Maintenance of microalgal master stock-cultures by routine serial transfer

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Additional Notes

This approach has been successfully employed for a wide variety of algae, see CCAP website www.ccap.ac.uk for a comprehensive list of algal taxa/ groups.

Apparatus: Incubators with controlled light/dark cycle; a class I biological safety cabinet; Bunsen burner; autoclave for sterilising medium.

Culture medium: liquid and/or agarised medium appropriate for the taxon and its osmotic origin.

Plasticware/glassware: disposable pipettes; disposable loops; suitable culture vessels (test-tubes, flasks etc.

All chemicals should be Analytical grade.

Additional information:

***Day JG, Achilles-Day U, Brown S and Warren A** (2007) Cultivation of algae and protozoa. In: manual of environmental microbiology. Hurst C.J. et al. (eds) ASM Press, Washington DC. pp 79-92.*
***Watanabe MM** (2005) Freshwater culture media. In: Algal Culturing Techniques. Andersen R.A. (ed.) Academic Press, New York. pp 13-20.*
***Harrison PJ and Berges JA** (2005) Marine culture media. In: Algal Culturing Techniques. Andersen R.A. (ed.) Academic Press, New York. pp 21-34.*
***Lorenz M, Friedl T and Day JG** (2005) Perpetual maintenance of actively metabolizing microalgal cultures. In: Algal Culturing Techniques. Andersen R.A. (ed.) Academic Press, New York. pp 145-155.*

The most common approach used to conserve micro-algal cultures *in vitro* is by their maintenance under controlled environmental conditions. Routine serial sub-culturing is performed using aseptic microbiological technique and involves transferring an inoculum generally from a late log /stationary phase culture into fresh, pre-sterilised medium. This leads to metabolically active cultures that can be used at short notice. The objective is to retain a healthy, physiologically, morphologically, genetically and functionally representative culture. Whilst the method cannot provide an absolute guarantee of the long-term maintenance of some algal taxa, for many it is successful, with some organisms, such as the standard ecotoxicological test-strain *Chlorella vulgaris* CCAP 211/11B having been maintained in culture since the 1890's.

Medium preparation

Generally, a medium is chosen with characteristics broadly comparable to the ecological niche of the isolate. However, most algae will grow significantly "better", and remain "healthy" for longer, on media with higher nutrient levels than in their original niche. This can produce much higher levels of biomass (by a factor of 100 or more), but may in some cases result in phenotypic shifts and loss of specific characteristics, e.g. loss of spine in *Desmodesmus*, or pigment shifts in some cyanobacteria.

1. Medium formulation will be dependent on the organism to be maintained. In general for long-term maintenance, clearly defined synthetic media are employed based on deionised water with the addition of salts/nutrients, vitamins, metal chelators, trace elements and buffers, or enriched media. Alternatively, primarily for marine taxa, enriched media based on natural seawater with the addition of nutrients, vitamins, metal chelators, trace elements and buffers may be employed. For some, mostly freshwater taxa complex media such as traditional bi-phasic media, where soil is over-layered by deionised water, or EG:JM www.ccap.ac.uk/media/documents/EG_JM.pdf where a 50:50 formulation of a synthetic mineral medium (JM) is supplemented with a complex organic cocktail (EG) containing sodium acetate and a variety of protein digestates. All algal media are normally formulated through the addition of defined quantities of stock-solutions and other ingredients to the deionised water or seawater base. Many media formulations require the pH to be adjusted and the recipes/ SOP for medium formulation should be rigidly adhered to, in order to minimise potential errors, or the risk of excessive precipitate formation. For a list of widely used media see www.ccap.ac.uk/pdfrecipes.htm

Note: many algae are auxotrophic for vitamins and so require vitamins, soil extract, or small quantities of organic material to be included in the medium. Diatoms require additional silica in the medium. Where possible employ defined media, as complex constituents such as soil extract may vary radically in composition. From the author's experience soil extracts from different origins may have either a positive or a negative effect on growth and culture longevity.

Where organic materials such a protein digestates are added to medium, the culture will inevitably and rapidly become overgrown by bacterial contaminants if it is not axenic.

The quality of seawater can vary widely in osmotic potential and nutrient profile between locations, at the CCAP collection based at SAMS where natural seawater is used as the basis of media off-shore water is collected to maximise consistency of the media base.

