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Standard Operating Protocol Manual for Seaweed Biomass Cultivation and Analysis.

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Standard Operating Protocol Manual

For seaweed biomass cultivation and analysis

Edwards M.D., Mooney-McAuley, K., Gorman, E. and Champenois, J.

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Chapter 1: Introduction

Modern methods of macroalgal cultivation of the large brown seaweeds commonly and collectively referred to as 'kelp' began in the early 1950's in China, with research spearheaded by 'the father of mariculture', Prof. CK Tseng and his team. Since this time, kelp aquaculture has steadily grown in China and other Asian countries such as Japan, and the Republic of Korea. Now, almost all of the seaweed produced for human consumption, alginates and other purposes comes from aquaculture, with the extent of cultivation clearly visible on satellite images in some regions. Research in these countries has focused on brown algal species including *Saccharina japonica* and *Undaria pinnatifida*, concentrating primarily on developing a number of commercial strains that demonstrate temperature-tolerance of warmer seawater, and latterly, on improved productivity. In China, it is common practice to have centralised seaweed hatchery facilities that produce plantlets for further on-growing at a number of local sea sites. The development of centralised facilities using controlled seaweed strains most likely lends itself to a higher degree of standardised operations during the production cycle.

In direct contrast to the mass culture of seaweed in China and other Asian countries, commercial cultivation production in Europe remains on a small scale. While European research on kelp has existed at least as long as for that conducted in China, seaweed cultivation techniques have been known (generally only within research facilities) for approximately thirty years. For most of this time, cultivation has existed at a demonstration/pilot-scale only, although this is now changing in a number of countries, primarily across NW Europe. It was recognised early on that the Chinese method of growing seaweed by inserting small plantlets into ropes for on-growing at sea would not be economically efficient in Europe, given the cost of labour. Alternative cheaper seeding techniques, as described in this document, have been developed instead. Longline design is also different in Europe, where sea conditions are generally more challenging than the more sheltered areas that typically support Chinese aquaculture. This influences design and of course the cost of equipment that is used. The concurrent European research on wild strains of kelp species, partnered with a strong requirement for developing site specific cultivation systems has naturally led to some variance in production protocols and systems.

The EnAlgae project has allowed its research partners in NW Europe the opportunity to use hindsight of the expansion of global seaweed cultivation to full advantage. A theme of the project (WP1, Action 5) has been 'the development and exchange of best practice for mass production of macroalgae'. For this action to be completed satisfactorily, demonstration/pilot sites (WP1, Action 2) were constructed at the macroalgal partner facilities. The subsequent development of a close working relationship enabled production of comparable data for identified species (*Saccharina latissima* and *Alaria esculenta*) on cultivation methodology, data collection (WP1, Action 3) and macroalgal growth results. The standardisation of all of these elements was designed to produce reliable data and share common experiences between macroalgal cultivation stakeholders in NW Europe, culminating in the production of this manual, and the accompanying Macroalgal Best Practice document.

This document is a manual of collated Standard Operating Protocols (SOPs) that were developed in the three EnAlgae macroalgal hatcheries in the National University of Ireland, Galway (NUIG),

Queen's University, Belfast (QUB) and the Centre d'Etude et de Valorisation des Algues (CEVA). It describes the set-up/requirements of each seaweed hatchery, the hatchery cultivation process, the sea on-growing process, as well as biomass and environmental parameter measurements and sampling. While some SOPs were subject to some inherent differences within each hatchery (e.g. pre-existing seawater pumping and distribution systems), an effort was made to ensure that as many elements of the SOPs were standardised across the partners. For example, the biomass sampling SOP was particularly important to enable the collection of comparable data in Ireland, Northern Ireland and France.

The SOP manual has been designed for reading with the **EnAlgae Macroalgal Best Practice** document. This sister document offers further advice and observations of the methods used, including elements of the processes that worked, and equally importantly, where system failures occurred, and revisions were made. It is hoped that both documents will become a valuable resource for those interested in developing European kelp cultivation in NW Europe and beyond, providing information on techniques that can further refined, leading to ever-increasingly efficient seaweed production at sea.

Chapter 2: Basic Algal Hatchery Techniques

2.1 System Requirements

The basic algal hatchery setup for the first phase of kelp cultivation requires a number of key infrastructures. These include the provision of a temperature controlled hatchery space, additional work/storage space, as well as the equipment to provide sterilised seawater, aeration and lighting. System requirements have been described for hatchery facilities at NUIG Carna Research Station, QUB Marine Laboratory, Portaferry and at the CEVA laboratory. To avoid SOP duplication, only the most relevant differences and observations of each facility's infrastructure are described. Equipment manufacturer names are described where known, however specific machinery sizes/capacities are generally excluded. This is because equipment is often location-specific and can often over-specified because they are also used concurrently in other aquaculture/hatchery systems at the three hatchery facilities.

2.1.1 Water

Purpose

Fully saline seawater (34-35) is extracted from an unpolluted coastal source and used in the hatchery cultivation phase of the kelp species, *Alaria esculenta*, *Saccharina latissima* and *Laminaria digitata*.

Principle

Through a series of pumping, filtration and sterilisation systems, seawater is used as the growing medium for free-living gametophyte phase and the seeded collector phase of the hatchery process for the kelp species. For algal cultures, water quality is extremely important, and should reflect the species cultured.

Requirements and Procedure

NUIG

At the NUIG Carna Research Station, seawater is supplied by 2 Fibroc centrifugal pumps of 15 kW (one operating and one standby) at a continuous mode with a speed of approximately 21 L s^{-1} . A Liquivac Priming pump is necessary to start the 2 main Fibroc pumps. The water then passes through a Bernoulli pneumatically controlled filter system ($250 \mu\text{m}$) to remove suspended solids and compressed air is delivered by Atlas Copco compressors. There are 2 filters and 2 compressors available, but only one of each is on duty at the time. Water quality is further improved by 2 in-line TMC cartridge filters of $10 \mu\text{m}$ and $1 \mu\text{m}$ mesh size, running under pressure from the incoming water, and a TMC UV sterilizer to eliminate harmful microorganisms.

Waste seawater is removed from the algal hatchery through a drainage system to the treatment plant mentioned below. Wastewater is chemically sterilised within the tank by a sodium hypochlorite solution followed by neutralisation with sodium metabisulphite where required (e.g. when culturing strains of algal species not local to the hatchery). The floor of the hatchery is designed with the drain located centrally in the lowest point in the floor so all water flows to this point.

QUB

QUB has an external seawater supply coming directly from Strangford Lough. This seawater is pumped daily into two large settlement tanks where large particles sink. The water is subsequently pumped through a sand filter and around the QUB Marine Laboratory site. PVC piping is used to direct the seawater into the hatchery facility and several tap units are fitted along the pipe lengths to allow isolation of the water supply should leaks develop.

The water supply is directed into two particle filter housings containing a 5 μm spun wound filter and 1 μm pleated filter. From the filters the water is directed into a UV sterilisation unit and then supplies a bank of taps that lead to individual tank units (Figure 2.1).



Figure 2.1. (a) UV sterilisation unit and double filters in housing. Inflow comes via the orange taps (circled) (b) First two tanks in a series of 5 supplied with water by blue taps behind (circled). The tubing below the water line is a fixed air-line.

CEVA

CEVA has an external seawater supply. This seawater is pumped into two large settlement tanks where large particles sink (Figure 2.2 a and b). Time of pumping depends on the tide as the pump emerges at low tide. A second pump and PVC piping are subsequently used to direct the seawater into the hatchery facility. The water supply is directed into filtering cartridges for a two-step filtration procedure (10 μm and 1 μm). From the filters the water is directed into a UV sterilisation unit (Figure 2.3).



Figure 2.2 a) and b) Tanks for storage of seawater at CEVA. Total capacity of 45 m³ (a) and 20 m³ (b).

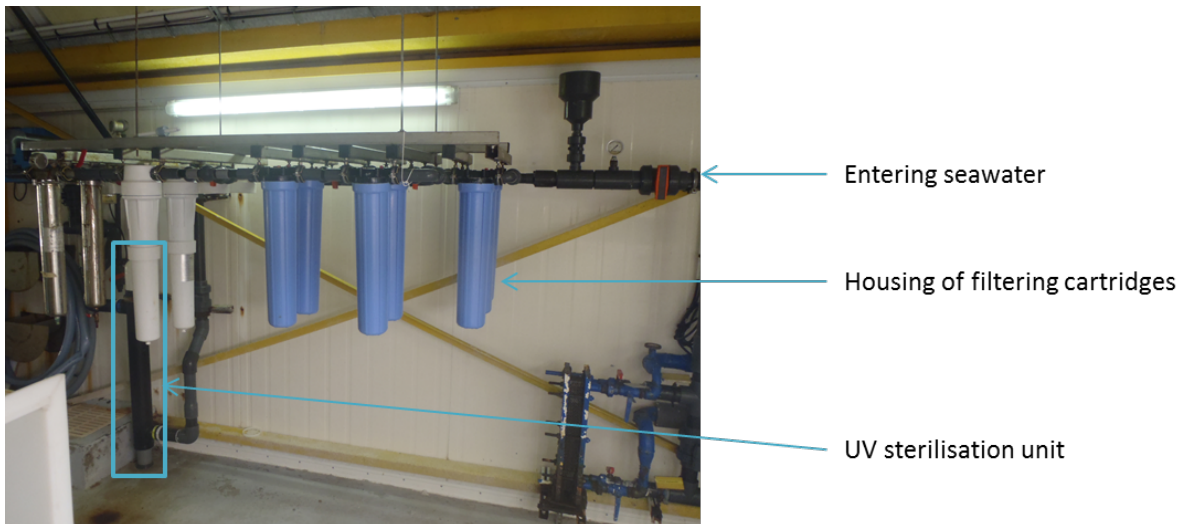


Figure 2.3. Filtration system at CEVA

Hazards and precautionary statements

Maintenance of all pieces of equipment should be carried out by trained and qualified personnel, or by service contract. Maintenance logs should be kept for all equipment, especially as rust can weaken and destroy equipment quickly without proper care and attention. Risk assessments should be carried out on machinery as they age and prior to maintenance. Care should be taken to avoid electrical shocks when operating/maintaining pumps and filters when surrounded by seawater. Always switch machinery off at the mains electrics before maintenance, and isolate units from the seawater supply. In particular, always turn off UV filtration systems and allow them to cool down before handling, taking care not to break the fragile quartz sleeves when cleaning/replacing. These systems should never be turned on during this time, as there is a significant risk of radiation burns from the high-voltage units. In general, filtration and pumping equipment can be extremely heavy. Always wear suitable protective/safety clothing when using such equipment and practise good

manual handling skills where required. Ensure adequate first aid facilities are always nearby, and never work alone where possible.

Quality Control

The quality of the incoming seawater depends on keeping the complex pumping and filtration systems in good working order by trained and qualified personnel. This includes the regular (daily) back-flushing of filters and cleaning pipes using pressurised water forced behind a bullet-shaped brush (sometimes known as a 'pig'). This removes build-up of unwanted organic material (dead and alive), which will ensure no development of high bacterial loads etc. Light penetration should be avoided in reservoirs and pipework as this encourages algal growth. Avoid using stagnant water from pipes by running water to waste for at least 20 minutes before filling tanks.

Despite high levels of filtration contaminating species can invade and grow in tanks (e.g. *Ectocarpus* spp. at QUB). Ensure that tanks, filters, taps, hoses and airlines are regularly cleaned to limit the spread of these species. Filters can be removed from housings and left to dry between water changes to further limit the growth of contaminants.

Waste stream and proper disposal

NUIG

At the Carna Research Station, all wastewater from the hatchery is collected and pre-filtered in a hydrotech drumfilter. A Grundfos pump is installed for backwashing. Suspended solids are removed in order to improve the efficiency of the Wedeco UV disinfection unit where bacteria and viruses are destroyed by high intensity UV before wastewater is released back into the sea. Dispose of obsolete equipment as per manufacturer's recommendations.

CEVA

Seawater effluents are discharged directly to sea (any seawater with non-native organisms is pre-treated and disposed of separately). Freshwater effluents are collected and treated as a municipal sewage at the wastewater treatment plant of the municipality where the facility is located.

References

- Edwards, M.D. and Watson, L. (2011). Cultivating *Laminaria digitata*. In: Aquaculture Explained No. 26 (Edited by Watson, L.). 71pp. Bord Iascaigh Mhara, Dublin, Ireland.
- Golven. P., Le Goff, T. and Champenois, J. (2014). Seeding techniques used at CEVA for cultivation of *Saccharina latissima*. Report produced by Centre d'Etude et de Valorisation des Algues, Pleubian, France.
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2.1.2 Temperature Control

Purpose

A temperature-controlled, insulated room is required as an integral part of an algal hatchery. Seawater held in tanks and in other culture vessels needs to be maintained at a constant temperature, often when seasonal temperature variations occur across winter and summer. Water

temperature can be controlled directly in the tanks/vessels, or indirectly through control of air temperature surrounding the vessels.

Principle

The insulated algal hatchery room should be held at a constant temperature of approximately 10-13 °C (± 1 °C) for kelp cultivation of *Alaria esculenta*, *Saccharina latissima* and *Laminaria digitata*, depending on local sea conditions. Multiple culture vessels/tanks are exposed to chilled air, which ensures large amounts of water can be chilled to the same temperature at the same time.

Requirements and Procedure

NUIG

At the NUIG Ryan Institute Carna Research Station, a ceiling-mounted Searle room air chiller maintains an all-year round temperature of 10 °C for a series of tanks ranging from 250 L to 1000 L as well as numerous 6 L glass culture vessels held on enclosed plastic shelving units.

QUB

Adequate insulation lines the ceiling and door units to help maintain room temperature control. A Qualitair Invicta ICCe refrigeration unit (Figure 2.4) is attached to the exterior wall and provides the airflow and chilling for the entire room to 10°C \pm 2°C.



Figure 2.4. Qualitair Invicta ICCe Refrigeration unit attached to external wall of hatchery in QUB.

An incubation unit/culture cabinet is required if gametophyte cultures are desired. The unit must have in-built lighting, lighting timer control, chilling capacity to 10 °C, shelving and air-line inputs. At QUB, an LEEC incubation cabinet was purchased for this purpose. The cabinet doors are screened internally with either red or blue cellophane depending on what stage in the life cycle is required for gametophyte cultures (Figure 2.5).



Figure 2.5 a) LEEC incubation cabinet external view and (b) Internal view with gametophyte cultures on shelves behind red light at QUB hatchery.

CEVA

Whenever there is a need to decrease the temperature of the water in tanks containing collectors, individual aquarium chillers are used (temperature setting: 13°C) (Figure 2.6).

For smaller vessels (e.g. gametophyte flasks), culture cabinets were used which have lighting, temperature control, shelving and air-line inputs. CEVA has 5 culture cabinets (2 x 500L; 3 x 260L), allowing different temperature and/or light settings, depending on species requirements (Figure 2.7).

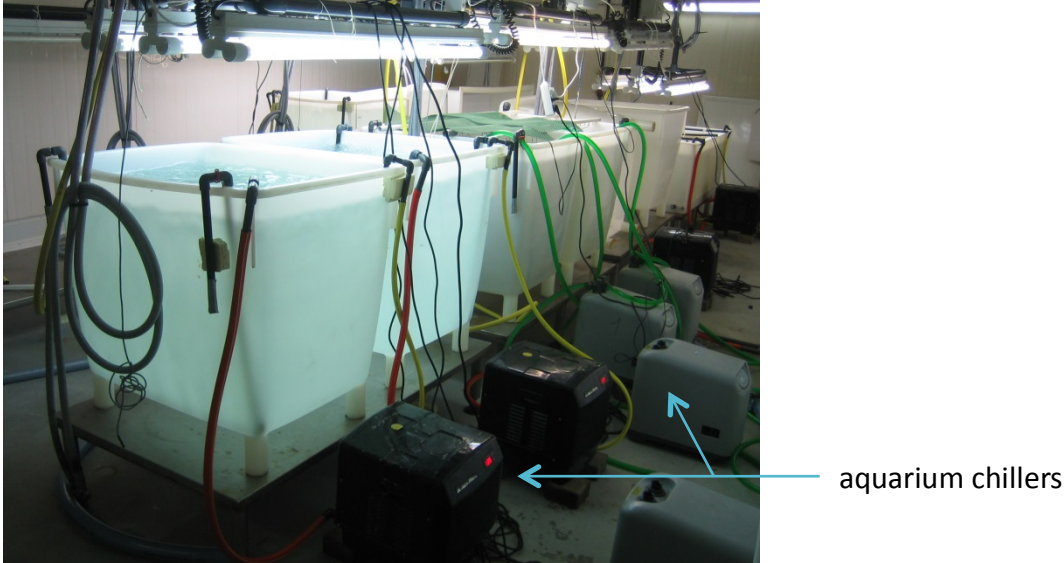


Figure 2.6. View of CEVA hatchery tanks in the hatchery, with seawater in individual tanks controlled by dedicated aquarium chiller units.



Figure 2.7 (a and b). Culture cabinets of 260L (a) and 500L (b), the latter being operated under red light for vegetative growth of *Saccharina latissima* gametophytes at the CEVA hatchery.

Hazards and precautionary statements

Maintenance of all pieces of equipment should be carried out by trained and qualified personnel, or by service contract. Maintenance logs should be kept for all equipment, especially as rust can weaken and destroy equipment quickly without proper care and attention. Risk assessments should be carried out on machinery as they age and prior to maintenance. Whilst the chilled hatchery room is needed for the algae, this environment can be difficult for the operator to work in for any length of time (e.g. greater than 1-2 hours). Wear suitable warm and waterproof clothing and footwear at all times, and take regular breaks in a warmer area.

Quality Control

Maintenance of all pieces of equipment should be carried out by trained and qualified personnel, and should be carried out on an annual basis to ensure continuity of chilled air supply. A temperature logger can be installed in the room to monitor air chiller performance on a daily or weekly basis.

Waste stream and proper disposal

Dispose of obsolete equipment as per manufacturer's recommendations.

References

- Edwards, M.D. and Watson, L. (2011). Cultivating *Laminaria digitata*. In: Aquaculture Explained No. 26 (Edited by Watson, L.). 71pp. Bord Iascaigh Mhara, Dublin, Ireland.
- Golven. P., Le Goff, T. and Champenois, J. (2014). Seeding techniques used at CEVA for cultivation of *Saccharina latissima*. Report produced by Centre d'Etude et de Valorisation des Algues, Pleubian, France.

2.1.3 Aeration

Purpose

Seawater culture media used in the hatchery phase of the kelp species, *Alaria esculenta*, *Saccharina latissima* and *Laminaria digitata* is provided with an essential air supply for successful large-scale cultivation.

Principle

A dedicated air supply for free-living gametophyte cultures is essential to keep large stocks of this microscopic kelp phase in suspension and exposed to the available nutrients and light. Air supply to the larger tanks containing gametophyte-sprayed collectors is important. Lack of adequate air quickly develops 'dead spaces' in tanks, resulting in the degradation and loss of cultures. Air supply also has an important developmental role to play in strengthening developing sporophyte holdfasts before deployment at sea.

Requirements and Procedure

NUIG

A small to medium-sized air blower is required for an algal hatchery. While small electric 'aquaria' air pumps are useful for cultures flasks with volumes of approximately 10 L, a dedicated air blower will provide the necessary air supply for much larger tanks. In the Carna Research Station, air from the air blower is delivered to the algal hatchery through a pipe network that runs around the room in a ring.

