mg
$FeCl_3 \cdot 6H_2O$	4.9	mg
$MnSO_4 \cdot 4H_2O$	16.4	mg
$ZnSO_4 \cdot 7H_2O$	2.2	mg
$CoSO_4 \cdot 7H_2O$	480	µg
Distilled water	100	mL

94. P IV metals* (772)

$Na_2EDTA \cdot 2H_2O$	100	mg
$FeCl_3 \cdot 6H_2O$	19.6	mg
$MnCl_2 \cdot 4H_2O$	3.6	mg
$ZnCl_2$[1)]	2.2	mg
$CoCl_2 \cdot 6H_2O$	0.4	mg
$Na_2MoO_4 \cdot 2H_2O$	0.25	mg
Distilled water	100	mL

1) In the NIES-Collection, $ZnCl_2$ is replaced by $ZnSO_4 \cdot 7H_2O$.

95. P_N metals (1058)

$Na_2EDTA \cdot 2H_2O$	100	mg
H_3BO_3	113	mg
$FeCl_3 \cdot 6H_2O$	6.3	mg
$CoSO_4 \cdot 7H_2O$	0.093	mg
$ZnSO_4 \cdot 7H_2O$	4.66	mg
$MnCl_2 \cdot 4H_2O$	3.2	mg
Distilled water	100	mL

96. PRO-99 trace metals*

$Na_2EDTA \cdot 2H_2O$	0.145	mg
$FeCl_3 \cdot 6H_2O$	0.422	mg
$ZnSO_4 \cdot 7H_2O$	2.9	mg
$CoCl_2 \cdot 6H_2O$	1.3	mg
$MnCl_2 \cdot 4H_2O$	1.3	mg
$Na_2MoO_4 \cdot 2H_2O$	0.85	mg
Na_2SeO_3	0.173	mg
$NiCl_2 \cdot 6H_2O$	0.36	mg
Distilled water	100	mL

97. Soil extract (771)

To 1000 mL distilled water add 200 mL of soil (soil from undisturbed deciduous woodland is best) and heat by autoclaving for 1 h at 105°C. When cool, heat by autoclaving for 1 h at 105°C again. Pass the supernatant through a GF/C filter and Celite, and then pass the filtrate through a GF/F filter. Adjust to 1000 mL by adding distilled water. Dispense 10 mL of the final filtrate into each test tube and sterilize by autoclaving for 20 min at 121°C. Keep in a cool place.

98. Trace metal mix A_5 + Co*

H_3BO_3	286	mg
$MnCl_4 \cdot 4H_2O$	181	mg
$ZnSO_4 \cdot 7H_2O$	22.2	mg
$Na_2MoO_4 \cdot 2H_2O$	39	mg
$CuSO_4 \cdot 5H_2O$	7.9	mg
$Co(NO_3)_2 \cdot 6H_2O$	4.9	mg
Distilled water	100	mL

99. Vitamin mix S_3 (769)

Thiamine HCl	5	mg
Nicotinic acid	1	mg
Calcium pantothenate	1	mg
p-Aminobenzoic acid	0.1	mg
Biotin	0.01	mg
Inositol	50	mg
Folic acid	0.02	mg
Thymine	30	mg
Distilled water	100	mL

2.6. Media for protozoa
(原生動物用培地)

Media indicated with asterisks (*) are available for distribution. Reference numbers are shown in parentheses after medium names.
(* 分譲可能な培地を示す。培地に関する文献番号を培地名の後のカッコ内に示した。)

100. ESM + mTYGM-9 + Rice

Beforehand, sterilize polished rice by dry heating (150°C, 30 min). Keep in a cool place. For use, add 500 µL mTYGM-9[1)] and a grain of sterile rice to 10 mL ESM[2)] medium.

1) See mTYGM-9
2) See ESM

101. f/2 + mTYGM-9 + Rice

Beforehand, sterilize polished rice by dry heating (150°C, 30 min). Keep in a cool place. For use, add 500 µL mTYGM-9[1)] and a grain of sterile rice to 10 mL f/2[2)] medium.