For solidified media non-nutrient agar (generally 15-g l^{-1}) is added to the completed medium. A common oversight is the use of nutrient agar rather than non-nutrient agar, where strains are not axenic this will result in a rapid overgrowth by contaminants.

2. After formulation the medium is dispensed into culture vessels, which for the maintenance of master stock-cultures are most commonly test-tubes or small (< 100-ml) Erlenmeyer flasks. The culture vessels are then capped. In the case of test-tubes, these are generally produced in batches and placed in a basket covered with aluminum foil prior to sterilisation. For flask, the bungs are generally covered with aluminum foil prior to sterilisation.

   Choice of cap/bung can be critical to the successful long-term maintenance of algae as poor fitting closures may allow the ingress of contaminants (bacterial, fungal or even mites) as well as excessive evaporation that may influence the viability of the alga. At CCAP siliconised foam bungs have been found to be best as these also allow for adequate gas-exchange without significant evaporation, or compromising the closure. Furthermore, they can be heated/passed through a Bunsen flame to kill any bacteria adhering to them without being damaged. Traditional caps and foam bungs have been found to be unsuitable for long-term security of algal cultures.

3. Sterilise by autoclaving (normally 15 minutes or longer at 121^{o}C).

   Note. The scientific literature usually advises autoclaving algal media without the addition of vitamins, which should be filter sterilised and added after the medium has cooled post autoclaving. However, our experience is that for most algal taxa vitamins may be autoclaved in situ *in the complete medium, with no deleterious effects on the culture.*

4. Allow to cool to room temperature and then store until required.

   To produce agar slopes test-tubes containing molten agar should be placed on a racking system, which allows the tubes to be cooled at an angle (25-45^{o}). Where possible this should be performed in a pre-sterilised laminar flow cabinet, or clean room.

   Note: if you store prepared materials in a refrigerator then you should take out the media the day before to (a) allow it to equilibrate to room temperature and (b) particularly in the case of complex media, any contaminant growth may be spotted prior to the tubes being inoculated. This is mostly an issue with biphasic (soil/water) tubes where fungal spores have been known to survive the autoclaving process. To minimise the likelihood of this occurring at the CCAP collection based at SAMS biphasic tubes are autoclaved twice.

Transfer techniques and culture maintenance

Methodology and approach are dictated by the facilities available, but absolutely central to successful maintenance is good aseptic technique.

1. Prior to initiating the process prepare all materials in advance. Ensure that all fresh, sterile test-tubes or flasks are appropriately labelled. Choice of culture vessel will be dependent on the growth characteristics of the alga and some general suggestions are listed below in Table 1.

   The minimum information on any receiving culture vessel should be the algal unique identifier (strain number), the medium and the date of transfer. Where practicable additional information such as the name of the alga and replicate number should be included.

2. Perform all manipulations in a laminar flow cabinet that has been thoroughly disinfected with 70% (v/v) ethanol. Standard microbiological methods **MUST** be applied and the work has to be done under aseptic conditions.

3. Transferring cultures growing on agar. All containers should be opened for the shortest time possible to limit the risk of contamination. Transfer equipment includes a Bunsen burner for flaming, an inoculation wire loop, or disposable plastic loop. The vessel neck of the current culture is flamed after removing the cap/ bung. Then the culture vessel with the fresh medium is uncapped and the neck flamed. For transferring algal colonies from agar, an inoculation loop is sterilised by holding in a Bunsen flame, at such an angle that the whole wire glows red, and is then allowed to cool in air or by placing the wire on the agar at a site where no algal colonies are growing. Alternatively use sterile plastic loops. The inoculum is usually 1 - 10% (v/v) of the original culture and should be distributed evenly across the surface of the agar streaking from the bottom of the slant in loops toward the top. All tube and vessel necks are flamed again before the cap/bung is replaced.

   It is important to allow even distribution (no clumping) so that the newly inoculated cells receive even illumination.

   With agar cultures, colonies may stick rather firmly to the agar surface (e.g., some benthic diatoms) or may even grow into the agar (e.g., some filamentous cyanobacteria). These cannot be transferred without removing agar that contains the algal material. Sometimes it is useful to excise blocks of the agar using a small lancet or a sterile scalpel. The agar block is then placed upside down on the surface of the fresh agar.