Air supply from the ring system is via adjustable taps or a manifold inserted into the pipe and connected to air hose (e.g. silicon, PVC or vinyl), connecting to air stones of varying sizes depending on the tanks. Air supply is controlled by the taps themselves, or by using clamps on the air hose.

QUB

Air supply is controlled by an industrial air pump, which supplies all of the QUB outbuildings. A smaller scale air pump with sufficient power could also be used. Each tank in the hatchery will require an air supply, as will the incubation unit for cultures.

CEVA

In the hatchery, an air blower provides air for both seaweed and microalgae productions. Air is delivered in seaweed tanks through PVC piping. For smaller vessels up to 10-20L electric 'aquaria' air pumps are used.

Hazards and precautionary statements

Air blowers generate a considerable amount of heat when in use. If this piece of equipment is placed in a room at ambient temperature and is pumping air into a much colder room such as a hatchery, a significant amount of condensation forms inside the air pipes. It is therefore useful to incorporate a filter and a number of valves into the pipe network to drain the condensation from the system.

Quality Control

Drain condensation water from inside the air pipes as part of a weekly routine. Insert reusable in-line air filters (e.g. Whatman Hepa-Vent) onto culture flasks to prevent aerial-borne contamination of cultures. Clean air hoses and air stones with sodium hypochlorite (subsequently neutralised or rinsed thoroughly in fresh water) as part of the tank water exchange/cleaning process.

Errors and interferences

A standby blower connected to the system is recommended to prevent interruption in supply.

Waste stream and proper disposal

Dispose of obsolete equipment as per manufacturer's recommendations.

References

- Edwards, M.D. and Watson, L. (2011). Cultivating *Laminaria digitata*. In: Aquaculture Explained No. 26 (Edited by Watson, L.). 71pp. Bord Iascaigh Mhara, Dublin, Ireland.
- Golven. P., Le Goff, T. and Champenois, J. (2014). Seeding techniques used at CEVA for cultivation of *Saccharina latissima*. Report produced by Centre d'Etude et de Valorisation des Algues, Pleubian, France.

2.1.4 Light

Purpose

Supply of appropriate irradiance at a particular light intensity, photoperiod and spectrum is an essential requirement for photosynthesis and therefore production of gametophyte and sporophyte cultures in the algal hatchery.

Principle

Photosynthetically active radiation (PAR) at suitable intensity in the range of 400-700 nm is required for algal photosynthesis. However, maintenance of gametophyte growth and stimulation of reproduction are controlled by both day length (photoperiod) and defined parts of the visible light spectrum.

Requirements and Procedure

NUIG

At the Carna Research Station, lights are suspended on chains from the ceiling in a grid pattern so that tanks of varying sizes can be placed underneath. The lights are then moved closer or further away from tanks by lengthening or shortening the chains, allowing variation in light intensity for different stages of gametophyte and sporophyte development. Double-bulb fluorescent lights are used in the algal hatchery as they do not emit a large amount of heat and provide a relatively stable light at the correct light spectrum. Waterproof housings are fitted to the bulbs so that the lights can be used near water; these housings are essential for safety reasons. The lights are used in conjunction with timers so that day length can also be manipulated. For gametophyte cultures, the colour of the light transmitted to cultures can also be manipulated by covering the light fitting in a layer of coloured cellophane (see Section 2.2.2 and 2.2.3).

For specific day length, and light intensity requirements for cultures, see Sections 2.2.2, 2.2.3 and 2.3.2 and 2.3.3.

QUB

Lighting units are suspended from chain from the ceiling and are height adjustable (Figure 2.8). They are single units (sufficient for QUB cultures) and contain T5 Linda bulbs with a dimmer capacity.

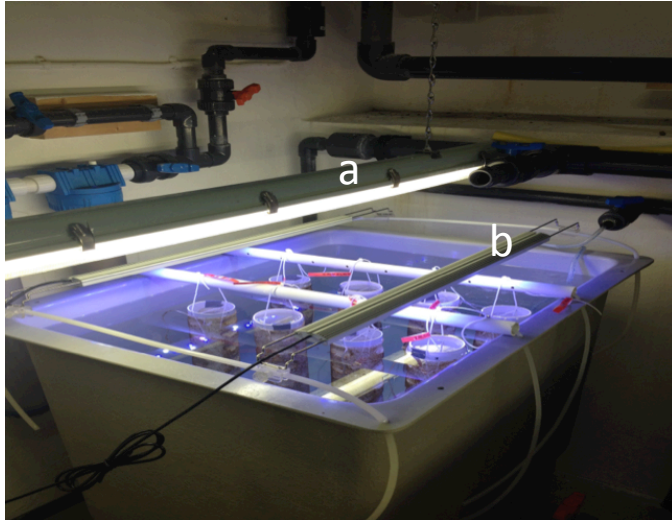


Figure 2.8. Overhead tank light fittings: (a) T5 Linda bulb lighting (b) Arcadia strip LED lighting

One lighting unit serves each tank. An alternative and more economic light option are Arcadia LED strip lights (Figure 2.8). Lights must be able to be timer programmed, either with simple plug timers or a custom made system (Figure 2.9). For light sensitive/manipulation experiments, medical curtains were added to surround each water tank. These are suspended on ceiling rails and allow screening between tanks exposed to different light regimes.



Figure 2.9. Custom made lighting unit at QUB for T5 Linda bulbs with dimmer and programmable clock system for each light unit.

CEVA

Lighting units are suspended from the ceiling and are height adjustable. They consist of double strips packed per two and contain fluorescent tubes delivering white light (some references: OSRAM L58W/880 Lumilux Skywhite; Polylux XLr F58W/840). Each fluorescent light unit serves two tanks,

while one tank is illuminated by 4 units (Figure 2.6). Waterproof housings are fitted to the bulbs so that the lights can be used near water. Lights are timer programmed.

Hazards and precautionary statements

Risk of electrocution when using electric lighting near tanks of water. Always use lights units as per manufacturers instructions, and get a qualified electrician to construct and/or check construction of the units/timer sets. Make sure that light units are attached securely to a supporting overhead frame or ceiling when used near/above water. Lights should also be used with a fully compliant IP66 waterproof plug, which should be connected to an IP66 electrical distribution box containing the light timers. Position the electrical distribution box on a wall as far away from accidental water spillages as possible.

Quality Control

Use a calibrated handheld PAR light meter to check irradiance received to each tank at the water surface. Replace old bulbs as they dim or expire.

Waste stream and proper disposal

Dispose of glass fluorescent bulbs as per regional recycling/disposal advice. Light fittings and other electrical components can be disposed of as WEEE rubbish.

References

- Edwards, M.D. and Watson, L. (2011). Cultivating *Laminaria digitata*. In: Aquaculture Explained No. 26 (Edited by Watson, L.). 71pp. Bord Iascaigh Mhara, Dublin, Ireland.
- Golven. P., Le Goff, T. and Champenois, J. (2014). Seeding techniques used at CEVA for cultivation of *Saccharina latissima*. Report produced by Centre d'Etude et de Valorisation des Algues, Pleubian, France.

2.1.5 Additional Information

Purpose

The main system requirements for kelp cultivation have been described in Sections 2.1.1 to 2.1.4. Section 2.1.5 briefly describes further useful system requirements and large pieces of infrastructure that are in use at the three macroalgal hatcheries.

Principle

Biological requirements have been discussed in terms of light, aeration, temperature control and provision of water. The following information discusses general technical requirements covering workspace, freshwater supply, and water sterilisation.

Requirements and Procedure

In addition to the temperature-controlled room, a nearby ambient temperature room is also used for microscope work, changing of culture media, storage of equipment, paperwork such as maintenance logs/schedules etc. This is a more comfortable environment for the hatchery operators, while it also aids the preservation of expensive equipment, and protects against degradation from salt water.

This room should come equipped with a freshwater supply of hot and cold water, as well as a deep sink and draining area. This is required for cleaning of culture flasks and other consumable equipment. Flasks are cleaned using a phosphate-free laboratory detergent (Decon 90) in advance of seawater sterilisation. A supply of distilled water is also extremely useful for the final rinse of glassware, therefore the still for generating this water should be located near the sink so that the water supply can be easily connected for distillation and cooling.

Seawater sterilisation (as well as equipment sterilisation) can be achieved by tyndallisation (Edwards and Watson, 2011), or by using an autoclave. In NUIG Carna Research Station, seawater required for flask culture of gametophytes is sent to a large Astell autoclave for complete sterilization (~0.3 m³ capacity which caters for approx. six 6-L culture flasks at a time. QUB have a similar sized autoclave, and both run on a standard sterilisation programme of 121 °C at 1 bar for 20 minutes.

Hazards and precautionary statements

Stills and autoclaves both require heavy-duty electrical wiring, and should be wired by professional electricians as per manufacturer's instructions. There is a risk of burns from equipment and water in culture vessels when using both the still and autoclave. Production of distilled water can take a long time; do not forget to turn off still after use, and do not allow still to run dry, as there is a risk of equipment failure. Do not open the autoclave before it has fully depressurised and cooled down (up to 12 hours later) and ensure that the autoclave water reserve is full at all times. Make sure that culture vessels do not become pressurised with steam – loosen caps or bungs on vessels to ensure steam vents. If a culture vessel does leak seawater into the autoclave, remove and replace the water reserve with fresh water to avoid undue corrosion.

Quality Control

Avoid potential contamination and culture crashes by ensuring the cleaning protocol for glassware is carried out properly and glassware is perfectly clean before subsequent use. Use autoclave tape on culture vessels to ensure that the autoclave has brought the seawater to the correct temperature for sterilisation.

References

- Edwards, M.D. and Watson, L. (2011). Cultivating *Laminaria digitata*. In: Aquaculture Explained No. 26 (Edited by Watson, L.). 71pp. Bord Iascaigh Mhara, Dublin, Ireland.

2.2 Gametophyte Culture Maintenance

Kelp cultivation requires the understanding and control of the microscopic gametophyte life stage of these algae, which for the purposes of EnAlgae, include *Saccharina latissima*, *Alaria esculenta*, and *Laminaria digitata*. The following generalised SOPs on culture maintenance can be used for any of the algae mentioned. Where species-specific tasks/conditions are required, this is stated. The culture maintenance starts with the selection and preparation of gametophyte cultures from wild fertile material, followed by the requirements for the development and maintenance of free-living gametophyte cultures. Finally, the process of inducing gametogenesis and initiation of

juvenile sporophytes are described. The requirements and procedures are adapted from Edwards and Watson (2011) and extended to cover all three species.

2.2.1 Selection and preparation of fertile material

Purpose

Under current practice, all kelp cultures start with the collection of fertile sporophytes. This is a seasonal activity, specific to each species that can also be geographically variable. It is important to be able to identify fully mature fertile sori of collected sporophytes for most efficient zoospore release, as this can also be variable across individuals, location and time.

Principle

Molecular studies of kelp populations are still underdeveloped in Europe and beyond. Therefore, it is still difficult to assess what impact kelp cultivation will have on the genetic diversity of native populations of the same species found nearby. Until more research can take place, a conservative, best practice approach is taken within the EnAlgae project to only develop gametophyte cultures for future seeding from fertile sporophytes collected locally to the cultivation site.

Requirements and Procedure

Fertile kelp species can be found around many Western European shores at different latitudes (see Algaebase.org for specific details of individual species). They grow on rocky shores (bedrock and or boulders) in the lower intertidal and subtidal regions, often in moderate to wave and current-exposed locations. Collection and release of zoospores from fertile material is a two-day process, with collection and preparation of the fronds containing sorus completed on day 1, followed by zoospore release on day 2.

When collection of fertile material is required, use the relevant tide and correction tables for the relevant area of coastline³, and identify the spring tides at the appropriate time of year (Table 2.1). In Ireland, choose a day when the height of water above chart datum at low tide is approximately 0.6-0.9 m. Collection of kelp is easiest on these days, as kelp beds are not generally exposed when the tidal height is above 0.9 m. This may vary according to country coastline and specific tidal range.

Table 2.1. Reproductive seasons of three species of kelp cultivated in the EnAlgae project in Ireland/Northern Ireland (North East and West coasts) and France (Brittany).

| Species | Location of sori | Main reproductive Season | Applicable Country |
|-----------------------------|---|---|----------------------------|
| <i>Alaria esculenta</i> | On sporophylls: specialised structures that develop on the holdfast | October to November | Ireland (W) |
| <i>Saccharina latissima</i> | Centrally, down much of the length of the frond | November to February November to January | Ireland (W) France (NW) |

³ For tide forecasts around Europe and the rest of the world, use either [Easytide](#) or [Tide-Forecast](#)

| | | | |
|---------------------------|--|--------------------------------------|--------------------------------------|
| <i>Laminaria digitata</i> | In small to large irregular patches on the distal ends of each blade | April to November May to December | Ireland (W) Northern Ireland (NE) |
|---------------------------|--|--------------------------------------|--------------------------------------|

Once on the shore, choose individuals with clearly defined patches of sorus (Table 2.1 and Figure 2.10). Cut only the pieces of blade with the sori that are required – it is not necessary to remove the whole plant. If the available kelp bed is far from the hatchery/laboratory, wrap the pieces of blades in laboratory tissue soaked in seawater and keep cool by placing in a sample bag in a cool box containing frozen ice blocks.

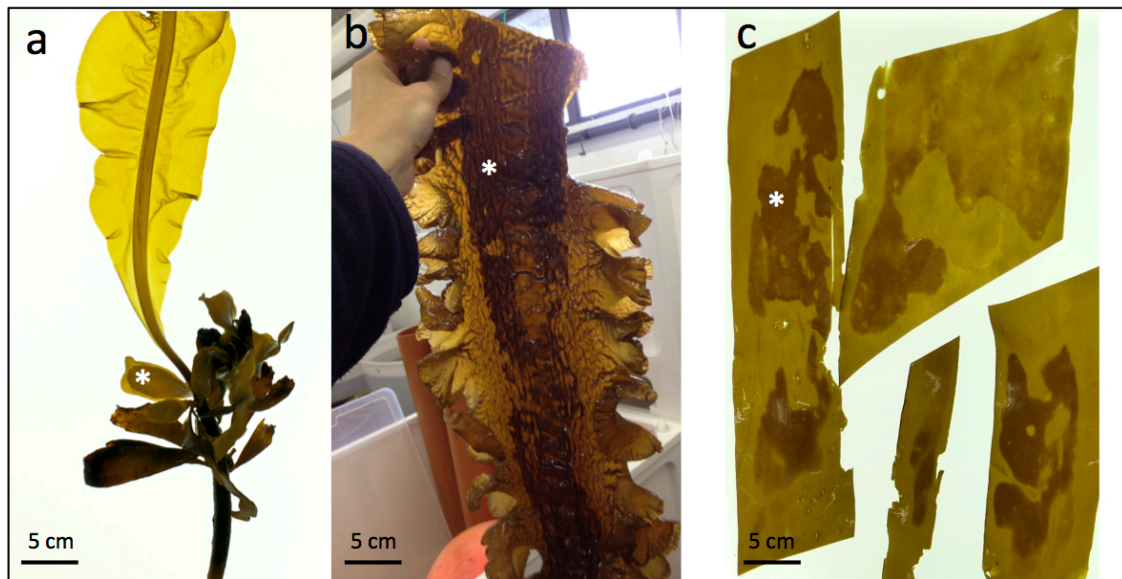


Figure 2.10. Photographs showing presence of mature sorus (marked with a white asterisk) on a) *Alaria esculenta*, b) *Saccharina latissima* and c) *Laminaria digitata*.

Back in the laboratory, the pieces of thalli/blade/sporophylls are prepared for spore release by selecting only the reproductive sori and cleaning these areas. Use a scalpel to cut around large areas of sorus roughly, to remove as much sterile tissue as possible. Ensure any small patches of fouling organisms are excised from the pieces. Discard the sterile tissue.

Clean the sori by dipping laboratory tissue into sterilised seawater (SSW) and wiping both sides of the piece of sorus firmly. Discard the tissue, and using a clean, dry piece of tissue, firmly wipe the sorus dry. Repeat this process 3-4 times. For species such as *S. latissima* that don't have a smooth thallus, use a soft toothbrush or small bottlebrush dipped in SSW to clean the sorus before wiping dry with tissue. Once all pieces of sorus are prepared, place between several layers of tissue in a tray, and keep in a dark, cool place (~ 10°C) for 18 to 24 hours.

QUB

If the return journey to the lab is anticipated to be a long one, kelp can be wrapped in damp lab roll and placed in a cool box with ice packs. On return to the lab, use a scalpel or razor blade to cut away all non-fertile material, and dry sori thoroughly with paper towel. Take care at this stage to remove any epiphytes that may be growing.

Immerse sori in a bath of 5% bleach and autoclaved seawater for no more than 5 min. Remove from bleach solution and rinse 3 times in autoclaved seawater (you may see spores already releasing at this stage). Wipe dry with paper towel between each rinse.

Use a soft bottlebrush to perform a final clean on the sori then rinse and dry with paper towel. Wrap the sori in damp (UV seawater) paper towel, place into a plastic bag and refrigerate overnight.

CEVA

Twenty five to thirty small pieces (4 to 5 cm²) of ripe material are cut from the blades, avoiding edge part and dirty areas. The fragments are put in a first basin filled with sterile seawater and brushed to remove animals and epiphytes.

Fragments are bathed during 10 minutes in a second basin filled with 0.5% chlorinated sterile seawater (chlorinated seawater is achieved by adding 5 ml of bleach (2.6% of chlorine) into 1 L of sterile seawater). Then, the fragments are brushed carefully. This operation (10 min bath in 0.5% chlorinated sterile seawater + brushing) is repeated once more in a 3rd basin. NB: Bathing in bleach must not exceed 10 minutes per fragment.

Fragments then undergo successive rinsing in basins filled with sterile seawater. At the end, fragments are carefully wiped with tissue paper. Fragments are placed in a trail, covered with aluminum and stored at room temperature (10 - 12°C) in the dark for 18 to 24h.

All the operations are carried out with a pair of tweezers thoroughly disinfected between each step with acetone (or alcohol). Fragments that would fall on the laboratory bench are thrown away. Coloring of the water and brown marks on the tissue paper during the cleaning procedure are signs of good maturity of the blades, but too much color, especially in water bath indicates improper crop conditions or storage (too much time before arrival at the laboratory or prolonged exposure to drying).

Hazards and precautionary statements

Collection of fertile material can be carried out by foot on spring low tides, or by SCUBA. Extreme care must be taken when collecting material using either method. Normal shore work common-sense applies: wear suitable footwear and wet gear; always carry a mobile phone; check the times of the tide carefully and always work down the shore with a dropping tide. Do not go on the shore unaccompanied, and always tell someone where you are going and what time you expect to return. When diving, always go sampling with a buddy, sample in fine weather to minimise unnecessary complications (e.g. reduced visibility, large swell etc.), and always tell someone where you are going and what time you expect to return.

Quality Control

Much of the contamination that may occur in the gametophyte cultures referred to in Section 2.2.2 can be avoided with careful choice of initial reproductive material. Avoid fronds that have obvious epiphytes growing on them (e.g. filamentous brown algae), or have a rough texture. Sori and the sterile tissue surrounding it should be clean and smooth with as few nicks or cuts visible as possible

(source of potential refugia for epiphytic organisms). Once the sori are being prepared for release in the laboratory, always pay meticulous attention to the cleaning process to ensure a low to no contamination of microscopic epiphytes such as diatoms and ciliates.