1) See mTYGM-9
2) See f/2

102. f/2 + Wheat

Beforehand, sterilize wheat grains by dry heating (150°C, 30 min). Keep in a cool place. For use, add a grain of sterile wheat to 10 mL f/2[1)] medium.

1) See f/2

103. LE

L solution: White part of lettuce is dried at 90°C for 16–18 h without scorching; 300 mg of the dried lettuce is added to 100 mL boiling water (9:1 distilled water to tap water) and boiled for 30 min, while stirring. The supernatant is passed through cotton wool.

E solution: 300 mg of crushed yolk of hardboiled egg is added to 100 mL water (9:1 distilled water to tap water) and boiled for 30 min, while stirring. The supernatant is passed through cotton wool.

Equal quantities of L and E solutions are mixed. The pH is adjusted to 6.8–7.0 with 1 mol/L NaOH, and 100 mL of the solution is dispensed into each 200-mL Erlenmeyer flask and sterilized by autoclaving (121°C, 15 min).

104. SUY* (521)

Prepare as for 100 mL URO[1)] medium with seawater instead of distilled water. Add 10 mg yeast extract and 20 mg tryptone.

Indicated as " URO-YT " in reference.

1) See URO

105. SUY 1/10* (522)

Prepare as for 100 mL URO[1)] medium with seawater instead of distilled water. Add 1 mg yeast extract and 2 mg tryptone.

Indicated as " URO-1/10 YT " in reference.

1) See URO

106. SUY 1/10 + mTYGM-9 + Rice

Beforehand, sterilize polished rice by dry heat (150°C, 30 min). Keep in a cool place. For use, add 500 µL mTYGM-9[1)] and a grain of sterile rice to 10 mL SUY 1/10[2)] medium.

1) See mTYGM-9
2) See SUY 1/10

107. SUY 1/10 + Wheat

Beforehand, sterilize wheat grains by dry heating (150°C, 30 min). Keep in a cool place. For use, add a grain of sterile wheat to 10 mL SUY 1/10[1)] medium.

1) See SUY 1/10

108. URO + Wheat

Beforehand, sterilize wheat grains by dry heating (150°C, 30 min). Keep in a cool place. For use, add a grain of sterile wheat to 10 mL URO[1)] medium.

1) See URO

109. UYTS + Rice

Prepare as for 100 mL URO[1)] medium with 99.2 mL instead of 99.9 mL distilled water. Adjust to pH 7.5 with 0.1 mol/L HCl, and add 10 mg yeast extract, 20 mg tryptone and 0.3 ml horse serum (UYTS medium).

Beforehand, sterilize polished rice by dry heating (150°C, 30 min). Keep in a cool place. For use, add a grain of sterile rice to 10 mL UYTS medium.

1) See URO

2.7. Media for freshwater red algae
(淡水産紅藻用培地)

Media indicated with asterisks (*) are available for distribution. Reference numbers are shown in parentheses after medium names.

(* 分譲可能な培地を示す。培地に関する文献番号を培地名の後のカッコ内に示した。)

110. Bold 3N*

$NaNO_3$	75	mg
$CaCl_2 \cdot 2H_2O$	2.5	mg
$MgSO_4 \cdot 7H_2O$	7.5	mg
K_2HPO_4	7.5	mg
KH_2PO_4[1)]	17.5	mg
NaCl	2.5	mg
Vitamin B_{12}[2)]	0. 015	µg
PIV metals	0.6	mL
Soil extract[3)]	4	mL
Distilled water	95.4	mL

1) In the NIES-Collection, the amount of KH_2PO_4 is reduced from 17.5 mg to 10.5 mg.
2) In the NIES-Collection, the amount of vitamin B_{12} is increased from 0.015 µg to 0.02 µg.
3) See Soil extract

2.8. Media for Charales
(シャジクモ類用培地)

111. mSWC-2 (Modified SWC-2) (788)

Put leaf mould into a glass vessel to make a thin bottom layer, and add river sand onto the bottom layer up to one-

quarter to one-fifth from the bottom. Add a pinch of garden lime to the river sand before use.
Dampen the soil with deionized water (or distilled water). Cover the glass vessel with a plastic cap or aluminum foil, and autoclave it twice with overnight rest in between (121°C, 20 min). After cooling the mixture to room temperature, pour sterilized deionized water (or sterilized distilled water) into it carefully (so as not to disturb the soil). In the case of brackish water strains, deionized water is replaced by about one-third-diluted Herbst artificial seawater (1/3 Herbst ASW).