   Transfers from liquid to agar medium. Aseptically remove 0.5 – 1.0-ml from the original culture, then transfer/inoculate two or three drops of the culture by placing them at the top of the agar in a tube and let them run down and across the surface of the agar. As above, use appropriate microbiological techniques.

   Transferring liquid cultures. With liquid cultures, a few drops or millilitres of the original algal suspension are aseptically transferred to vessels containing fresh sterile media using sterilised pipettes/pre-sterilised Pastettes. As above, use appropriate microbiological techniques.

   Note: many planktonic algae grow with an uneven distribution in the water column/ within a test-tube (e.g., gas vacuolated cyanobacteria). Many euglenophytes are best grown in biphasic soil-water medium, and they grow primarily just above the soil. This uneven distribution may influence where you select your inoculum from in the original culture vessel.

   The inoculum volume is usually 1 -10% (v/v) of the receiving culture vessel, but some dinoflagellates, as well as the cyanobacteria Synechococcus *and* Prochlorococcus*, require inocula of up to 25% (v/v).*

   It is recommended to keep a number of empty sterilised tubes and vessels with caps in reserve in case one drops a tube or cap.

4. After inoculation cultures should be transferred to an appropriate environmental regime, which is dependent on the requirements of the alga. Whilst there are some algae with obligate requirement, e.g. some polar taxa will not survive above 10°C, the majority of strains have relatively wide tolerances and some general growth conditions are outlined in Table 1.

   As a general rule for long term maintenance sub-optimal growth conditions are employed such as lower than optimal temperature and light levels. In addition, photo period adjustments are often employed, e.g. where a light:dark regime of 16h:8h is generally considered optimal; however, major collections often use 12h:12h.

   Note; for many marine taxa there is a dramatic reduction in viability if the incubation temperature exceeds 30°C.

5. Transfer intervals need to be empirically derived for each organism, but some general guidance on transfer intervals is outlined in Table 1.

Also note recommendations in SOP on Best Practice for the Management and monitoring of serially transferred microalgal & cyanobacterial master stock-cultures.

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Table 1 Overview of different culture conditions for various taxonomic groups of microalgae

		Light/PAR [µmol m^2s^{-1}]	Temp. [°C]	Medium specificity/ preference	Culture vessel	Transfer cycle [weeks]	Additional comments
Cyanobacteria		10-20	10-25	Alkaline (e.g. BG11)	tubes + flasks	4-12	Low N-medium needed to induce heterocyst formation
Rhodophyta		10-20	15 -20	Medium+ soil extract	Erlenmeyer flasks + tubes	4-6	
Cryptophytes		10-20	15	Specific medium	tubes	4-6	
Desmids		40-100	15	Specific medium	Erlenmeyer flasks	8-12	Many prefer an acidic environment
Dinoflagellates		40-100	15-20	Specific medium + soil extract	cell culture bottles	4-6	Should be maintained in culture at high densities
Euglenoids		30-50	15-20	Axenic taxa mixotrophic	tubes	6-8	
Green Algae	freshwater	40-100	15-20	Many axenic taxa mixotrophic	slant agar tubes	12-30	Some colony forming strains only survive in liquid media (e.g. *Volvox*)
Green Algae	marine	40-100	15-20	Many axenic taxa mixotrophic	tubes	8-12	
Diatoms	benthic	40-100	15	+ SiO_2 + soil extract	slant agar, beads	12-30	Many taxa prefer alkaline environment. Irreversible cell size reduction observed in freshwater taxa
Diatoms	planktonic	40-100	15	+ SiO_2 + soil extract	flasks	4-6	Many taxa prefer alkaline environment. Irreversible cell size reduction observed in freshwater taxa
Chrysophytes		10-20	15	Specific medium	tubes	8-12	
Synurophytes		30-50	15	Specific medium	tubes	8-12	
Eustigmatophytes		30-50	15-20	Many axenic taxa mixotrophic	slant agar tubes	8-12	
Xanthophytes		30-50	15-20	+ soil extract	slant agar tubes	8-12	
Heterotrophic algae		constant darkness	15	Heterotrophic media; soil-water	tubes	4-12	