Waste stream and proper disposal

Do not throw waste pieces of kelp back into the sea after choosing fertile sori in case the pieces are from a distant location and introduce associated flora and fauna or release zoospores into local hatchery area. Garden composting is a safe way of disposal, whilst retaining some final value of disposed pieces.

References

- Edwards, M.D. and Watson, L. (2011). Cultivating *Laminaria digitata*. In: Aquaculture Explained No. 26 (Edited by Watson, L.). 71pp. Bord Iascaigh Mhara, Dublin, Ireland.
- Golven. P., Le Goff, T. and Champenois, J. (2014). Seeding techniques used at CEVA for cultivation of *Saccharina latissima*. Report produced by Centre d'Etude et de Valorisation des Algues, Pleubian, France.

2.2.2 Establishment and maintenance of gametophyte cultures

Purpose

Gametophyte cultures are established from developing zoospores released from fertile sporophytes. For large-scale sea cultivation, moderately large amounts of gametophyte cultures are required. These take time to develop and grow, and must be maintained carefully to avoid culture contamination and subsequent crashes.

Principle

The initiation of gametophyte cultures is dependent on time of year that sporophytes become fertile. However, this time of year may not be suitable for developing and deploying seeded strings straight away as in a direct seeding deployment (see Section 2.3.3). Instead, gametophyte cultures are developed over a period of months until they are required for further use in a sea deployment at a suitable time of year (e.g. mid to late winter).

Requirements and Procedure

NUIG

At the NUIG Carna Research Station, all seawater required for gametophyte cultures is sterilised in an autoclave in advance. The autoclave is a large capacity model that can take 8 to 10 6-L vessels at a time, with an approximate cycle of 3-4 hours, making the sterilisation process more efficient. Glass (or plastic Nalgene®) vessels are filled with filtered and UV-sterilised water (SSW) (Section 2.1.1) to approximately $\frac{3}{4}$ capacity, and the mouth of the vessel covered either with the lid (only loosely screwed, so that steam can escape), or a folded piece of strong tinfoil. A standard autoclave programme of 121°C, 1.03 bar (15 lb in⁻²) for 15 minutes is used for the sterilisation process. Autoclave tape is used to indicate a full sterilisation programme has been successful.

To release the zoospores, remove the prepared pieces of sori from the dark cupboard or box and cut them into smaller pieces (4 or 5 cm²). Place in a 1-L beaker and add cool, sterilised seawater. Cover the beaker (e.g. with tinfoil or parafilm) and leave the sori to release zoospores for 30-45 minutes (or

up to an hour if release is slow), stirring occasionally with a clean spatula. The seawater becomes cloudy from spores released from the pieces of sori. Using the phytoplankton netting (e.g. 30 µm mesh size) in a funnel to remove the pieces of blade, strain the zoospore suspension into a suitable culture vessel containing SSW.

Add further SSW to the spore suspension to fill the vessel, if required. Approximately 10-15 cm air space is left between the water level and the neck of the vessel to ensure the culture does not come in contact with the bung when the culture is aerated. Nutrients are then added zoospore suspension in the form of a pre-prepared f/2 media (in powder form, from Varicon Aqua⁴). Dosage of media is administered at 0.1 g per litre SSW.

A pre-sterilised glass tube is inserted into the culture vessel, and secure with a cotton wool bung. If there is a doubt about the sterility of the glass tubing, it is passed through a flame before use. A piece of parafilm is placed over the bung and glass tubing to seal the top of the vessel. The glass tubing is connected to an air-supply via a length of plastic tubing to provide aeration and water movement for the culture.

The amount of biomass per culture flask must be increased in the next 3 to 5 months. This is achieved by keeping the cultures in motion and regularly exchanging the media, every 10-14 days. When cleaning cultures, the air tubing and cotton wool bung are removed, but the parafilm cover is retained over the mouth of the flask (to reduce air-borne contaminants). If the gametophytes have adhered to the flask walls, a sterilised spatula or piece of modified glass tubing is used to scrape the gametophytes into the main culture. Allow the gametophytes to settle onto the bottom of the culture vessel, which may take approximately 30 min, depending on amount of biomass in the vessel. Spent seawater is gently poured off without losing too many gametophytes with it. If the biomass has been disturbed, allow it to settle again, before continuing to pour off as much of the excess seawater as possible. For example, in a 6-L flask containing approximately 5 L of culture, 3.5 to 4 L of seawater is removed, leaving the biomass remaining in the final litre.

Decant the gametophytes and remaining medium into newly sterilised glassware, and fill with new SSW. Add the appropriate amount of nutrients for the volume, all the while maintaining a cover on the new flask between additions of seawater, nutrients and culture. Introduce an air supply to the culture and replace the vessel in the light regime described above.

Laboratory conditions in the NUIG Carna Research Station include the following: Light units are covered in red cellophane, with irradiance at the surface of the glassware between 15-20 µmol m⁻² s⁻¹ which is adjusted by moving the light source closer or further away from the culture. A timer sets the lights to function in long days; 16 to 24 hours of light per day are sufficient for gametophyte culture vegetative growth. Air temperature is maintained at 10 °C.

Moderate aeration of cultures is achieved by adjusting the position of the glass tubing within the culture vessel to obtain a steady, moderate aeration. For example, the tubing is lifted off the base of

⁴ All-in-one f/2 nutrient media in powder form available from:
<http://www.variconaqua.com/productsandservices/algalnutrients.php>

the vessel slightly to increase the airflow, or a clip or an adjustable tap is used on the plastic tubing from the air supply to reduce the airflow.

QUB

After 12-24 hrs refrigeration, remove sori. Use a scalpel or razor blade to cut into squares approx. 2 – 3 cm² and weigh the total mass. Transfer weighed material to a 2-litre beaker, and add enough cold autoclaved seawater to cover the sori. Cover with an aluminium foil lid to prevent spills, secure to a shaker table and leave to shake for around 30 - 45mins, with enough speed to suspend the sori in the seawater. The seawater should be brown in colour after this period. Filter the sori suspension through a 35µm mesh into a beaker to remove debris.

For gametophyte culture: prepare a flask containing UV filtered autoclaved seawater by adding 1ml L⁻¹ of F/2 medium and 0.5 ml L⁻¹ of GeO₂ solution. Growing gametophyte cultures are maintained within an incubator at approx. 10 °C, with the doors screened with red cellophane. Irradiance at the surface of the flasks should be approx. 15-20 µmol m⁻² s⁻¹ for 16-24 hrs day⁻¹, and flasks are constantly aerated.

CEVA

Additional glassware that is required to be sterilised (e.g. beakers, additional empty glass culture vessels) can be heated to 170°C for 15 min in the oven. For the equipment that cannot be sterilized by heating or autoclave, disinfection is done with acetone or alcohol.

Spore release is similar to the NUIG protocol, however according to Golven et al. (2014), the fragments are immersed in a beaker containing 200-250 mL of sterile seawater stirred by a magnetic stirrer at room temperature. The emission of spores is immediate and massive and zoospores can be seen under x10 magnification. Fragments of seaweeds are removed after 10 to 15 min.

Germination of spores into gametophytes can occur only after spores attach to a support. Here, the provided support is the precipitate produced by mixing solutions Miquel A and Miquel B. The day prior to seeding the flask with gametophytes, set up the 2L round-bottom flasks filled with sterile seawater in the thermostatic room at 15°C. Add nutrients to the flasks at the following concentrations:

- Miquel A: 2mL/L
- Miquel B: 1mL/L
- Provasoli P6: 1mL/L
- Germanium oxide GeO₂ solution: 5mL/L

Miquel solutions (Annex 1) lead to the formation of a precipitate, which will provide a support for attachment of the spores. Thus, it is important not to move the flask so the precipitate does not sediment at the bottom of the flask.

Seed the 2L flask by adding carefully 100 to 150 mL of the spore solution. Five days after seeding (time required for spore germination), slight air bubbling is allowed in the flask. The bubbles help to

break the precipitate so the filamentous gametophytes get free in the solution.

Use red light and maintain light intensity at $10 \mu\text{mol m}^{-2} \text{s}^{-1}$ continuously (photoperiod 24:0 L:D). The conditions of temperature (15°C) and light quality and quantity inhibit gametogenesis and allow vegetative growth of the filaments.

The general condition of the culture is monitored visually on a daily-basis. Air supply is adjusted so that the medium is homogeneous and flasks are manually agitated to prevent deposits on the inner surface. Culture medium is renewed every week and microscope observation allows controlling the general condition of the filaments, the proportion of both sexes and any source of pollution.

During maintenance of the gametophyte cultures, stop the air supply into the flask to allow gametophytes to deposit (30 min. to 1 h) and then remove the supernatant and blend the concentrated gametophyte solution in a beaker (30-40 seconds).

Pour the blended solution of gametophytes into a new flask filled with sterile seawater. If the density is too high, divide into 2 new flasks or inoculate in 6-liter flasks. (In the case of strain conservation, inoculate the new flask with half of the blended gametophytes solution and discard the excess). Enrich the medium by adding nutrients solution (Annex 1). Replace the flask in the thermostatic chamber and set up air supply again.

Hazards and precautionary statements

Culture establishment and maintenance requires the use of heavy glass or plastic vessels containing 6-20 kg of water, depending on the culture requirements and facilities. Ensure that correct manual handling is practised to avoid unnecessary damage to the operator's back. Take care not to use glassware that is chipped or broken. Throw out culture vessels with even the smallest of cracks or damage as these can easily shatter when handled too roughly, or during the pressurised and high temperature conditions of the autoclave. Care is also required when handling glassware after using the blowtorch as it will be extremely hot to the touch for several minutes afterwards.

Quality Control

Ensure that glassware is always perfectly clean; using a series of washes and rinses to achieve this. Culture vessels should be washed in a very small amount phosphate-free detergent, such as Decon90 in hot water. The inside of the vessels should be scoured with a good quality bottle brush, the outside should be scrubbed with a mildly abrasive pad, similar to those used in the kitchen to clean pots and pans. Vessels should then be rinsed thoroughly inside and out with hot tap water, inspected and cleaned and rinsed again if necessary. Once thoroughly clean, rinsed inside and out with distilled water and invert to drain on a clean surface (e.g. draining rack or tissue paper).

Always be aware of contamination and cross-contamination throughout the whole maintenance process. Practice good laboratory techniques as per Edwards and Watson (2011) and Golven *et al.*, (2014).

Waste stream and proper disposal

Discard the rehydrated sori used for spore release as per other pieces of sporophyte in Section 2.2.1. A gametophyte culture needs to be disposed of when it becomes contaminated, or it is no longer needed. Do not dispose of any gametophyte cultures by pouring them down the drain or into the sea. They can be placed in the autoclave and killed by heat or they can be killed by addition of sodium hypochlorite (bleach). Alternatively, very hot water (e.g. ~70 °C) may also kill off smaller amounts of culture.

References

- Edwards, M.D. and Watson, L. (2011). Cultivating *Laminaria digitata*. In: Aquaculture Explained No. 26 (Edited by Watson, L.). 71pp. Bord Iascaigh Mhara, Dublin, Ireland.
- Golven. P., Le Goff, T. and Champenois, J. (2014). Seeding techniques used at CEVA for cultivation of *Saccharina latissima*. Report produced by Centre d'Etude et de Valorisation des Algues, Pleubian, France.

2.2.3 Estimation of zoospore density using a Coulter Counter or haemocytometer

Purpose

This Standard Operating Procedure (SOP) will specify the method employed by Queen's University Belfast (QUB) to quantify the density of spores released by a given weight of fertile adult macroalgal sporophytes for their subsequent use in a macroalgal hatchery. This SOP has been applied to the species *Laminaria digitata*, *Alaria esculenta* and *Saccharina latissima*.

The procedures outlined in this SOP will aid in ensuring accuracy and completeness of datasets as defined by the users in the EnAlgae project.

Principle

The method for release of kelp spores from fertile adult kelp sporophytes which are either used for direct seeding of culture twine, or are used to develop gametophyte cultures has been outlined in SOP 2. This SOP outlines the method of quantifying these spore releases. Spore releases can be highly variable and are not necessarily correlated to the weight of fertile material from which they are released. Variability can also occur seasonally. This method should be employed with every spore release to give an accurate quantification of spore density.

Requirements

Equipment and materials:

- Microscope with x10 objective
- Hand tally counter clicker
- Neubauer haemocytometer slide
- Lugol's iodine
- Glass Pasteur pipette
- Approx. 20-50ml sample of spore suspension.

Procedure

Preparation:

A representative 20 – 50ml sample of a freshly released spore suspension should be preserved in Lugol’s iodine to fix spores and subsequently should be kept in a brown bottle or in darkness until ready to be counted.

Density is measured on a Neubauer haemocytometer slide (Figure 2.11). Make sure the slide and coverslip are clean before use.

The slide consists of a glass base and coverslip. The slide has a counting chamber in the middle and finely ruled grids can be seen within this area. The coverslip should be gently pushed onto the slide base over the top of the slightly depressed counting chamber until it sticks. Refraction rings (a rainbow colouration) will be visible on each side of the coverslip when it has successfully attached.

Preparation of Samples:

For each sample:

Shake the preserved spore suspension well and dip a Pasteur pipette into the sample. A small amount of sample will be drawn up the pipette. Touch this lightly to the bottom edge of the coverslip where it should be drawn up into the counting chamber. Repeat until the counting chamber is full.

Place the slide under x10 objective and focus until the grid structure of the counting chamber and the spores themselves are visible. The spores will be small, round, and red/brown stained at this point.

Spores are then counted by using either a high or low density method depending on the sample. The most accurate samples are where 120 – 200 spores can be counted in total within the 4 – 5 squares selected. The counting grid consists of 9 large squares in a 3 x 3 grid and is further subdivided in the central square into a 5 x 5 grid. Each of the large squares has a surface area of 1mm² and a volume of 0.1mm². Each of the 25 smaller central squares has an area of 0.04mm² and a volume of 0.004mm²:

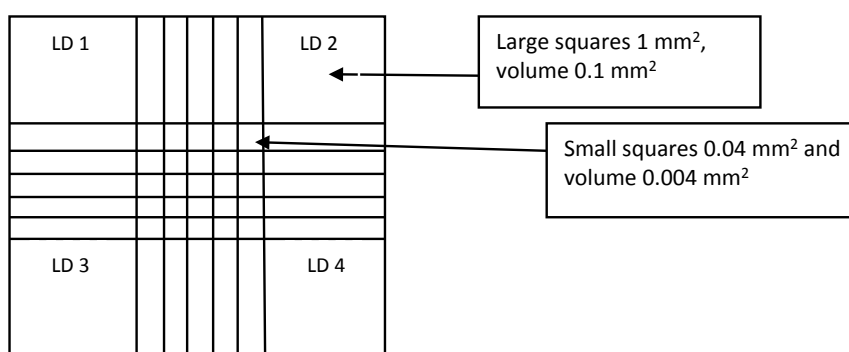


Figure 2.11. Neubauer Haemocytometer Slide and Subdivisions – Squares labelled LD 1-4 represent counting areas for low-density samples.

Low Density Method: The four squares labelled LD 1-4 in Figure 2.11 above represent the area within which spores are counted if the sample is reasonably low enough in density to accurately count all spores within these boundaries. If the sample density is too high to reliably count these, revert to the high sample density method outlined in below. Using the hand tally counter clicker,

scan the square LD1, working along the rows and counting spores, which fall within the LD1 square and on its left and top boundary lines. Do not count those on the right and bottom boundary lines. Note the total number of spores and repeat for each of squares LD 2-4.

High Density Method: The principle of the high-density method is the same as the low density above, but utilizing the smaller internal squares in the central 5 x 5 grid of the haemocytometer (Figure 2.12). Within each of the squares on this grid there is further subdivision giving 16 smaller squares in a 4 x 4 grid. Each has a volume of 0.00025 mm². Using the same method as for low-density samples, count and note the total number of spores in each of the squares marked HD 1-5.

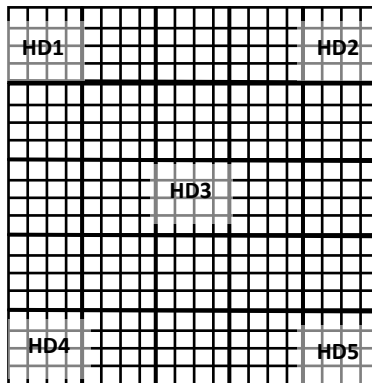


Figure 2.12. Further Subdivisions of Neubauer Haemocytometer Slide - Internal 5 x 5 grid. Squared labelled HD 1-5 represent counting areas for high density samples

The slide can be cleaned with distilled water and the process repeated twice to improve accuracy of counts.

Calculation of results

Results are calculated by taking an average spore count across the counted squares and multiplying by the known volume of the sample:

Low-Density Calculation:

Total number of cells counted in LD1 -4 as shown in Figure 2.11 = X

Number of spores per ml = X/4 x 10000

High-density calculation:

Total number of cells counted in HD1 -5 as shown in Figure 2.12 = X

Number of spores per ml = X/5 x 25 x 10000

Errors and interferences

Samples may have extremely high densities – if densities exceed 200 spores in the total number of squares sampled, then original samples can be diluted to make results more accurate.

Ensure that the coverslip is securely attached each time. This can be determined by inverting the slide with coverslip attached before adding samples. Loose coverslips will affect the volume retained within the counting chamber and may lead to inaccuracies.

Addition of the staining agent will have a dilution effect on the original sample. Use known measured amounts of Lugol's iodine to allow calculation of the dilution factor on your final results.

Lugol's iodine breaks down in light and should be stored in a brown bottle. Likewise, any samples being preserved in Lugol's iodine must be kept in dark bottles, wrapped in foil, or kept in darkness.

Waste stream and proper disposal

The coverslip and haemocytometer slide can be washed in distilled water and reused. Any broken glass should be deposited in the glass bins provided in the lab. Lugol's iodine can be neutralized with thiosulphate and disposed of down drains with excess water. In the event of a spillage, absorb with paper towels and dispose of in normal bin collection.

Hazards and precautionary statements

Take care when attaching coverslips in case of glass breakages. Lab coats and gloves should be worn throughout as Lugol's iodine leaves a dark black stain on fabrics. Lugol's iodine is not normally considered to be harmful, but can in some cases cause contact dermatitis. In case of skin contact flush well with tap water for 5-10 min. In the event of eye contact flush well with water for 15 min and seek medical advice. In the event of ingestion vomiting may be induced if the casualty is alert and able, otherwise drink 2 glasses of water and seek medical advice. In the case of a Lugol's iodine spill, mop up well with tissue paper. This can be disposed of in the normal waste bin.

References

- Andersen R.A. (2005). Algal culturing techniques. Elsevier Academic Press.
- Edwards, M.D. and Watson, L. (2011). Cultivating *Laminaria digitata*. In: Aquaculture Explained No. 26 (Edited by Watson, L.). 71pp. Bord Iascaigh Mhara, Dublin, Ireland.
- Punched N.A. – <http://www.inflammation.ndo.co.uk/Haemocytv3.doc>

2.2.4 Estimation of gametophyte density in cultures using the Wintrobe tube method

Purpose

This Standard Operating Procedure (SOP) will specify the method employed by Queen's University Belfast (QUB) to quantify and track the density of developing kelp gametophyte cultures in a macroalgal hatchery. This SOP has been applied to the species *Laminaria digitata*, *Alaria esculenta* and *Saccharina latissima*.

The procedures outlined in this SOP will aid in ensuring accuracy and completeness of datasets as defined by the users in the EnAlgae project.