112. SWC-1 (788)

Put leaf mould into a glass vessel to make a thin bottom layer, and add black soil onto the bottom layer up to one-quarter to one-fifth from the bottom.
Dampen the soil with deionized water (or distilled water). Cover the glass vessel with a plastic cap or aluminum foil, and autoclave it twice with overnight rest in between (121°C, 20 min). After cooling the mixture to room temperature, pour sterilized deionized water (or sterilized distilled water) into it carefully (so as not to disturb the soil).

113. SWCN-1

Put bottom mud from a paddy field into a glass vessel up to one-quarter to one-fifth from the bottom. Dampen the mud with deionized water (or distilled water). Cover the glass vessel or jar with a plastic cap or aluminum foil, and autoclave it twice with overnight rest in between (121°C, 20 min). After cooling the mixture to room temperature, pour sterilized deionized water (or sterilized distilled water) into it carefully (so as not to disturb the soil).

114. SWCN-2

Put leaf mould into a glass vessel to make a thin bottom layer, and add a mixture of black soil and river sand onto the bottom layer up to one-quarter to one-fifth from the bottom. Dampen the soil with deionized water (or distilled water). Cover the glass vessel or jar with a plastic cap or aluminum foil, and autoclave it twice with overnight rest in between (121°C, 20 min). After cooling the mixture to room temperature, pour sterilized deionized water (or sterilized distilled water) into it carefully (so as not to disturb the soil). In the case of brackish water strains, deionized water is replaced by about one-third-diluted Herbst artificial seawater (1/3 Herbst ASW).

115. SWCN-3

Put a mixture of black soil and bottom mud from a paddy field into a glass vessel up to one-quarter to one-fifth from the bottom. Dampen the soil with deionized water (or distilled water). Cover the glass vessel or jar with a plastic cap or aluminum foil, and autoclave it twice with overnight rest in between (121°C, 20 min). After cooling the mixture to room temperature, pour sterilized deionized water (or sterilized distilled water) into it carefully (so as not to disturb the soil).

116. 1/3 Herbst ASW* (726)

NaCl	3	mg
KCl[1)]	81.4	mg
$CaCl_2$[1)]	132	mg
$MgSO_4$[1)]	660	mg
$NaHCO_3$[1)]	504	mg
Distilled water	100	mL

1) In the NIES-Collection, the amount of KCl is reduced from 81.4 mg to 80.0 mg, 132 mg $CaCl_2$ is replaced by 172 mg $CaCl_2 \cdot 2H_2O$, 660 mg $MgSO_4$ is replaced by 1.35 g $MgSO_4 \cdot 7H_2O$, and the amount of $NaHCO_3$ is reduced from 504 mg to 49.5 mg.

3. Subculture methods

3.1. Microalgae, protozoa, and freshwater red algae

You will receive the culture strains in a screw-cap test tube. Slightly loosen the screw cap and keep the test tube in an appropriate place, as indicated in individual strain data. If you want to maintain the culture strain, please transfer the culture into fresh medium according to the following methods.

i) Before you receive the strains, prepare the appropriate medium according to the media list.
ii) Adapt the fresh medium to the culturing temperature.
iii) Transfer an appropriate quantity of cell suspension to the fresh medium by an aseptic technique. In the NIES-Collection, we transfer cell suspensions by using a sterilized pipette with a cotton plug (Plate 7-1, 2). Agitate the culture liquid by pipetting if cells settle out or become attached to the container when you are sucking up the cell suspension. In the case of cells such as those of *Chattonella*, which are weak and lack cell-coverings, gently suck up a concentrated part of the

cell suspension without agitating to prevent breakage of the cells during the pipetting. The quantity of cell suspension differs with the species and the condition of the strain: to 10 mL fresh medium, we usually transfer 1 or 2 drops of cell suspension for small strains that grow well, whereas we use 4 or 5 drops for large strains and sparse cultures. In the case of agar slants, scratch a mass of cells off the surface of the agar with a sterile platinum loop and spread it on a fresh agar slant.