Principle

The method for establishing a gametophyte culture from the capture of kelp spores has been outlined in Section 2.2.2. As these cultures are maintained in a macroalgal hatchery they will grow and increase in density. Gametophyte development can be highly variable and individual male and female gametophytes may grow in long strands or bind together to form large clumps of random size. Consequently it can be extremely difficult to ascertain culture density using cell counter slides or coulter counters. The method outlined here allows the comparison of these highly variable gametophyte cultures and provides a method for tracking their growth and development.

This protocol has been derived from Dring (1967).

Requirements

Equipment and materials:

45 µm mesh sieve/netting

Wintrobe tubes 10mm

Mortar and pestle

Centrifuge/Falcon tubes, 14 mm

UV Autoclaved seawater in a wash bottle

200 µm Pipette and tips

Measuring cylinder

50-70 ml samples taken from well-mixed gametophyte culture.

Centrifuge

Procedure

Preparation:

A representative 50 – 70ml sample is taken per strain when water changes are carried out on gametophyte cultures. Sample bottles should note the species, strain, date and the volume of the culture from which they have been taken.

Estimation of density is made from the compacted volume of filaments (wet measurement) in a Wintrobe (blood sedimentation) tube. These are 10-cm long and graduated in mm. Total volume of tubes is variable, so each tube should be numbered and the following measurements taken:

- Empty tube weight
- Tube weight when filled to the 10cm mark with distilled water

Tubes should then be emptied for samples to be added.

Table 2.2 should be used to ensure that all appropriate details are noted during the density analysis:

Table 2.2. Example of data table to gather necessary data for estimation of gametophyte biomass.

| Date of sample | Species/Strain no | Volume of sample being processed (ml) | Volume of original culture flask (ml) | Centrifuge and Wintrobe tube no | Wintrobe weight empty (g) | Wintrobe weight full (filled to 10cm)(g) | Final Wintrobe reading (cm) | Final Wintrobe weight (filled to 10cm)(g) |
|----------------|-------------------|---------------------------------------|---------------------------------------|---------------------------------|---------------------------|--|-----------------------------|---|
| Example: | | | | | | | | |
| 12/12/12 | Alaria #6 | 54 | 1500 | 1 | 3.14 | 4.23 | 1.2 | 4.33 |

Preparation of Samples

For each sample:

Note the volume of the culture flask to be sampled, and then pour a sample into the measuring cylinder to record an exact volume.

Using a filter funnel positioned over a waste container, strain the sample from the measuring cylinder through the 45 μm mesh. The gametophyte clumps will collect on the mesh. If you need to rinse the measuring cylinder to remove all the gametophytes, do so with UV seawater until all the clumps are collected on the mesh.

Transfer gametophyte clumps to the mortar. Larger clumps fall off the mesh quite easily with a little agitation whereas smaller particles will need to be washed in using UV seawater. At this stage try to use as little seawater as possible as it makes the grinding and transfer to the centrifuge tubes an easier process.



Figure 2.13. (a) Grinding the gametophyte clumps, and (b) transfer to Wintrobe tubes.

Thoroughly grind the clumps with the mortar and pestle, and transfer the ground contents to a centrifuge tube (Figure 2.13a). Rinse the mortar to get all particles into the centrifuge tube, and fill to the top with UV seawater (Figure 2.13b).

Centrifuge at 3600rpm for 10-15 minutes or until settled.

Pour away the supernatant, and transfer the pellet material into the Wintrobe tube. QUB have found the most effective method for this to be a 200 μm pipette with the end of the tip slightly cut to

make a bigger aperture. You may need to tap the tubes to eliminate air gaps. This stage requires care to be taken as some parts of the pellet may stick to the inside of the pipette tip and need to be rinsed out with UV seawater.

Wrap the Wintrobe tube in paper towel or an equivalent for cushioning and reinsert into the centrifuge tube (Wintrobe tubes should fit perfectly in into a standard 14ml centrifuge/falcon tube). Centrifuge at 1200rpm for 5 min – any faster than this will snap the tubes. Often the pellet will be slanted after this first round, so rotate tubes by 180 degrees and repeat to flatten the slant. If there is still an angled pellet, readings are estimated by taking the reading in mm in the middle of the slant.

- Note the reading on the Wintrobe tube in mm.

Check that the tube is filled to the 10mm mark by either adding or removing excess water and record the weight of the tube including the pellet.

Calculation of results

Use Microsoft Excel or a similar package to calculate the density of samples from the data recorded in the methods stage. Two outputs are generated from the analysis:

1. True sample density in g/l – the density of gametophytes, which will be found in each flask
2. Condensed to 1 l sample density - density of the culture if it was condensed to 1 l, which allows development of the culture to be quantified, as it is up-scaled to larger containers. For example, both a 1 l and a 2 l culture of *A. esculenta* may have the same sample density of 6g gametophytes/l, but the condensed sample densities would be 6g/l and 12g/l respectively. This data is solely used for tracking culture growth and is not a true representation of gametophyte density.

Table 2.3 can be used to generate these two outputs:

Table 2.3. Example of data table to generate final estimation of gametophyte biomass.

| A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P |
|---------|--------------------|----------------------------|------------------|-----------------------|----------------------|----------------------------|-----------------------------------|------------------------------|-------------------------|---|-------------------------------|---------------------|----------------------|--|------------------------------------|
| Strain | Sample volume (ml) | Total original volume (ml) | Wintrobe tube no | Tube weight empty (g) | Tube weight full (g) | Tube weight difference (g) | Wintrobe Volume per cm of tube(g) | End water weight in tube (g) | Wintrobe weight end (g) | Wintrobe tube measurement after centrifuging (cm) | Sample weight in Wintrobe (g) | True density (g/ml) | True density (g/vol) | Adjusted original sample ml for condensed density (ml) | Condensed to 1 litre density (g/l) |
| #9 1 | 32 | 800 | 1 | 3.4 97 | 4.4 04 | 0.907 | 0.09 07 | 0.743 74 | 4.441 | 1.8 | 0.200 26 | 0.006 258 | 6.258 125 | 40 | 5.0065 |

All data in red are calculated by simple excel formula; all data in black are the inputs.

$$G2 = F2 - E2$$

$$H2 = G2 / 10 \text{ (as tubes are filled to 10 cm mark for weighing)}$$

$I2 = H2*(10-K2)$ (this calculates the space taken up by water by eliminating the space taken by sample and is required to calculate sample weight)

$L2 = J2-I2-E2$

$M2 = L2/B2$

$N2 = M2*1000$

$O2 = (B2/C2)*1000$

$P2 = (L2/O2)*1000$ (this is a useful measurement to track growth as up scaling or diluting of cultures will make the true density measurement variable, whereas condensed density is consistent over time)

Errors and interferences

Ensure that Wintrobe tubes are weighed at the start of every sample session, even if the tubes have been used for previous sampling to ensure accuracy. If samples are particularly dense they may be difficult to transfer to Wintrobe tubes. In the case of very dense samples consider using smaller sample volumes of 10-20ml. Angled pellets, as described in section may lead to some inaccuracy in results. Some cultures may be characterized by extremely large clumps, making obtaining a representative sample more difficult. It is acceptable to break down large clumped cultures with a hand blender prior to sampling if it is thought that this may improve accuracy.

Waste stream and proper disposal

Used glass Wintrobe tubes should be disposed of in appropriate glass bins or cleaned and retained for future use. The small volumes of gametophytes used can be safely disposed of by placing in standard waste bins.

Hazards and precautionary statements

Take care when handling fine glass Wintrobe tubes as these may snap. This is a particular risk when removing from the centrifuge so extra care should be taken at this time.

References

Dring, M.J., 1967 "Effects of Daylength on Growth and Reproduction of the Conchocelis-Phase of *Porphyra Tenera*", *J. Mar.Biol. Association UK* **47**, 501-510

2.2.5 Induction of reproduction

Purpose

Gametophyte cultures are held in laboratory conditions that promote vegetative growth over reproductive development. Once cultures are required for use (late autumn/early winter), laboratory conditions must be changed to allow the development of oogonia and spermatia, fertilisation, and development of juvenile sporophytes in culture.

Principle

Light intensity and spectrum play an essential role in the development of sporogenesis. Higher light intensity delivered with more of the blue part of the light spectrum will encourage the gametophyte cultures to become reproductive.

Requirements and Procedure

NUIG

Induction of a kelp sporophyte culture begins by refreshing the medium in the culture flask of gametophytes as described in Section 2.2.2, to ensure that development is not affected by any lack of nutrients.

The light requirements for sporophyte development change, where the red cellophane on fluorescent lighting units are exchanged for blue cellophane (white light can also be used). Irradiance at the surface of the glassware is held at $15\text{-}20 \mu\text{mol m}^{-2} \text{s}^{-1}$, however the photoperiod is reduced from long days to 12:12 hours, light: dark. The temperature of the culture should be kept at 10°C as before and aeration should be provided.

Maintain the culture flask(s) in these conditions for 12-15 days or until reproductive structures can be observed. These will either be the developing unfertilised eggs still attached to the female gametophyte (Figure 2.14), or the fertilised egg/developing sporophyte (Figure 2.14c). The reproductive state of the culture is assessed by following egg development, as it is much more difficult to observe the smaller male reproductive structures.

Once a large number of reproductive structures are observed, the culture is prepared for spraying onto culture string.

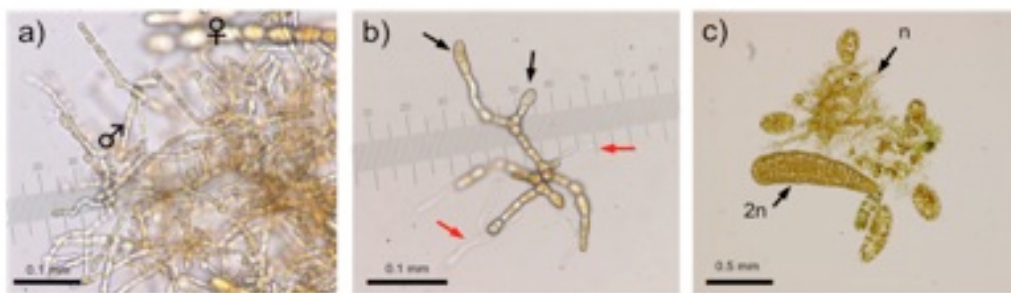


Figure 2.14 (a-c). Stages of gamete and sporophyte development. Male (foreground, ♂) and female (background, ♀) gametophytes showing differences in cell size (a). Reproductive female gametophyte with developing oogonia and eggs (black arrows), and previously released eggs (red arrows) (b). Clump of gametophytes (n) with developing juvenile sporophytes (e.g. 2n) (c). Reproduced from Edwards and Watson (2011).

QUB

To begin, set a timeline for when you wish to deploy seaweed on the open water longlines. The following protocol should be started approximately 8-10 weeks before the anticipated deployment date. Decide which gametophyte cultures are intended for cultivation on the longlines, ensuring that they are locally derived strains.

These strains will be transferred to blue light conditions. It may be sensible to retain some of the cultures under red light conditions for use in the future, if required. Before transferring the culture to the blue light section of the incubation cabinet, a water change should be carried out to ensure cleanliness of samples, following the maintenance of gametophyte culture procedure outlined in Section 2.2.2. In this case twice as many flasks will be required for the water change as one will be retained under red light and one moved to blue. The ratio of nutrients and GeO_2 remains the same in both flasks.

The volume of culture placed under blue and red light flasks is dependent on the number of collectors to be sprayed and the density of culture on each collector. Approximately 1200 ml of high-density gametophyte culture will be sufficient to spray approx. 8 – 10 40-cm collectors.

Prior to spraying of collectors, measure the gametophyte culture density using the Wintrobe density estimation (Section 2.3.2.) Record the strain, date and total volume of the flask from which the sample came.

Label the new flask in permanent marker with the species, strain number and date of water change. Place the old flask under red light and the new flask under blue. Wipe down airlines with ethanol and lab roll and add a sterile 5 ml glass pipette to bubble each flask. Light intensity of 15-20 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and light periods of 12:12 L: D should be maintained, with the temperature at 10 °C.

The blue light will begin to induce fertility in the gametophytes. Eggs will become visible anytime from 10-30 days, so the flasks should be regularly sampled and observed under a microscope for the presence of these reproductive structures. Spermata are smaller and difficult to see, so the development of either developing eggs at the end of gametophytes, or developing sporophyte juveniles should be used to track fertility in the cultures. Once a large number of these structures are observed, the culture for spraying onto collectors.

CEVA

This technique is not used in CEVA; the non-reproductive gametophyte cultures are sprayed onto collectors and held in tanks (Section 2.3) under the conditions stated, which lead to the development of juvenile sporophytes.

Hazards and precautionary statements

Hazard statement as per Section 2.2.2.

Quality Control

Quality Control as per Section 2.2.2.

Waste stream and proper disposal

Waste stream disposal as per Section 2.2.2.

References

- Edwards, M.D. and Watson, L. (2011). Cultivating *Laminaria digitata*. In: Aquaculture Explained No. 26 (Edited by Watson, L.). 71pp. Bord Iascaigh Mhara, Dublin, Ireland.

2.2.6 Artificial inducement of fertile tissue from sterile material

Purpose

An alternative method to collecting wild, fertile sori is to carry an artificial sorus induction procedure on non-fertile blades. This method has been used in CEVA to obtain fertile sori (Golven et al., 2014).

Principle

In fact, for *Saccharina latissima*, it has been shown that sorus formation is based on a genuine

photoperiodic effect, with sori visible within a few weeks after transfer from long-day (LD; 16 h light per day) to short-day (SD; 8 h light per day) conditions (Lüning, 1988). Furthermore, induction of sorus formation in *Saccharina latissima* can be enhanced by removing the meristematic part from the blade, which prevents the distal transport of putative sporulation inhibitors (most likely auxin) that are produced by the meristem (Kai et al. 2006).

Requirements and Procedure

Duration:

Collection of blades in the wild: 1 hour

Sorus induction experience at the hatchery: 1-2 months

Equipment:

- Plastic tank
- Aquarium chiller
- Light source: 2 x OSRAM L58W/880 Lumilux Skywhite + 4 x Polylux XLr F85W/840
- Walne's solution (Annex 1)
- Air supply

Blades of non-fertile *Saccharina latissima* are collected in the wild. 50-cm long pieces are cut from the middle part of these blades (the meristematic part has to be removed) and put into cultivation tanks (10 pieces per m³).

Seeding techniques used at CEVA for cultivation of *Saccharina latissima*:

Tanks are filled with fresh seawater and have a continuous water flow, at a rate of around 1 volume per day. Water temperature is kept at 13-15°C (using the aquarium chiller). Light intensity at the water surface is 65 $\mu\text{mol}/\text{m}^2/\text{s}$ with a short day photoperiod of 8:16. 0.3 mL/L of Walne solution is added weekly, to keep the blades as healthy as possible. Air bubbling allows pieces to be kept in motion. Under these conditions, it takes around one month to get sorus on the pieces of seaweed (Figure 2.15).

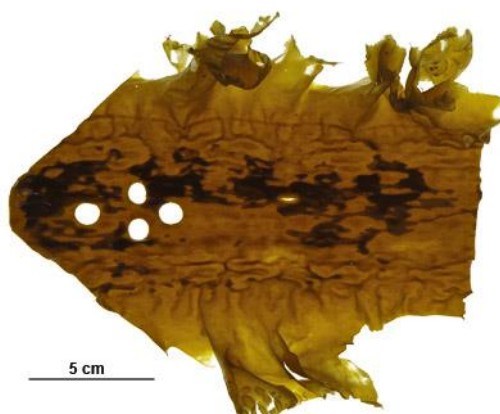


Figure 2.15. Result obtained after a sorus induction experiment carried out on *Saccharina latissima*.

Hazards and precautionary statements

Risk of electrocution: ensure lighting is secure above tanks. Avoid touching any electrical equipment with wet hands. Avoid injury to back by practising correct manual handling of tanks and other equipment as required. Always wear wet and waterproof clothing.

Quality Control

Choice of healthiest individuals from the wild will ensure best survival in tanks. Keep tanks and filters very regularly cleaned (at least once per week) to avoid any contaminating organisms settling on tank walls.

Waste stream and proper disposal

Wastewater is disposed of through CEVA effluent discharge process.

References

- Golven. P., Le Goff, T. and Champenois, J. (2014). Seeding techniques used at CEVA for cultivation of *Saccharina latissima*. Report produced by Centre d'Etude et de Valorisation des Algues, Pleubian, France.
- Lüning K. (1988). Photoperiodic control of sorus formation in the brown alga *Laminaria saccharina*. *Marine Ecology-progress Series*, **45**, 137-144.
- Kai T., Nimura K., Yasui H., Mizuta H. (2006). Regulation of sorus formation by auxin in laminariales sporophyte. *Journal of Applied Phycology* **18**, 95-101.

2.3 Seeding Methods

Kelp cultivation at sea starts with the attachment of juvenile sporophytes to culture strings in the hatchery phase (Section 2.2). Two methods for seeding kelp sporophytes are used; this depends on how much control is required over the developing crop. Once seeding is completed, the maintenance of cultures from each method is exactly the same. The seeding methods are a) the gametophyte culture method and b) the direct zoospore method. The advantages of using the gametophyte culture method is when the hatchery is required to produce seeded strings from gametophytes from specific populations, or when the availability of zoospores do not coincide with the start of the on-growing season at sea. The direct zoospore method advantages are that the gametophyte culture phase is completely eliminated, making the culture process easier, faster and cheaper. However, this method is best suited to species that are reproductive at the correct time of year for on-growing, and also for hatcheries that are geographically close to the populations of kelp that they want to use as seedstock for their cultures.

2.3.1 Sporophyte production from gametophyte cultures: method and maintenance

Purpose

In NW Europe, the busiest phase of the seaweed hatchery is approximately 1-3 months prior to the deployment of seeded strings at sea in late autumn/early winter. Juvenile sporophytes produced from gametophytes or zoospores are grown on many kilometres of culture string in tanks and are subject to controlled light, temperature and nutrient environment of the hatchery.

Principle

Mass cultivation of gametophytes are used in NW Europe to produce juvenile sporophytes for attachment to culture string on many kilometres of culture string. Section 2.1 provides information on the hatchery system requirements, while Section 2.2 describes the induction of eggs and spermatia and ultimately the juvenile sporophyte ready for spraying.

Requirements and Procedure

NUIG

As reproduction is being induced in the gametophyte cultures (Section 2.2.5), preparation of collectors and tanks takes place. Collectors can be made from anything that can be easily cleaned, holds a sufficient amount of culture string for deployment (~30-50 m) and is an appropriate size for the culture tanks to be used. At the Carna Research Station (NUIG), collectors are made from square plastic drainpipe with large, circular holes cut into each plane (allowing water to diffuse through the structure surrounding the culture string; Figure 2.16). Collectors are approximately 50 cm in length, and designed to be suspended vertically in tanks. Hence, in the case of collector length is dictated by tank depth (Figure 2.16).

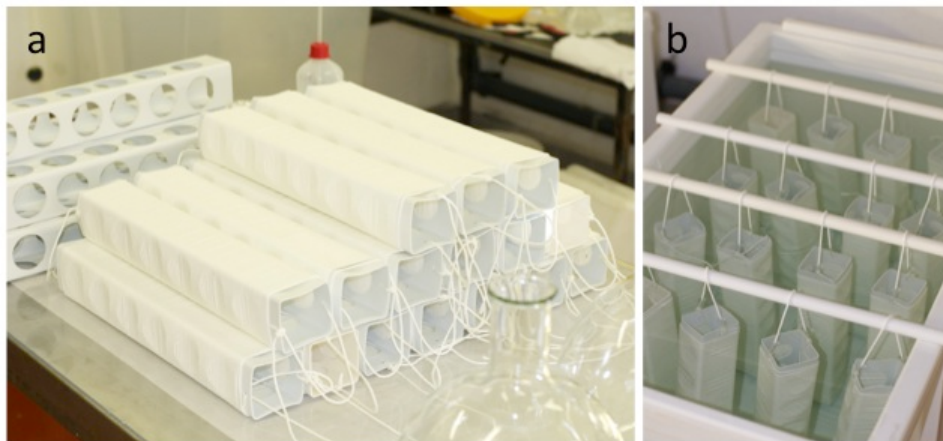


Figure 2.16. a) Empty NUIG culture collectors (background) and wound with culture string (foreground) b) Collectors suspended vertically in tanks (250 L) at NUIG.

The culture string wound around the collectors is made of polyvinyl alcohol fibres ⁵

⁵ Further information on PVOH can be found at <http://www.kuraray.eu/en/produkte/product-ranges/kuralon/>

(PVA or PVOH) and is known commercially as Kuralon (also as Vinylon). PVA fibres are used routinely in Korea and other countries for different forms of seaweed aquaculture. The strength, flexibility and hydrophilic nature of these fibres are some of the main benefits of using it as culture string in seaweed cultivation. However, the texture of Kuralon can vary according to producer, so it is worth ordering a test batch for trials before investing in many kilometres of string. NUIG, QUB and CEVA use a similar PVA soft spun fibre mixed with Dyneema from Schappe Techniques⁴ (France), 1.7 mm diameter. Approximately 50 m is wound neatly around the collectors as the collector is rotated on a hand drill. The string is tied off, and two extra loops were made, one at each end. These are used to suspend the collector on a pole in the tank (Figure 2.16b), and have an additional length of string incorporated, which can be snipped off at intervals to check on sporophyte development under the microscope. The texture of Kuralon can often be described as fluffy or pilose because of the presence of loose fibres. These can prevent the rhizoid-like structures of the kelp sporophyte protoholdfast from attaching properly. Therefore in NUIG, string wound around collectors are carefully and lightly singed with a hot flame to remove the fibres. Finally, wound collectors are soaked for several days in a couple of exchanges of hot fresh water (60-70 °C) to remove any chemical residues that may persist from manufacturing processes.

Tanks are set up in the hatchery and filled prior to use with filtered seawater (Section 2.1.1) that is allowed to equilibrate with the temperature of the hatchery. The Carna Research Station hatchery temperature is maintained at 10 °C (Section 2.1.2). Tanks must be set up under the adjustable fluorescent lights (Section 2.1.4) to maximise control of light to the culture. Air tubing and stones must be clean and set up ready for use (Section 2.1.3). Finally, nutrients must be added to the water as per gametophyte cultures in flasks at a dosage of 0.1 g per litre of seawater.

Gametophyte cultures as prepared in Section 2.2.2 are judged to be ready for spraying onto culture string when microscopic analysis of culture samples show multiple developing sporophytes and eggs. The culture to be used is blended by a thoroughly cleaned handheld or jug blender for approximately 1 minute. This homogenises the culture by reducing large clumps of gametophytes but doesn't appear to destroy the developing sporophytes.

The blended culture is loaded into a thoroughly cleaned plastic sprayer (Figure 2.17), which is pressurised and used to spray the collectors with culture. Collectors are placed upright in a thoroughly cleaned plastic box for spraying. The nozzle of the bottle is adjusted to produce a fine spray, and the collectors are covered evenly from top to bottom with culture. The bottle is shaken regularly to ensure a homogeneous mixture of culture. All four planes of the collectors receive 2-3 full sprays of the bottle. Further sprays of each plane are judged to wash more culture off than is added. Excess culture that has dripped off the collectors into the box are collected and used again in the plant spray bottle. In this way, a culture vessel holding 4-5 L of gametophytes will be more than sufficient for 12-15 collectors (approximately 600-750 m). Sprayed collectors are allowed to stand in the cool hatchery air for 10 minutes while the culture absorbs into the fibres of the string. From here,

⁴ Schappe Techniques, Allée des Prunus, 01150 Blyes, France; www.schappe.com.

they are threaded onto a pole through the collector’s top loop of string and very slowly and carefully lowered into the filled tank of seawater.



Figure 2.17. Pressurised spray bottle (capacity 7 L) used by all three EnAlgae hatcheries.

Cultures are kept in the laboratory on average of approximately 1 month in order for sporophytes to develop strong holdfast attachments to the string. Light and aeration conditions are gradually changed over this time (Table 2.4) to increase sporophyte growth and prepare them for deployment at sea.

Table 2.4. Culture conditions and practices for seeded sporophytes in tanks during pre-deployment hatchery phase (adapted from Edwards and Watson, 2011).

| Day Number | Light intensity* ($\mu\text{mol m}^{-2} \text{s}^{-1}$) | Aeration | Cleaning and nutrient exchange |
|------------|--|-----------------------------------|---|
| 0-3 | 35-40 | None | No cleaning |
| 4-14 | 60-70 | Low, increasing to moderate | Scrub tanks with hot fresh water, replace seawater and add nutrients every 4-5 days |
| 15+ | 60-70 | Moderate, increasing towards high | Scrub tanks with hot fresh water, replace seawater and add nutrients every 4-5 days |

*at the tank water surface

QUB

Collectors are very similar to the square drainpipe collectors described in NUIG, using the same culture string. However, a hacksaw or tile cutter is used to cut the collector from top to bottom on one side (Figure 2.18). This cut is then resealed with duct tape to allow the easy removal of the collector from the longline after deployment of culture string.



Figure 2.18. A simple collector prepared at QUB from 65mm drainpipe with holes for water flow. A lateral cut has been made and resealed to allow later removal from longlines during deployment.

CEVA

Two different types of collector are used in CEVA (Figure 2.19). Collector Type 1 is made with 4 pieces of plexiglass (1cm wide x 1.5cm large x 33cm long) + 2 electrical sheathing, all glued together. Collector Type 2 is made with iron rods, PVC tube, 2 electrical sheathing and heat-shrink* tube for sealing (* “thermorétractable” in French).

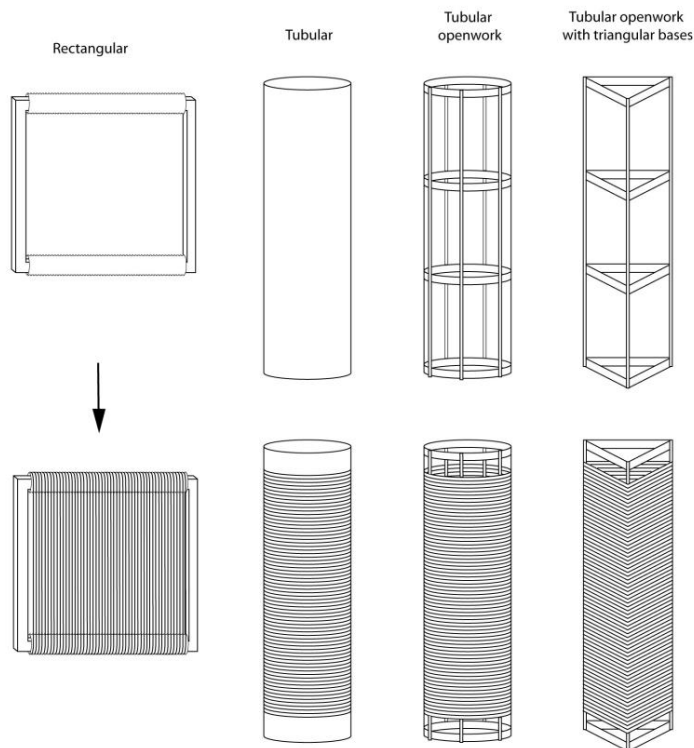


Figure 2.19. Various shapes of collector frames used in CEVA for seeding *S. latissima*.

For seeding from the gametophyte method, CEVA uses a string manufactured by French company, Schappe Techniques (see footnote number 4). Reference for the string is follows: 1.7 mm diameter,

33% Dyneema (a Ultra-High Molecular Weight Polyethylene allowing high resistance/strength) + 67% PVA (Poly Vinyl Alcohol for hydrophobic property).

Preparation and storage of collectors:

Culture string is twisted round the collector frame using the electrical sheathing as a guide. Total length of string per collector is around 65 m (Figure 2.20). The whole collectors (string + frames) are stored at room temperature in polyethylene bags, to avoid moisture and dust. If necessary, collectors are dried at 50-60°C in a ventilated oven prior to seeding.



Figure 2.20. CEVA device used to twist the culture string round the collector frame.

Spraying and loading collectors carefully into tanks is very similar to the NUIG method described above.

Preparation of tanks before collectors are added (the day before the seeding of collectors):

Clean the 300L tank with brush and freshwater, wipe with tissue paper and disinfect with acetone. Then, rinse the tank. Fill the tank with filtered seawater (obtained after a two-step filtration procedure using 10 and 1 μm filtering cartridges). Set up the aquarium chiller (allowing temperature regulation at 13°C) and air supply. Set up light tubes. Light intensity at the water surface is 65 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with a photoperiod of 12:12, L:D.

On the day of the seeding:

Add bleach to sterilize the water: 0.1 mL of bleach per 1L of seawater. Allow vigorous bubbling in the tank for 10 min. Add sodium thiosulfate to neutralize bleach: 0.1mL per 1L of seawater. Allow strong bubbling in the tank for 10 min. Add ammonium nitrate and disodium hydrogen phosphate to enrich the culture medium:

- Ammonium nitrate NH_4NO_3 at 160g/L
- Disodium hydrogen phosphate $\text{PO}_4\text{HNa}_2, 2\text{H}_2\text{O}$ at 22.4g/L
- Add 0.25 mL per L of each

- Allow strong bubbling in tank for 5 minutes to stir well.

Just after the seeding of collectors:

Stop air supply into the tank. Take extra care when inserting collectors into tank for the first time.

Collectors are maintained in tank in the hatchery during a few weeks (4 or 5 weeks), until they reach 3 to 5 mm. The conditions are changed progressively to reach those found at sea. This allows plantlets to get acclimated to outdoor conditions (Table 2.5). Once they have reached 3 to 5 mm, they must be quickly transferred at sea.

Table 2.5. Example of monitoring of tanks in the hatchery for collectors seeded from a solution of gametophytes.

| Week Number | Light intensity* ($\mu\text{mol m}^{-2} \text{s}^{-1}$) | Aeration | Temperature ($^{\circ}\text{C}$) | Photoperiod (L:D) | Cleaning and nutrient exchange |
|-------------|---|----------|------------------------------------|-------------------|--------------------------------|
| 1 | 20-40 | No | 12-13 | 12:12 | Refresh every week (PES; 100%) |
| 2 | 20-40 | Low | 12-13 | 12:12 | Refresh every week (PES; 100%) |
| 3 | 40-60 | Strong | 14-15 | 12:12 | Refresh every week (PES; 100%) |
| 4 | Natural | Strong | 14-15 | 12:12 | Refresh every week (PES; 100%) |

Hazards and precautionary statements

Always wear appropriate warm and wet weather gear in the hatchery when maintaining cultures in tanks. Practice good manual handling techniques at all times, particularly when moving tanks that contain water. Siphoning wastewater out of tanks using a wide diameter hose or installing an outflow tap at the base of the tanks are good options to reduce the amount of manual handling required when it comes to cleaning tanks. Take care when using the gas torch to sterilise glass equipment: depending on the model used, the flame can be up to 1800 $^{\circ}\text{C}$, and glass/other surfaces can be extremely hot to the touch for several minutes after application of heat. Ensure that only electrical items that are waterproof (e.g. fluorescent lighting) are near water.

Quality Control

While large-scale kelp cultures are not grown in axenic conditions, quality control of cultivation conditions is still of utmost importance to ensure a successful production of seeded collectors. Much of this control will come from only maintaining and using (i.e. spraying) cultures that are not contaminated with other microalgae, macroalgae or zooplankton (contaminants can include diatoms, *Ulva* sp., and ciliates; see Section 2.2 for advice on prevention). The remaining quality control comes from ensuring incoming water quality is of a high standard (Section 2.1.1), and that tanks are very thoroughly cleaned on a regularly basis to prevent the development of biological populations (bacterial, microalgal etc.) other than the kelp sporophytes themselves. Chemicals such as bleach can be used to clean the tanks, however, very hot fresh water and an abrasive scrubbing pad is just as effective and with no risk of leaving any chemical residues behind. Finally, attention to detail

regarding light and nutrients and avoiding the holding of collectors for extended periods of time in the hatchery before deployment is also practiced to ensure that the sporophytes are of optimal quality at deployment.

Health and development of sporophytes on collectors can be observed under the microscope on a weekly basis by snipping off a small length (less than 5 cm) of excess culture string from one or more of the collectors hanging loops.

Waste stream and proper disposal

Waste seawater from the tanks is the biggest waste stream to dispose of, which must be in accordance with discharge regulations of each particular country/legislative region. See waste stream of water in Section 2.1.1 for further details. If waste seawater contains sporophytes of kelp from distant populations to the hatchery location, then the water must be chemically sterilised to destroy detached individuals to avoid the possibility of these sporophytes surviving the disposal process and establishing themselves in local algal populations outside the hatchery. Water sterilisation can be achieved by sodium hypochlorite (bleach) followed by neutralisation with sodium metabisulphite.

References

- Edwards, M.D. and Watson, L. (2011). Cultivating *Laminaria digitata*. In: Aquaculture Explained No. 26 (Edited by Watson, L.). 71pp. Bord Iascaigh Mhara, Dublin, Ireland.
- Golven, P., Le Goff, T. and Champenois, J. (2014). Seeding techniques used at CEVA for cultivation of *Saccharina latissima*. Report produced by Centre d'Etude et de Valorisation des Algues, Pleubian, France.

2.3.2 Sporophyte production from direct zoospore release: method and maintenance

Purpose

In NW Europe, the busiest phase of the seaweed hatchery is approximately 1-3 months prior to the deployment of seeded strings at sea in late autumn/early winter. Juvenile sporophytes produced from gametophytes or zoospores are grown on many kilometres of culture string in tanks and are subject to controlled light, temperature and nutrient environment of the hatchery.

Principle

The alternative to mass cultivation of gametophytes for the production juvenile sporophytes is the direct zoospore method, where all phases of the kelp life cycle takes place on the culture string over a shorter period of time in the hatchery. Section 2.1 provides information on the hatchery system requirements required to support the production of seeded collectors by the direct zoospore method.

Requirements and Procedure

Much of the techniques for the direct zoospore method have already been described in this SOP document, the only difference in this procedure is one of scale.

NUIG

Collectors and tanks are prepared in the Carna Research Station as described in Section 2.3.1, prior to the collection and preparation of fertile mature sporophytes from wild populations (Section 2.2.1

and 2.2.2). Sporophytes with mature sori are collected when required, approximately 1-3 months prior to deployment of collectors at sea, with sori from approximately 4-5 individuals used per tank release (in the NUIG hatchery 250 L white HDPE rectangular tanks are used; Figure 3.1b). Zoospores from prepared sori are released in a large beaker, added to the tank of seawater (with additional nutrients; Section 2.3.1) having been strained through a piece of phytoplankton netting (mesh size anywhere between 30 to 60 μm).

Culture conditions for the directly settled collectors follow gametophyte-sprayed collector conditions as summarised in Table 2.2 in the following 1+ months.

QUB

In the hatchery, place prepared collectors into an empty tank, and cover with UV-filtered seawater. Add an air stone with moderate aeration. Close curtains or add a lid for darkness, and pour in the spore suspension. Leave for 1.5 – 2 hrs to allow attachment to the twine, turning the collectors regularly while avoiding touching the twine itself. Moderate aeration should only allow attachment of strong spores.

After 2 hrs, collectors can be lifted out of the container, and the container can be filled with UV filtered seawater. Suspend the collectors with string in the filled container. Add gentle aeration and light intensity of approx. $35\text{-}40\mu\text{mol m}^{-2} \text{s}^{-1}$ on a 12 hour light to dark cycle. Add F/2 medium to the tank in the ratio of 1ml L^{-1} .

Water in the tank should be emptied and refreshed every 3-4 days, cleaning the tank with a dilute bleach solution and rinsing thoroughly before refilling. F/2 medium should be added at every refill. Gradually increase the light intensity over the next 3-4 days to $60\text{-}70\mu\text{mol m}^{-2} \text{s}^{-1}$, and increase aeration from low to moderate in the first 14 days, then from moderate to high from day 14 onwards. Regularly take samples from the loose twine at the collector ends to track development of the blades. Collectors should be ready for deployment after 4-8 weeks, when the blades are 2-5mm in length (Figure 2.21).

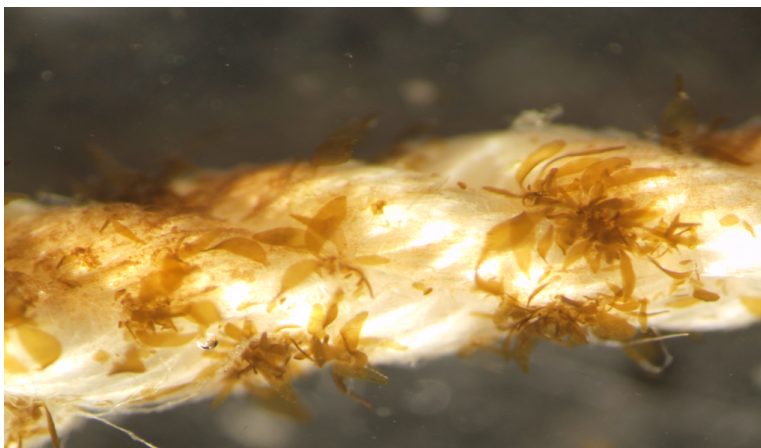


Figure 2.21. Developing sporophytes (2-5 mm) of *Alaria esculenta* from direct spore release on culture string.

Collectors used by CEVA in direct spore seeding are detailed in Figure 2.19. The culture string used is 2-mm nylon usually used for net repair and found in sailor shop. This kind of string is coated with silicone to help the manufacturing process. Thus, it is important to rinse thoroughly the collectors to eliminate that compound. After that, the collectors are dried at 50-60°C in a ventilated oven.

Set up a tank in the dark. Fill the tank with sterile seawater. Immerse collectors and microscope slides. Pour the spore solution into the tank. Allow a strong agitation by air bubbling into the tank, to select the strongest spores. After 30 min in dark conditions, the light is turned on. Fixation of spores can be monitored using the microscope slides. When there are 20 spores under x10 magnification, we consider that seeding has succeeded and collectors are removed from the tank. Fixation time must not exceed 2 hours.

Direct seeding requires an additional week of culture to allow time for the spores to germinate into gametophytes and gametophytes to grow (Table 2.6). During this period the conditions must fit the requirements for gametophytes growth.

Table 2.6. Example of monitoring of tanks in the hatchery for collectors seeded from a solution of spores.

| Week Number | Light intensity* ($\mu\text{mol m}^{-2} \text{s}^{-1}$) | Aeration | Temperature (°C) | Photoperiod (L:D) | Cleaning and nutrient exchange |
|-------------|---|----------|------------------|-------------------|--------------------------------|
| 1 | 5-10 | Low | 12-13 | 12:12 | Refresh every week (PES; 50%) |
| 2 | 20-40 | Medium | 12-13 | 12:12 | Refresh every week (PES; 50%) |
| 3 | 40-60 | Strong | 14-15 | 12:12 | Refresh every week (PES; 100%) |
| 4 | 40-60 | Strong | 14-15 | 12:12 | Refresh every week (PES; 100%) |
| 5 | Natural | Strong | Natural | Natural | Refresh every week (PES; 100%) |

Quality Control

Quality control of the direct zoospore method is broadly similar to that described in Section 2.3.1. Instead of working with a good quality gametophyte culture for spraying onto collectors, similar care must be taken to select zoospores from the best mature sori, as described in Section 2.2.1.