iv) Incubate the culture at the temperature and light conditions indicated in the individual strain data (Plate 7-3, 4). The light-dark cycle should be 12 h light: 12 h dark. The screw cap on the tube should be slightly loosened. Transfer to new medium at the intervals indicated in the individual strain data (sometimes shorter or longer depends on your laboratory conditions).

v) In the NIES-Collection, we visually check the cultures every week and when needed with a microscope. If the culture does not grow well, we transfer again, and sometimes test other media and light conditions.

For heterotrophic strains, pay attention to the following points.

i) Some strains need cereal grain or other algae as food sources added to each medium during transfer (Plate 7-5). Others need algae multiplied in advance, in accordance with individual strain data.

ii) Incubation of these strains does not need light, except in the case of cultures that contain algae as food.

iii) Always agitate the culture liquid by pipetting before transferring. In the case of adherent strains, strong pipetting is needed.

3.2. Charales

You will receive several pieces of thallus. As soon as you receive them, transplant them into fresh culture media according to the following methods.

i) Prepare appropriate culture media before you receive the strains. Add 1 to 2 mL of germanium dioxide solution (1 mg/L) to a 900-mL glass vessel, each containing fresh medium. For unialgal strains, germanium solution is not necessary.

ii) Inoculate individual thalli gently into soil in a glass vessel by using a bamboo skewer or tweezers (Plate 7-6). Make sure that one or more nodes of the thallus (root bulbils in the case of *Lamprothamnium*, stellate bulbils in the case of *Nitellopsis*) are embedded into the soil.

iii) Incubate transplanted cultures at the temperature and light conditions indicated in the strain data. About 2 weeks after the transplantation, the thalli should start to grow. (You may place the cultures near a window in the laboratory, provided that the cultures are not exposed to direct sunlight or extremely high or low temperatures.)

iv) Transfer into new media at the intervals suggested in the strain data, by using the following methods.
   a) Cut 3 or 4 apical internodes from a well-developed thallus with scissors or tweezers (Plate 7-7).
   b) Remove microalgae from the surface of each piece with a paintbrush (Plate 7-8) and rinse with deionized water (or distilled water). (For unialgal strains this process is not necessary.)
   c) Inoculate the rinsed pieces into a fresh medium as described in ii) to iii).

4. Methods of cryopreservation

A two-step freezing protocol is used in the NIES Collection: algal culture is cooled to –40°C by a programmable freezer and then cooled rapidly to –196°C in liquid nitrogen. Most cyanobacterial strains, some strains of green and red microalgae, and some strains of freshwater red algae are cryopreserved by the methods described in 4.1 and 4.2. Detailed methods for microalgae are also explained in Mori *et al.* (2002) and Mori (2007).

REFERENCES

Mori, F., Erata, M. & Watanabe, M. M. 2002. Cryopreservation of cyanobacteria and green algae in the NIES-Collection. Microbiol. Cult. Coll. 18: 45-55.

Mori, F. 2007. Cryopreservation methods of microalgae. Microbiol. Cult. Coll. 23: 89-93. (In Japanese)

4.1. Cryopreservation of microalgae

4.1.1. Materials and instruments

i) Culture: late log or early stationary phase cultures.

ii) Medium: appropriate sterile medium for each strain.

iii) Cryoprotectant: 6% dimethyl sulfoxide (DMSO) for cyanobacterial strains, and 10% DMSO for green and red algal strains dissolved in the appropriate media. These concentrations are double the final concentration. DMSO is previously sterilized by filtering through an alcohol-stable filter (Millex-LG).

iv) Laminar-flow cabinet and materials for aseptic treatment.

v) Cryovials: 2-mL presterilized polypropylene cryovials, pre-labeled with the strain number and date.