Waste stream and proper disposal

See Section 2.3.1

References

- Edwards, M.D. and Watson, L. (2011). Cultivating *Laminaria digitata*. In: Aquaculture Explained No. 26 (Edited by Watson, L.). 71pp. Bord Iascaigh Mhara, Dublin, Ireland.
- Golven. P., Le Goff, T. and Champenois, J. (2014). Seeding techniques used at CEVA for cultivation of *Saccharina latissima*. Report produced by Centre d'Etude et de Valorisation des Algues, Pleubian, France.

Chapter 3: Cultivation Techniques at Sea

3.1 Deployment Techniques For Seeded Material

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The hatchery phase (Chapter 2) of the kelp life cycle is completed once the seeded collectors have developing juvenile sporophytes growing evenly over all surfaces. In NW Europe, transfer of collectors to the sea site(s) generally occurs in the late autumn/early winter. This coincides with a drop in summertime water temperatures and light levels and ensures maximum survival of the juvenile kelp. Transfer and deployment is a brief but critical phase for these sporophytes, and maximum care and attention is required to obtain a consistent crop in the spring. Common challenges during this time include long distance transportation (e.g. greater than 200 km) and unfavourable weather conditions for deployment (e.g. warm and windy). These factors must be minimised where possible to ensure the best results.

3.1.1 Preparation of seeded material for transport

Purpose

The hatchery phase finishes with the preparation of seeded collectors for transport. Juvenile seeded collectors are carefully wrapped up and placed in suitable containers for transportation, bearing in mind method of transport may be by road and sea, or even road, air and sea, depending on final cultivation site location.

Principle

Preparation for transport requires very simple equipment, all of which should be readily available within the hatchery and used at other times for other purposes. The principle of the preparation and transportation of seeded collectors is to prevent excessive removal of kelp juveniles by abrasion or desiccation.

Requirements and Procedure

NUIG

Prior to the day of transport, a number of key pieces of equipment/consumables are prepared for the journey at the Carna Research Station. The hatchery plant spray bottle (Figure 2.14) is filled with UV-filtered seawater and allowed to reach the same temperature of the seawater surrounding the collectors in the tanks (10 °C). Ice blocks are frozen, and wrapping material is laid out in preparation for use. Finally, depending on the size and number of the collectors, a suitable container is cleaned thoroughly also ready for use. Containers range from lidded boxes of approx. 50 L, to carriage of one of the 250 L tanks used in the hatchery phase (Figure 3.1).

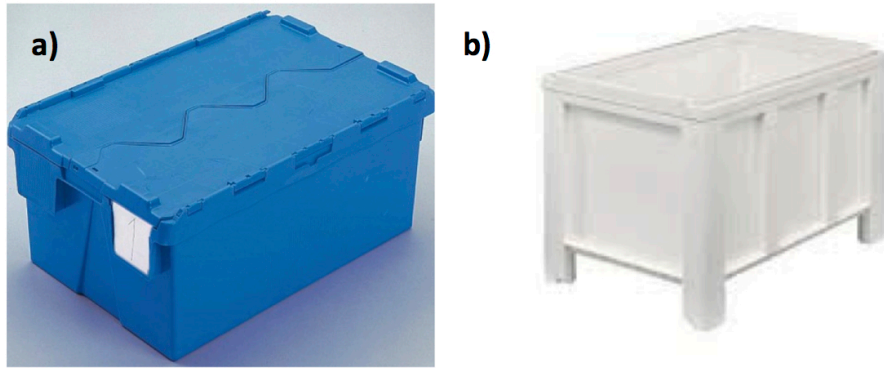


Figure 3.1. a) Lidded container (~50 L) and b) tank (250L) used to transport seeded collectors of various sizes from NUIG hatchery to sea site.

Wrapping material consists of an inner layer that is placed on top of the collectors; either an open textured, non-woven cloth such as a J-Cloth™ (Jay-Cloth; Figure 3.2), or simply strong laboratory paper roll. The outer layer of wrapping material is bubble wrap. Both jay-cloth and laboratory roll are used as they absorb seawater very well, are non-toxic, and wrap efficiently around the collectors preventing desiccation. The bubble wrap is an efficient insulator, slowing down increases in temperature and desiccation during transport.

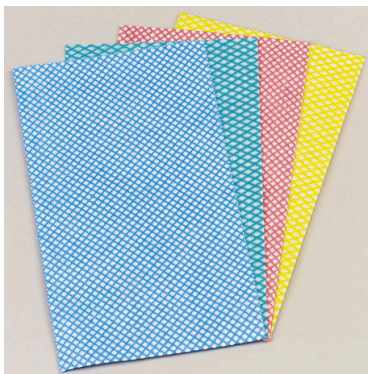


Figure 3.2. Example of wrapping material for collectors; Jay-Cloths.

Just prior to transport, long strips of laboratory roll or cloths are soaked by spraying them with seawater from the sprayer. Each collector is lifted out of the tanks, one by one, and individual layers of paper or cloth are gently and loosely wrapped around the length of the collector. Each collector takes approximately 3-4 lengths of paper/cloth for complete wrapping (no gaps), and 3-4 minutes application time.

Once all collectors are carefully wrapped, they are placed in a box (Figure 3.1) and sprayed with a little additional water. The collectors are laid upon layers of bubble wrap and wrapped up. Several ice blocks can be placed above and below the bubble wrap (but *not* directly in contact with the wrapped collectors) to aid temperature control before the box lid is closed.

QUB

At QUB, on the day of deployment, cool boxes are prepared by lining with lab roll soaked in filtered and UV-sterilised seawater. Collectors are removed slowly from tanks and placed upright into the coolbox, ensuring they are not touching. Additional damp lab roll is placed over the top of the collectors and the lid closed ensuring the collectors are held secure. The boxes are transported with great care onto the boat, ready for transport to sea.

CEVA

At CEVA, the seeded string from collectors is wound around a larger carrier rope (Figure 3.3) for further deployment at sea. The header rope is 12 or 14 mm 3-stranded PE. The work, as detailed below, takes 2 persons 2 hours to seed 200 m of culture rope.

A 25m long culture rope is set between an electric drill and a swivel (*émérillon*). One end of the seeded string is attached to that culture rope with a knot. The drill is switched on and the seeded string is wound around the culture rope. At the end of the 25-m culture rope, the seeded string is cut and attached to the culture rope with a knot. Total length of seeded string per collector is around 65m, which allows the seeding of 2 x 25-m of culture rope.



Figure 3.3. Fixation of the seeded culture string on the culture rope at CEVA before transportation and deployment.

Plantlets are very sensitive to desiccation and freshwater. They have to stay a minimum of time outside the seawater so you must work quickly. As soon as the seeded string has been deployed around the culture rope, the rope is immersed in a tank filled with seawater, at a temperature below 15°C, and covered with a fabric. After what, the ropes must be transferred at sea within 24 hours.

Quality Control

Excessively rough handling of the collectors and inner wrapping material can remove many thousands of plants unnecessarily. Collectors are lift and held by their string carrying handle (Figure 2.15), and utmost care is used when the wrapping occurs. If the wrapping is too tight, it makes it difficult to efficiently unwrap the collectors at the sea site and causes more damage.

References

- Edwards, M.D. and Watson, L. (2011). Cultivating *Laminaria digitata*. In: Aquaculture Explained No. 26 (Edited by Watson, L.). 71pp. Bord Iascaigh Mhara, Dublin, Ireland.
- Golven. P., Le Goff, T. and Champenois, J. (2014). Seeding techniques used at CEVA for cultivation

of *Saccharina latissima*. Report produced by Centre d'Etude et de Valorisation des Algues, Pleubian, France.

3.1.2 Transfer of seeded material to sea

Purpose

Transfer of cultivated macroalgae from hatchery phase to sea phase for further complete on-growing and crop harvest.

Principle

Hatchery and sea cultivation sites may be distant to each other, and so transportation by road (or air, or both) is necessary. Section 3.1.2 assumes transportation lengths of no longer than 8 hours by road for the conditions described. Impact of longer transit times on the viability of seeded collectors cannot be guaranteed under the stated SOPs.

Requirements and Procedure

NUIG

Collectors prepared as per Section 3.1.1 are transported from the NUIG Carna Research Station algal hatchery to the EnAlgae pilot facility at sea in Co. Kerry. Depending on the availability of vehicles and the amount of seeded material to be deployed, transport is with van or car by NUIG personnel.

Travel time is often no less than 5.5 hours by road (Figure 3.4), so it is necessary to stop and briefly open up bubble wrap to spray more cooled seawater on to the wrapped collectors.

Collectors are taken directly to sea and deployed on the longlines (Section 3.1.3).

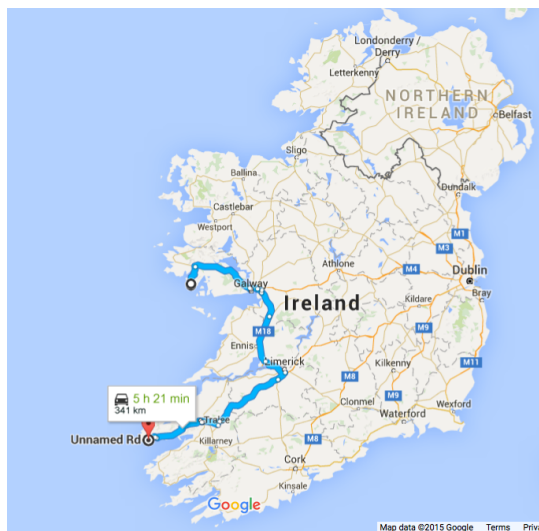


Figure 3.4. Indication of standard distance and length of transit for seeded collectors from NUIG hatchery (Carna Research Station, Carna, Co. Galway) to the EnAlgae pilot grow-out facility, Ventry Harbour, Co. Kerry); 341 km, 5 h 21 min.

QUB

Collectors are transported in the cool boxes to the site via boat (a 10 minute journey from hatchery to site at QUB). Lab tissue is gently soaked with seawater occasionally if starting to dry out.

CEVA

In the best case, seedlings are maintained in seawater in tanks all along. If it is not possible, the ropes are covered with wet towels. In that case the rope must not be out of water more than 2 hours and must be dampened regularly.

Quality Control

As mentioned above, desiccation and fluctuations in temperature is minimised where possible by regularly water sprays.

Where it is not possible to place the collectors at sea immediately upon arrival at site (normal procedure), it is important to be able to re-suspend the cultures in a tank of seawater (unfiltered is acceptable) with aeration or on flow-through until conditions are suitable to go to sea. Maximum length of time for this to occur should be no longer than 2 days in case quality of collectors is compromised.

References

- Edwards, M.D. and Watson, L. (2011). Cultivating *Laminaria digitata*. In: Aquaculture Explained No. 26 (Edited by Watson, L.). 71pp. Bord Iascaigh Mhara, Dublin, Ireland.
- Golven, P., Le Goff, T. and Champenois, J. (2014). Seeding techniques used at CEVA for cultivation of *Saccharina latissima*. Report produced by Centre d'Etude et de Valorisation des Algues, Pleubian, France.

3.1.3 Deployment of seeded material onto longline systems

Purpose

Further on-growing of juvenile sporophytes into harvest-sized individuals occurs entirely at sea over a period of months. Seeded collectors are deployed on longlines at sea. This process begins during late autumn/early winter.

Principle

Longlines are structures designed for the practice of aquaculture, supporting the cultivated species in a suitable location at an optimal water depth. Seaweed longlines consist of an anchoring system that maintains horizontal ropes within the water column, assisted by floats to provide buoyancy and control. Design of longlines can vary, depending on the requirements. EnAlgae pilot facility structures can be found in the accompanying **Macroalgal Best Practice report**. Juvenile sporophytes are commonly sprayed onto culture string or thin ropes wrapped around collectors, making best use of often small hatchery facilities. These require transfer to the larger ropes of the longlines, where growth and development continues and where the string supports sporophyte holdfasts initially before they enlarge and extend onto the rope of the longline itself for maximum anchorage.

Requirements and Procedure

NUIG

Upon arrival at the pier in Ventry Harbour, the box of seeded collectors is transferred from car/van to boat and the on-growing site is accessed immediately. Boat time is approximately 10 minutes. Seeded collectors are carefully unwrapped, one at a time (Figure 3.5a) and can be threaded onto the

header rope and unravelled by hand; the string uncoils from the collector when pulled from one end and twists around the larger header rope (Figure 3.5b). Alternatively, through support by Dingle Bay Seaweeds, EnAlgae collectors are mechanically deployed (Figure 3.6).

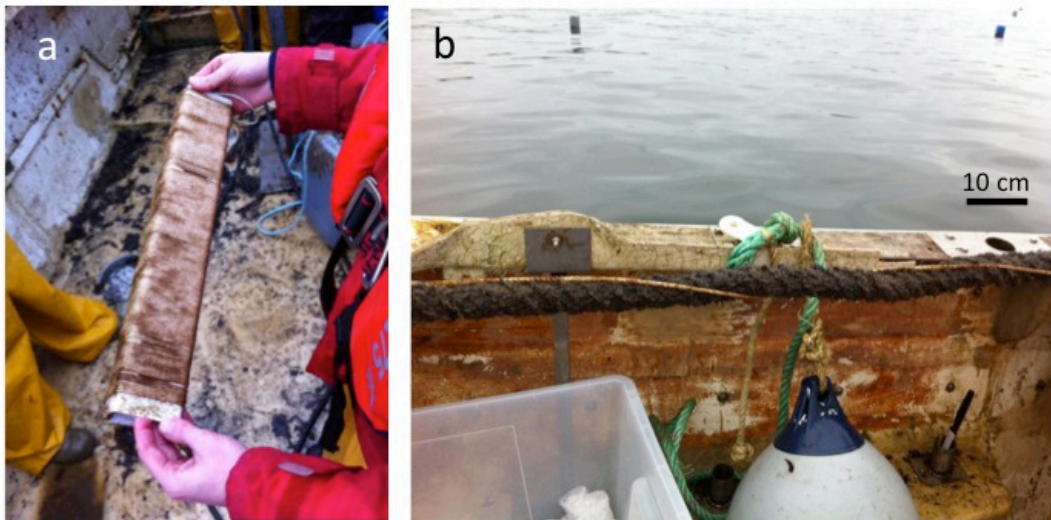


Figure 3.5 a) Seeded collector containing approx. 50 m seeded culture string unwrapped and ready for deployment b) culture string wrapped around the heavier header rope.



Figure 3.6. Seeding a longline header rope with culture string supporting kelp juveniles.

The header rope is pulled into the boat at the stern and runs the length of the boat, with newly seeded rope going back into the water over a roller at the bow. The seeded collector is loaded onto the custom-built rig, which is driven by a small motor. The string is untied from one end and attached to the header rope before the machinery wraps the string around it. Buoyancy is added continuously approximately every 14 m to ensure proper suspension of sporophyte cultures at the correct depth. Each longline is 280-300 m in length, and takes 6-7 collectors (as seen in Figure 2.15). Each longline can be seeded within approximately 20 minutes.

Hazards and precautionary statements

Moving mechanical machinery can be dangerous, and so should only be operated by trained operatives, such as from Dingle Bay Seaweeds. Life jackets and warm and waterproof clothing are worn by all

personnel on the boat. Suitably calm weather conditions are critical for a safe and successful deployment; therefore activities should be scheduled accordingly.

Quality Control

Like all aspects of Section 3.1, utmost care and attention to detail is required at this stage to ensure a successful harvest. Again, handling of the collectors is minimised by holding the carrying handle, gently unwrapping the inner paper/cloth and inserting onto the machine as soon as possible.

Deployments only take place when extremely good conditions occur. For deployment purposes, sites should only be accessed on a flat calm (where possible), on overcast, lower temperature days to avoid unnecessary abrasion and desiccation.

Waste stream and proper disposal

After deployment, 'waste' is produced in the form of empty collectors and wrapping/packing materials. Apart from wet laboratory tissue paper, which is disposed of in landfill, all other plastic and synthetic fibres (e.g. cloths) are washed, dried and stored for use again in the next season.

3.1.4 Estimation of seedling density

Purpose

This method employed by Queen's University Belfast (QUB) is designed to quantify and track the density of developing juvenile kelp sporophytes on seeded string in a macroalgal hatchery and has been applied to the species *Laminaria digitata*, *Alaria esculenta* and *Saccharina latissima*.

The procedures outlined in this SOP will aid in ensuring accuracy and completeness of datasets as defined by the users in the EnAlgae project.

Principle

It is useful to determine the density of seeded plantlets prior to deployment to obtain an indication of how successful seeding techniques have been, and to get an estimate of future productivity. Building up a dataset of culture densities from haemocytometer (Section 2.2.3) and Wintrobe counts (Section 2.2.4), density of plantlets prior to deployment, and density of adult plants during the on-growing season will help to determine which cultures are performing well and ensure maximum productivity of the hatchery by allowing removal of non-performing cultures.

Requirements

Equipment and materials:

- Microscope
- Scissors
- Seeded string
- 2 x Petri dishes
- Seawater
- Tweezers
- Tally counter
- Pen and paper

Procedure

Prior to deployment, snip off 3 x 1-cm sections of the excess seeded string at top and bottom of the collectors. Place the first 1-cm section into a petri dish with some seawater and looking through a microscope, gently remove the individual plantlets from the string using the tweezers and place them into a separate petri dish with seawater, recording each plantlet removed. Do this for each of the three top and bottom samples, giving a total of 6 counts for each collector. This can then be averaged to give an average density for each collector.

Errors and interferences

Some plantlets can be very clumped together and difficult to count individually upon removal. If this happens, then the second dish containing all removed plantlets can be counted to verify the number from the first dish.

Waste stream and proper disposal

The small volumes of juvenile kelp plantlets and string can be safely disposed of by placing in standard waste bins.

Hazards and precautionary statements

Take care when handling the seeded string so as not to dislodge any plantlets prior to counting.

3.2 Monitoring of the seaweed biomass

Once deployment of seeded material has occurred, the natural environment of the sea encourages approximately exponential growth of the juvenile kelp sporophytes. The sporophytes are typically deployed as very small to microscope sizes (1-10 mm), and can reach lengths of 100-200 mm in just a few months, resulting in plants of several metres in growth by harvest time (May-June). The only direct support that is required for this process to occur is to ensure that the growth systems/longline structures are kept in good condition in the correct location. Monitoring and subsequent maintenance is required on approximately a monthly basis and/or directly after stormy conditions.

3.2.1 Monitoring and maintenance of growing systems

Purpose

To ensure the longline structures are maintained in good order to provide optimal growing conditions for the attached seaweeds.

Principle

During the grow-out phase, algal cultures require suitable access to light and nutrients in water of the correct temperature. In NW Europe, culture systems such as longlines provide this by suspending seaweed approximately 1-2 m below the surface of the water.

Requirements and Procedure

NUIG

The most suitable longline structures developed for each EnAlgae pilot grow-out site have been discussed in the **accompanying Best Practice document**. While the structures are different, the principle of anchorage and buoyancy remain the same. Therefore monitoring and maintenance of structures are largely similar across sites too. It is assumed that all equipment has been thoroughly checked for damage/wear and components replaced before deployment at sea. Monitoring and maintenance as described in this section refers to that of the grow-out phase only.

All sites require at least a boat journey for access; therefore suitable maintenance and monitoring preparations and provisions must be made prior to departure. Table 3.1 describes basic necessary equipment to bring to the site and reasons for replacement.

Table 3.1. Maintenance equipment for seaweed grow-out sites

| Equipment Description | Number/Amount | Reason for Replacement |
|--|--|---|
| Buoy/Drum/Flotation device | 2-3 per longline (more after storm events) | Loss due to rope abrasion or puncture/leakage |
| Buoy rope (1.5-2m polypropylene rope) | 5-6 per longline (more after storm events) | Rope abrasion very common and can lead to loss of buoy (=additional expense). Quick replacement of fraying ropes saves money, longline integrity and biomass |
| Length of heavy duty rope (of similar weight to header rope), 5-10 m | 1 | 1) Occasionally the header rope will fail at one or other end where attached to anchor. A heavy-duty rope can be used to temporarily reconnect the longline to save further entanglement. 2) A heavy duty rope is also useful for towing longline anchors back into position if they have been moved in heavy storms |
| Serrated Knives, marlinspike, scissors | 1 each (plus spare knife) | Serrated knives are useful for sawing through rope and efficiently removing frayed components. Knives with blunt/rounded tips are safer for use on boats (especially inflatable craft such as RIBs). A marlinspike is inserted into the twist of a tightly coiled header rope and makes an opening through which a smaller buoy rope can be inserted. |
| Waterproof notebook, spool of twine/string, pack of cable ties | 1 each | Notebook useful to be able to make notes on work completed and future work required, especially in a large site of many lines. String and cable ties invaluable to re-tie culture string to header rope in places where it may have |

| | | |
|---|--------------------------------|--|
| | | become loose. |
| First aid kit, food, hot drink, mobile phone/boat communication devices e.g. radio and safety clothing/life jackets | Enough for each boat operative | Safety is first consideration during sea operations. Ensure adequate preparation for care of operatives. |

Hazards and precautionary statements

Do not attempt to do strenuous repairs or lifting of longlines at high tide when the structures are most taut/stretched. The weight of biomass and structure should not be underestimated and can lead to back damage and crushing injuries to hands, arms and legs if trapped within or under ropes. Where possible, use a mechanical motor to lift the longline up for inspection, and always use a boat or barge of suitable stability and size for the job. Always ensure there are an adequate number of personnel to carry out the work. Lone working is not advisable.

As with any other activity at sea, ensure the weather conditions are calm and safe enough to conduct maintenance and repairs. If underwater repairs are required, ensure that the dive team employed to carry out the work is suitably qualified, knowledgeable and insured before work begins. Always make sure that boat operatives are wearing warm and waterproof clothing and life jackets/personal flotation devices.

Quality Control

Regular trips to the longline structures of at least one per month and always as soon after storm events as possible ensure that small maintenance issues do not escalate into more serious/costly procedures. The greatest loss of biomass happens due to abrasion of structure rubbing together, sinking and then scraping across the seabed as well as abrasion around buoyancy. Initial deployment of structures at the correct tension and spacing and orientation for the particular site conditions is essential to understand (see **Macroalgal Best Practice Document** for further details), while regular maintenance as described in this section maintains the status quo.

As the biomass increases, note that it may be important to add additional buoyancy to support the header rope. This will most likely occur from April onwards.

Waste stream and proper disposal

Any waste ropes and buoys can be collected over the season in a bin/crate for recycling alongside other fishing gear if this is supported within the area/country by national schemes or local companies. For example, in Ireland, the Irish Sea Fisheries Board (BIM) find alternative green methods for the disposal of fishing gear⁵

⁵ <http://www.bim.ie/our-services/technology-services/for-fishermen/>

3.3 Harvesting Strategy and Techniques

Harvesting of the macroalgal crop generally occurs from late spring to early summer in NW Europe. Depending on the site, there is often an optimal harvesting period within which to remove all or some of the biomass. This usually coincides with an increase in water temperature, which is correlated to a decrease in biomass quality and quantity. Harvesting strategy based on the dynamics of the site and what the crop will be used for is therefore important, as are the harvesting techniques themselves to ensure an efficient process, and optimised quality of biomass.

Purpose

The removal of growing biomass for further downstream processing

Principle

Harvesting occurs during a relatively short window during late spring to early summer in most sites around NW Europe. It is often limited by the amount of material that can be removed per day, the method and type of transport, the distance from the processing facilities, as well as the requirements of the biomass in these processes and beyond.

Requirements and Procedure

NUIG

Biomass is removed from the Ventry Harbour site at one or sometimes two times per year. Most algal growth is typically removed in a single harvest during a period from late April to the end of June. All biomass is removed from the header rope; nothing remains. Alternatively, fronds only are cut in February/March, leaving the holdfast, stipe and some of the frond intact (Figure 3.7a). These portions are allowed to regrow until early summer (typically late May), after which time all growth is removed. A third option was investigated within the EnAlgae project, whereby the fronds only were cut in early summer, but the holdfast, stipes, and portions of fronds were left on the longlines until the following year and were known as the 'regrowth crop'. It was successfully demonstrated that although the seaweeds appeared to die back over the course of the summer, and became heavily fouled with epifauna, regrowth of fronds from the meristematic tissue did occur (Figure 3.7b), allowing for a second crop the following season without reseeding the longlines.

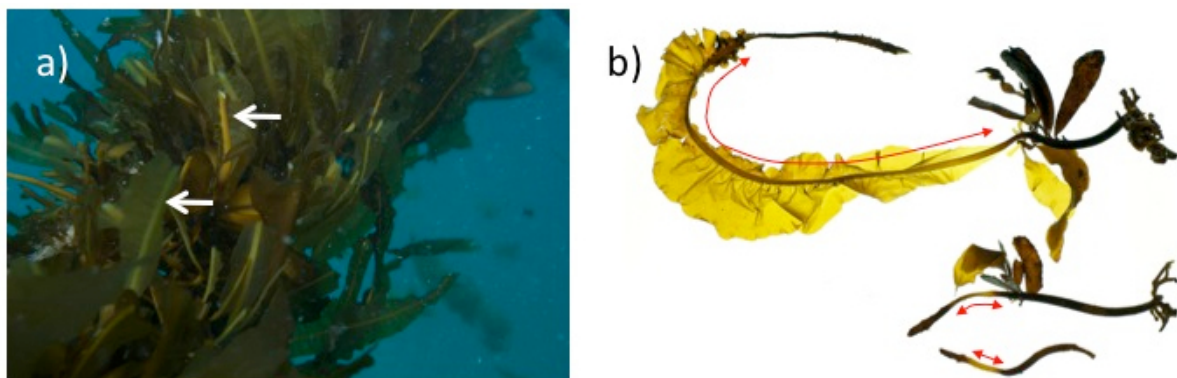


Figure 3.7 a) Freshly harvested algal biomass (*Alaria esculenta*) with holdfasts, stipes and partial frond (white arrows) remaining on longline for future regrowth b) Regeneration of *Alaria esculenta* fronds from meristematic region with regrowth sections indicated by red double-headed arrows.

Regardless of the harvesting strategy as described above, the main aim of the harvest period is to remove the biomass before the water temperature starts increasing quickly in the spring. This signals a speedy development of epifauna on the fronds of the seaweeds, which can quickly reduce the quality of the biomass. Common fouling organisms include hydroids (e.g. *Obelia geniculata*) and bryozoans (e.g. *Membranipora membranacea* and *Electra pilosa*).

Harvesting at Ventry Harbour during the EnAlgae project was done by hand and guided by the project's external experts, Dingle Bay Seaweeds. The header rope is lifted on board an adapted boat at the bow, over a rotating wheel that gently feeds the biomass into the boat without stripping it from the line. Operatives place a large net bag in the bottom of the boat, and use sharp knives to partially or totally remove the biomass (Figure 3.8a). The header rope is pulled along by a small motor at the stern of the boat, allowing the boat to move from one end of the line to the other. Harvesting of a longline typically takes 30-40 minutes.

Once the boat is full of seaweed, it returns to the pier, where the four corners of the net bag are securely gathered together and lifted out onto the pier by tractor (Figure 3.8b). Nets of seaweed are loaded into a waiting high-sided trailer or lorry for transport to a processing facility.

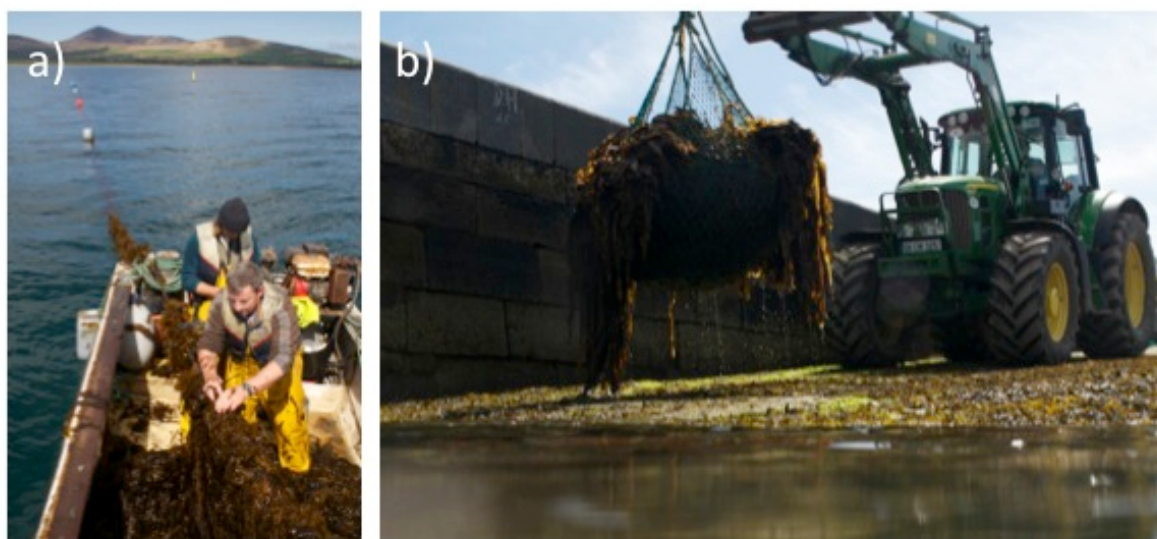


Figure 3.8 a) Harvesting algal biomass in Ventry Harbour, Co. Kerry b) Algal biomass lifted out of boat in nets (c. 750-1000 kg per net), allowing water to drain away from biomass.

Hazards and precautionary statements

Hazards and precautionary statements as described in Section 3.1.1 also apply when working on a seaweed site at harvesting time. Further care must be taken when using sharp harvesting knives and when moving about a boat that is full of slippery seaweed. Take great care not to overload the vessel with biomass as capsizing due to a top-heavy vessel may occur.

Quality Control

Quality has already been discussed above in the form of the harvesting strategy in relation to biofouling organisms. Regarding the actual harvesting process, ensure that the harvesting vessel or

containers are acceptably clean (e.g. it doesn't come in contact with engine oil, fuel or other contaminants), particularly if the seaweeds are going for food-grade products.

It is also important that harvesting occur as quickly as possible, preferably on a cooler day, so that the biomass does not heat up and start to 'cook' or ferment in the heat. Similarly, transport onwards from site must also ensure that the biomass does not deteriorate further where possible.

Waste stream and proper disposal

Typically, there is little in the form of waste during the harvesting process. All biomass cut is taken away for further processing, with only a very small proportion of biomass remaining in the vessel. Any liquid waste seawater and/or exudate from biomass is generally rinsed out of the boat at sea.

3.4 Environmental Monitoring Procedures

Environmental monitoring was an important part of the EnAlgae project, gathering data for inclusion in growth models and the understanding of what (measured) environmental factors were important for the optimal development of algal biomass. Directly measured parameters at most/all macroalgal pilot sites included seawater temperature, underwater irradiance (PAR; Photosynthetically Active Radiation), nutrients and turbidity.

3.4.1 Seawater Temperature

Purpose

To generate a long-term seawater temperature dataset at each macroalgal grow-out site

Principle

Temperature loggers were suspended off longlines to gather *in situ* seawater temperature data

Requirements and Procedure

A long-term monitoring device, such as a Tinytag® Aquatic 2 (TG-4100) logger (Figure 3.9) from Gemini Dataloggers (UK) was set up using manufacturer's software and instructions. The loggers were set to record the average seawater temperature after the end of every 10 minutes. Loggers had to be set up on the pier using a laptop, or back at the hatchery before deployment, therefore a start delay was included to allow for the logger to be deployed and equilibrate with the surrounding seawater before the first measurements were taken.



Figure 3.9. Tinytag® Aquatic 2 (TG-4100) temperature logger used to collect seawater temperature data at Ventry Harbour.

The capacity of the logger was set to measure at a 10 minute frequency which enabled 4-6 months of continuous data to be recorded before the logger memory was full. Loggers were always removed after approximately every 1.5 months to download the data.

Deployment and retrieval of temperature loggers occurred concurrently with the PAR meter (Section 3.4.2). In Ventry Harbour, both loggers were attached to a frame (Figure 3.10 a and b) at the start of longline 1. The frame was made of aluminium, approximately 1 kg in weight. Loggers were held on either by a threaded bolt (temperature logger) or a jubilee clip (PAR logger). The frame was designed as a 'T', with the horizontal bar supporting the loggers, and the 'tail' of the 'T' frame providing a counter-balance to ensure the loggers remained pointing upright towards the sea surface at all times. The whole structure was securely tied on to the longline header rope with slim nylon rope, and almost completely covered in electrical tape for further protection (Figure 3.10a). This structure remained in the sea attached to the longline with only minimum maintenance (cleaning off of algal growth) for the length of the project without failure, i.e. 4 years.

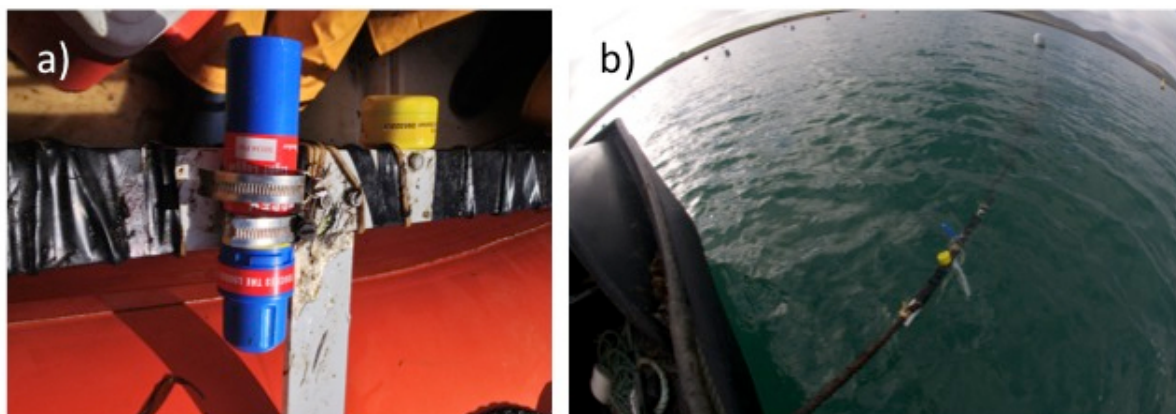


Figure 3.10 a) and b). Deployment of temperature (yellow) and light (blue) loggers a) on frame and b) attached to longline in Ventry Harbour, Co. Kerry.

Hazards and precautionary statements

Use and calibrate as per manufacturer's instructions. Buy and use multiple units (where possible) to ensure against failure of a single device. Thoroughly test the design of frame before placing out to

sea with equipment attached. Once out at sea, ensure inspection and any maintenance is carried out regularly to prevent damage/loss. Warm and waterproof clothing and lifejacket must be worn at all times during deployment/retrieval of equipment as per previous sections.

Quality Control

Regular retrieval of loggers and inspection of data is vital to detect irregularities in the dataset due to logger malfunction, or some other technical malfunction with the longline that leads to inaccurate data recording (e.g. the unlikely event that logger gets exposed for long periods of time to air temperature). Regular cleaning of fouling organisms from logger is also suggested as best practice.

Waste stream and proper disposal

These are long-lasting and reusable loggers. Periodically dispose of batteries and whole item at end of life in WEEE recycling.

References

- <http://www.gemindataloggers.com/data-loggers/tinytag-aquatic2>

3.4.2 Underwater PAR

Purpose

To generate a long-term seawater irradiance (PAR) dataset at each macroalgal grow-out site

Principle

PAR loggers were suspended off longlines to gather *in situ* irradiance data within the wavelength range of 400-700 nm; the range of the light spectrum utilised by the photosynthetic apparatus by plants and algae.

Requirements and Procedure

All three macroalgal partners purchased the same Odyssey PAR loggers from Dataflow Systems (New Zealand). The loggers measure light in the form of Photosynthetically Active Radiation (PAR) at 400-700 nm using a planar cosine-corrected sensor (Figure 3.11). The loggers were set up and calibrated using manufacturer's software and instructions. Calibration requires the use of a reference PAR meter. NUIG used a Skye Instruments Ltd. waterproof PAR sensor (SKP215 sensor with SKP200 meter). Once calibrated, the Odyssey PAR logger was set to record the average underwater light conditions after the end of every 10 minutes. Loggers had to be set up on the pier using a laptop, or back at the hatchery before deployment, therefore a start delay was included to allow for the logger to be deployed and equilibrate with the surrounding seawater before the first measurements were taken.

See Section 3.4.1 for further longline deployment and setup.



Figure 3.11. Odyssey light data logger

Hazards and precautionary statements

Unlike the temperature loggers, which were sealed units, as described in Section 3.4.1, the light loggers had to be opened up for set up and retrieval of data. Therefore, it is essential to correctly and completely tighten the cap of the logger to ensure water-tightness. In addition, a little silicone grease applied sparingly to the 'O' ring seal will give added reassurance of water-tightness. See Section 3.4.1 for further hazards and precautionary statements.

Quality Control

See Section 3.4.1

Waste stream and proper disposal

See Section 3.4.1. The light logger requires very regular cleaning to ensure the sensor does not become fouled with algae etc., which will steadily reduce the capacity to accurately record the underwater light environment. A wipe with a cloth, toothbrush etc. is usually sufficient to remove any growth and should ideally be carried out every 10-14 days, although this may be site dependent.

References

- <http://odysseydatarecording.com/index.php?route=product/category&path=64>
- Long, M.H., Rheuban, J.E., Berg, P. and Zieman, J.C. (2012). A comparison and correction of light intensity loggers to photosynthetically active radiation sensors. *Limnology and Oceanography: Methods* **10**, 416-424.
- <http://www.skyeinstruments.com/products/minimet-weather-station/sensors/par-quantum-sensors/>

3.4.3 Nutrients

Purpose

Regular collection of water samples to generate a profile of seawater macronutrient concentrations (nitrate, nitrite, ammonium, phosphate).

Principle

To provide information on nutrient profile/availability of surrounding seawater for uptake by seaweeds

Requirements and Procedure

Due to logistical reasons, it was not possible to take water samples for nutrient analysis in all sites, however it was successful at the QUB grow-out site. Water samples (n=3) were taken using a Niskin bottle (Figure 3.12). The bottle is opened at both ends and rinsed through with 'site' water. Both ends are held open on rubber bands and a quick release mechanism, which is triggered to close by sending a heavy weight down a wire to hit the mechanism. The open bottle is lowered to the desired depth in the water (1 m), before the weight is dropped onto the mechanism to close the ends and capture the water sample. The water sample is then poured into a 500 ml clean plastic screw-cap bottle. The process is repeated a further two times. The water is brought back to the lab for storage in the freezer, before analysis occurs at a later date with a collection of samples.