vi) Programmable freezer (e.g. Planer Kryo 320-1.7 is used in the NIES-Collection).
vii) Liquid nitrogen Dewar vessel: 10-L wide-neck Dewar vessel (Shuttle Drum JIK-S10).
viii) Long forceps (19 cm), cryogloves, a cryoapron, and goggles.
ix) Nunc polycarbonate storage boxes, 8-decker stainless-steel racks, a liquid nitrogen tank (Taiyo Nippon Sanso DR-245LM; vapor phase).
x) Water bath (e.g. As-One-Corp. Thermal Robo TR-1).

4.1.2. Freezing

i) The processes ii)-iv) should be done under aseptic conditions.
ii) Dilute the cryoprotectant with appropriate medium to obtain double the final concentration, and cool it on ice.
iii) Dispense 0.5 mL of cell suspension (late log or early stationary phase culture) into each labeled 2-mL-cryovial.
iv) Add 0.5 mL of the cryoprotectant (diluted and cooled) to each cryovial and mix well.
v) Leave the cryovials at room temperature for 15 min.
vi) Place the cryovials in a programmable freezer (Plate 7-9), and start cooling at –1°C/min to –40°C.
vii) Hold the cryovials in the programmable freezer at –40°C for 15 min.
viii) Transfer the cryovials rapidly to the Dewar vessel containing liquid nitrogen (Plate 7-10).
ix) After 1 h, transfer the cryovials in the Dewar vessel to a storage box and place the box on a stainless-steel rack set in the vapor phase of liquid nitrogen in a liquid nitrogen tank (Plate 7-11).

4.1.3. Thawing

i) Preheat a water bath to 40°C.
ii) Shake the cryovials well in the water bath until the last ice crystal in the cryovials has melted (Plate 7-12).
iii) Under aseptic conditions transfer the contents of the cryovials into test tubes each containing fresh liquid medium. Incubate under dim light for a few days (depending on the strain), and transfer to ordinary culture conditions as suggested in the strain data.

4.2. Cryopreservation of freshwater red algae

4.2.1. Materials and instruments

i) Culture: several thalli cultured for at least 2 weeks after the last transplantation. If a thallus is large, cut it into small pieces with scissors or tweezers, and culture for more than 2 weeks (for recovery), before use.
ii) Medium: sterile Bold 3N medium.
iii) Cryoprotectant: 40% dimethyl sulfoxide (DMSO) for cryopreservation of *Thorea okadae*, *T. hispida*, and *Nemalionopsis tortuosa*; and 30% methanol for *N. tortuosa*. These concentrations are double of the final ones. DMSO and methanol are previously sterilized by filtration through an alcohol-stable filter (Millex-LG), and dissolved in sterile Bold 3N medium.
iv) Instruments: same as the instruments for microalgae.

4.2.2. Freezing

i) Dilute the cryoprotectant (DMSO or methanol) with medium to obtain double the final concentrations (40% or 30%, respectively), and cool it on ice.
ii) Dispense a 0.8 mL aliquot of culture into each of the cryovials.
iii) Add 0.8 mL of 40% DMSO or 30% methanol to ii), and mix well. In the case of DMSO, leave the cryovials at room temperature for 15 min.
iv) Then same as 4.1.2 vi) to ix).

4.2.3. Thawing

i) Preheat a water bath to 40°C, and cool appropriate amount of medium in ice water.
ii) Shake the cryovials well in the water bath, and transfer the cryovials into ice water just before the last ice crystals have begun melting.
iii) Transfer the contents of the cryovials quickly into 50-mL centrifuge tubes, add 40 mL of cold medium, and leave the tubes until the thalli have settled to the bottom.
iv) Remove the supernatant with a pipette.
v) Add 40 mL of cold medium again, and again remove the supernatant with a pipette after the thalli have settled.
vi) Transfer the thalli into 60 mL of fresh media in 100-mL conical flasks, and incubate under the culture conditions suggested in the strain data.
vii) All manipulations from iii) to vi) should be done under aseptic conditions.