Analysis takes place using a nutrient analyser (Bran and Luebbe Autoanalyzer 3) using standard seawater chemistry analytical methods (Parsons *et al.*, 1984).

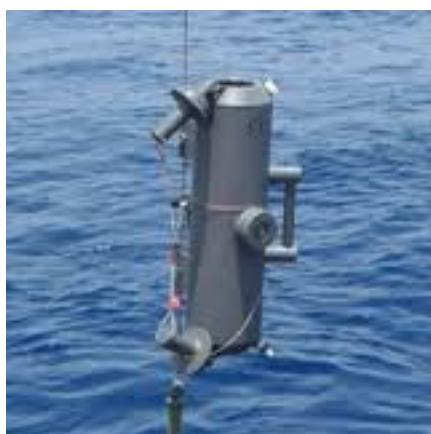


Figure 3.12. Example of an opened Niskin bottle used to take water samples (image from <http://benthicsolutions.com/equipments>).

Hazards and precautionary statements

See Section 3.4.1

Quality Control

Ensure multiple samples are taken at each sampling time, and ensure the protocol is followed correctly. Water samples must be either frozen or analysed within a couple of hours of collection, otherwise nutrient concentrations vary as living organisms (e.g. bacteria) within the sample will consume them.

Waste stream and proper disposal

NA

References

- Parsons, T.R., Maita, Y. and Lalli, C.M. (1984). *A manual of chemical and biological methods for seawater analysis*. Pergamon Press, Oxford.

3.4.4 Turbidity

Purpose

A simple, regular measurement of turbidity to assess general light conditions at the site.

Principle

The underwater light environment is complex, with suspended solids in the water column absorbing and reflecting rays of light, which can significantly alter light penetration to algae. Increased turbidity is caused by many things, including sediment particles, zooplankton, phytoplankton, plant detritus etc., particularly after storm events and spring blooms. Turbidity measurements were made at sites where possible, but preferably during each sampling trip to build up a seasonal dataset.

Requirements and Procedure

While turbidity can be measured by environmental probe sensors, it can also be simply and cheaply measured using a black and white quartered disc (approx. 30 cm diameter). This disc is weighted and lowered over the side of the boat as close to noon as possible. The rope attached to the disc is marked into metre sections. As the disc is dropped lower and lower, it reaches a depth where it finally disappears from sight. At this point, lift the rope gently upwards again, until the disc just reappears. When satisfied at having established the point at where the disc has just disappeared, measure the length of rope in the water (i.e. depth of the disc). Measure the metre section to the nearest centimetre. Take several readings, and take an average for the day. Additional information to record includes:

- Date and location of sample
- Time of sample(s)
- Depth of sample(s)
- General weather conditions
- Sea state

Hazards and precautionary statements

See Section 3.4.1

Quality Control

Where possible, make the measurements in as similar conditions as possible, e.g. using the same person, as close to noon as possible, slightly apart from the longlines to avoid entanglement/shadows etc.

Waste stream and proper disposal

NA

Chapter 4: Biomass Sampling

4.1 Biomass Sampling Methods

A central theme of the EnAlgae project was to establish a network of pilot facilities and to standardise the measurement and interpretation of results to add to the body of European (and global) research on algal cultivation. A macroalgal sampling protocol was developed for use at each of the three sites and is described below. It takes 3-4 months from deployment before seaweeds are large enough to handle and for sampling of biomass to occur. From this point until harvest, tissue samples were taken for morphometric measurements (Section 4.2) and further analysis (Chapter 5).

Purpose

To periodically collect seaweed samples in order to follow and understand the development and productivity of the biomass and to estimate/predict the final yield of crop.

Principle

Juvenile sporophytes are an average of 5-10 mm when deployed in late autumn/early winter and depending on species, can be on average 2.5-3 m in length in just 5-6 months. It is important to be able to monitor the crops to see a) whether the crop grows at all; b) the rate of growth in comparison to other years/sites; c) the quality of the fronds in relation to biofouling that can present from mid spring onwards.

Requirements and Procedure

Section 4.2 describes the shared protocol for sample measurements between the EnAlgae macroalgal partners.

Prior to measurement, sampling is very simple in all sites. General equipment include:

- Plastic sample bags
- Pre-written tags on waterproof paper/plastic (to include: date, site, species collected, longline number/location, sample number)
- Waterproof marker
- Cool box(es) and ice blocks
- Tape measure and ruler
- Knife (plus spare)

Samples are taken at each macroalgal site. Five samples are taken per species deployed on a particular date, which generally relates to five samples per longline, or other growth structure. Pre-determined longline positions are generated (to nearest metre), and a 15 cm sample (for high density) or 30 cm sample (for lower density) cultures is taken (Figure 4.1).



Figure 4.1. Taking a sample of *Saccharina latissima* biomass (from 15 cm of longline header rope) at Ventry Harbour, Co. Kerry.

Total seaweed biomass, including holdfasts are removed from the line, and put into plastic sample bags with appropriate sample tags. Bags are transported back to shore and packed into cool boxes for the trip back to the laboratory and morphometric measurements (Section 4.2).

Hazards and precautionary statements

See Section 3.1.1.

Quality Control

Ensure that samples are kept cool at all times after removal from lines and try to keep transportation time to a minimum. Process the samples for morphometric analysis as soon after the sampling as possible (i.e. max. 2 days afterwards, depending on the number of samples).

Waste stream and proper disposal

NA

4.2 Morphometrics

From the beginning of the EnAlgae project, morphometric measurements of the kelp were standardised between the three macroalgal pilots (where possible) to ensure direct comparisons of productivity could be made between the sites, and biochemical samples could be taken.

Purpose

To develop and share a set of morphometric measurements between the three macroalgal partners for use with *Saccharina lattissima* and *Alaria esculenta* (and any other kelp species cultivated).

Principle

To provide common measurement techniques that will enable direct comparison and analysis of macroalgal data between the EnAlgae project partners.

Requirements and Procedure

Samples are collected from the longline growth structures (Section 4.1). Sampling occurs monthly, from February to May or June, depending on final date of harvest.

Equipment required to make the measurements include:

- Tape measure (150 cm)
- Ruler
- Balance (to nearest 0.1 g)
- Trays
- Laboratory tissue
- Salad spinner
- Scalpel
- Tarpaulin/oilcloth (optional)

The following sampling measurements are made:

Productivity

A measurement of the seaweed biomass, as expressed per length of longline, i.e. kg m^{-1} culture⁶ rope. Measurements are made on the 5 biomass samples, and an average taken.

Density

The number of plants per biomass sample, expressed as number m^{-1} culture rope. Measurements are made on the 5 biomass samples and an average taken.

Individual plant measurements of largest plants per biomass sample

The 12 largest plants per biomass sample are measured, therefore total of 60 plants per deployment are measured. Measurements/observations (Figure 4.2) include:

- (1) Stipe length (to nearest 0.5 cm)

⁶ Culture rope in the case of EnAlgae pilots means the header rope of the longline as all deployments used for biomass measurements were deployed horizontally. However, if seaweed is grown on vertical rope droppers hanging from the longline header rope, then the culture rope in question may be a dropper. Productivity is standardised to per metre of culture rope, therefore in order to understand the productivity per area, the total amount of culture rope per site also needs to be known.

- (2) Blade length (to nearest 0.5 cm)
- (3) Blade width, at the widest point (to nearest 0.5 cm)
- Plant weight* (i.e. stipe and blade only, no holdfast) (to nearest 0.1 g)
- Blade weight* (to nearest 0.1 g)
- Sporophyll weight (to nearest 0.1 g; *A. esculenta* only)
- Reproductive status (Fertile; Yes/No)

* Before weighing the plant/blade weight, excess water is gently blotted from the blades using lab tissue paper when plants are very small, and by spinning individual plants in a salad spinner when plants are much bigger. Number of spins standardized to 10 revolutions.

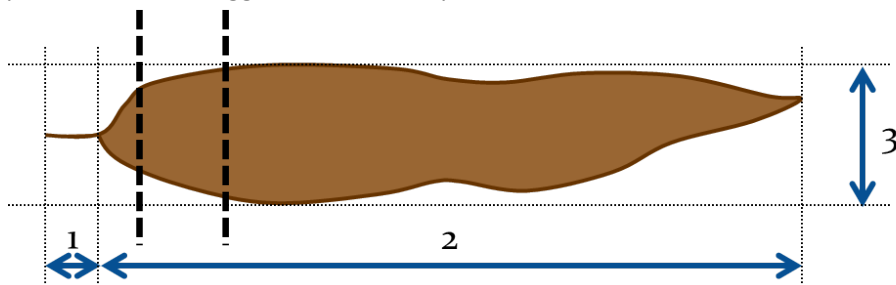


Figure 4.2. Measurements taken from kelp individuals (n = 12 per sample). Dotted lines represent approximate location of portion of blade taken for the dry weight sample (Section 4.3).

Sampling procedure requires two people minimum to ensure a quick process time. 3 people are preferable. Remove plants from sample bag onto a tray. Sort through sample and extract the 12 largest individuals for further morphometric measurements as described above.

Count the number of remaining plants, and record total number of plants (including 12 largest).

Spin the remaining biomass in a salad spinner, and weigh on balance to nearest 0.1 g and record. Remember to add weights of the 12 largest to this figure to obtain the final sample biomass.

On a table or on a tarpaulin/oilcloth on the floor once the individuals become too large, spread out the 12 largest individuals, ensuring full length is measurable, as well as the widest blade width. Record all measurements as described above, using a standardised record sheet (e.g. Figure 4.3).

EnAlage Biomass Sampling

Date:

Site:

| 12 Largest Plants per sample | | | | | | | |
|------------------------------|-----------|---------------|--------------|---------------|------------------|--------------|----------|
| Longline No. | Plant No. | F.Length (cm) | F.Width (cm) | S.Length (cm) | Plant Weight (g) | F.Weight (g) | Fertile? |
| | 1 | | | | | | |
| Sample No. | 2 | | | | | | |
| | 3 | | | | | | |
| Section No. | 4 | | | | | | |
| | 5 | | | | | | |
| Sample Biomass (kg) | 6 | | | | | | |
| | 7 | | | | | | |
| No. Plants in Sample | 8 | | | | | | |
| | 9 | | | | | | |
| | 10 | | | | | | |
| | 11 | | | | | | |
| | 12 | | | | | | |

Figure 4.3. Example of a data record sheet for kelp morphometric sampling.

Quality Control

Try to complete all measurements as quickly as possible after sampling to avoid decomposition of samples. During sampling, store samples in chilled conditions to slow down tissue deterioration. Any samples that are required for biomass analysis should be removed and stored (Section 4.4) as quickly as possible as a first priority before further measurements are made. If multiple people are taking measurements over the course of the season, ensure that some prior training and cross-checking of measurement technique takes place to minimise discrepancies between operatives.

Hazards and precautionary statements

If working in chilled conditions for measurements (e.g. in the temperature controlled wet lab), ensure that adequate warm and waterproof clothing is worn, and that regular rest breaks are included in the schedule.

Waste stream and proper disposal

Any waste sample biomass can be put in a compost bin. Sample bags can be disposed in the bin, or washed and dried and reused.

4.3 Dry Weight Estimation

The wet to dry weight ratio can vary from species to species, as well as age and point of growth in the cultivation cycle. Dry weights are a standardised way of reporting biomass, and therefore an important measurement within the EnAlage project.

Purpose

To obtain completely dried samples of kelp frond/blade tissue for further analysis and comparison

Principle

Wet weight can be enormously variable, and while useful (particularly when describing large amounts of fresh biomass), dry weights are internationally used/recognised and will aid in the description of the dry weight productivity from the sites.

Requirements and Procedure

Select blades for dry weight analysis from each sample (generally n=5 per sample resulting in n=25 per deployment, but minimum of n=3 per sample required) and rinse quickly in fresh water. Blot gently with laboratory tissue.

Meanwhile, prepare equivalent number of tinfoil pieces, numbered and weighed to nearest 0.0001 g. Weigh portion of blade (approx. 5-7 cm length) taken from a section as indicated by dotted lines in Figure 4.2 to nearest 0.0001 g, place on tinfoil and put in oven pre-heated to 60 °C for 24 hours or until constant weight has been achieved.

Remove from oven, weigh tinfoil and dried sample to nearest 0.0001 g and subtract tinfoil value from tinfoil+dried sample total to arrive at dried sample value.

Quality Control

Standardise to same location on blade; avoid using heavily fouled blades as this increases dry weight significantly.

Hazards and precautionary statements

Ensure correct operating protocols are used with the oven, and protective oven gloves used if required.

Waste stream and proper disposal

Samples and tinfoil can be safely disposed of in the bin.

4.4 Biomass storage for further biochemical analysis

Further chemical analyses were carried out on the biomass samples from the three macroalgal partners. All samples were stored in the following standardised way prior to the analysis.

Purpose

To provide a simple method of storing a repository of samples for further biochemical analysis.

Principle

Biochemical composition can change and degrade over time, giving false indications of results from cultivation conditions. Freezing, followed by freeze-drying is a recognised method for efficient stabilisation of material.

Requirements and Procedure

All samples are placed into clearly named plastic sample bags, with approx. 1 kg fresh material per sample bag. The sample is flattened out (not stuffed into the bag in a dense ball) to aid complete freeze-drying in the second stage. Samples are then placed in a standard -20 °C freezer.

After an appropriate period (which usually relates to access and capacity of freeze-drier), frozen samples are placed in a freeze-drier, and dehydrated according to manufacturer instructions. Samples are then removed and vacuum-packed in further plastic sample bags, which also contains appropriate sample identification details. The vacuum-packed samples are stored in cool, dry and dark conditions until required further.

Quality Control

Ensure adequate sample is retained for further analysis

Hazards and precautionary statements

Ensure freeze-drier is regularly serviced to ensure efficient freeze-drying processing. Always ensure that an appropriately trained operative uses this equipment.

Waste stream and proper disposal

NA

Annex 1 Nutrient Media Recipes

All recipes below are as used by CEVA in various macroalgal cultivation SOPs, as taken from Golven et al., 2014.

F/2

1) Solution 1.

Add the following to 1 L of distilled freshwater:

| | |
|-------------------|------|
| NaNO ₃ | 75 g |
|-------------------|------|

2) Solution 2.

Add the following to 1 L of distilled freshwater:

| | |
|---|-----|
| NaH ₂ PO ₄ , H ₂ O | 5 g |
|---|-----|

3) Metal Solution.

Add the following to 1 L of distilled freshwater:

| | |
|--|--------|
| FeCl ₃ , 6H ₂ O | 3.15 g |
| Na ₂ EDTA or Na ₂ EDTA, 2H ₂ O | 4.36 g |
| CuSO ₄ , 5H ₂ O (stock solution at 9.8g/L) | 1 mL |
| Na ₂ Mo ₄ , 2H ₂ O (stock solution at 6.3g/L) | 1 mL |
| ZnSO ₄ , 7H ₂ O (stock solution at 22g/L) | 1 mL |
| CoCl ₂ , 6H ₂ O (stock solution at 10g/L) | 1 mL |
| MnCl ₂ , 4H ₂ O (stock solution at 180g/L) | 1 mL |

4) Vitamin Solution.

Add the following to 1 L of distilled freshwater:

| | |
|--|-------|
| Vitamin B ₁ | 0.2 g |
| Stock solution of Vitamin B ₁₂ at 1 g L ⁻¹ | 1 mL |
| Stock solution of Vitamin H at 0.1 g L ⁻¹ | 10 mL |

5) Silicate Solution (optional).

Add the following to 1 L of distilled freshwater:

| | |
|--|------|
| Na ₂ SiO ₃ , 5H ₂ O | 30 g |
|--|------|

6) Utilisation.

Add the following to 1 L of culture medium (seawater):

| | |
|-------------------|--------|
| Solution 1 | 1 ml |
| Solution 2 | 1 ml |
| Metal Solution | 1 ml |
| Vitamin Solution | 0.5 ml |
| Silicate Solution | 1 ml |

Walne's solution

1) Principal solution.

Add the following compounds in this order and fill with distilled freshwater to 1L.

| | |
|--|--------|
| Na ₂ EDTA | 45 g |
| H ₃ BO ₃ | 33.6 g |
| NaNO ₃ | 100 g |
| NaH ₂ PO ₄ · 2H ₂ O | 20 g |
| MnCl ₂ · 4H ₂ O | 0.36 g |
| FeCl ₃ · 6H ₂ O | 1.3 g |
| Metals solution (see below) | 1 mL |

2) Metals solution.

Add the following compounds in this order and fill with distilled freshwater to 100 mL.

| | |
|---|-------|
| ZnCl ₂ | 2.1 g |
| CoCl ₂ · 6H ₂ O | 2 g |
| CuSO ₄ · 5H ₂ O | 2 g |
| (NH ₄) ₆ Mo ₇ O ₂₄ · 4H ₂ O | 0.9 g |

3) Vitamins solution.

Add the following compounds in this order and fill with distilled freshwater to 100 mL.

| | |
|--|--------|
| Thiamine (Vitamin B ₁) | 0.2 g |
| Cyanocobalamine (Vitamin B ₁₂) | 0.01 g |
| Vitamin H (Biotine) <i>DD</i> | 0.1 g |

4) Concentrations to be used in seawater.

| | |
|--------------------|------------------------|
| Principal solution | .1 mL L ⁻¹ |
| Vitamins solution | 0.1 mL L ⁻¹ |

PES medium

1) Fe solution.

Add the following compounds in this order and fill with distilled freshwater to 500mL. Store at 4°C.

| | |
|---|--------|
| Na ₂ -EDTA · 2H ₂ O | 330 mg |
| Fe(NH ₄) ₂ (SO ₄) ₂ · 6H ₂ O | 351 mg |

2) P-2 metal solution.

Add the following compounds in this order and fill with distilled freshwater to 500mL. Store at 4°C.

| | |
|---|---------|
| Na ₂ -EDTA · 2H ₂ O | 500 mg |
| H ₃ BO ₃ | 570 mg |
| FeCl ₃ · 6H ₂ O | 24.5 mg |

| | |
|--|---------|
| MnSO ₄ · 4H ₂ O | 82.0 mg |
| CoSO ₄ · 7H ₂ O stock solution (4.8 mg/mL) | 0.5mL |
| ZnSO ₄ · 7H ₂ O | 11.0 mg |

3) Preparation of the PES medium.

Add the following compounds in this order into 300mL of distilled freshwater.

| | |
|---|--------|
| Tris hydroxymethyl aminomethane | 5.0 g |
| NaNO ₃ | 3.5 g |
| Na ₂ -glycerophosphate | 500 mg |
| Fe solution | 250 mL |
| P-2 solution | 250 mL |
| Vitamin B ₁₂ stock solution (0.1mg/mL) | 1.0 mL |
| Thiamine-HCl stock solution (1.0mg/mL) | 5.0 mL |
| Biotine stock solution (0.1 mg/mL) | 0.5 mL |

Check the pH and adjust to 7.8 with 1N HCl or NaOH, and then filled to 1L with distilled freshwater. Dispense 20 mL of the PES medium into sealed tubes and sterilize the medium by autoclaving at 121°C for 20 min. Store at 4°C.