Asparagopsis taxiformis hatchery and cultivation manual

by Dr John Statton
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AgriFutures® Emerging Industries

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Developing *Asparagopsis* cultivation at scale for rapid industry growth

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Foreword

The emerging Australian seaweed industry is becoming prominent and attractive because of the role it can play in helping Australian agriculture realise its sustainability goals. The red seaweed *Asparagopsis* spp., when fed in small quantities, holds promise for mitigating methane emissions in cattle and helping producers achieve carbon-neutral meat production. Despite significant potential, however, technical challenges persist.

Understanding the potential the seaweed industry holds and the need for agriculture to reduce its emissions in line with Australian Government targets, AgriFutures Australia invested in the development of this hatchery and cultivation manual, aimed at people interested in exploring the production of *Asparagopsis taxiformis*.

The manual primarily provides guidelines for cultivating *A. taxiformis* tetrasporophytes. It serves as a starting platform for industry stakeholders and researchers to cultivate the species by providing practical insights to improve the efficiency of the hatchery-to-nursery production process and generate seaweed juveniles suitable for commercial production.

It is anticipated the manual will assist in advancing the commercial cultivation of this native seaweed in Australia. The availability of the manual and the information contained herein may stimulate further emergence of the domestic seaweed industry, particularly *Asparagopsis* cultivation, leading to increased standardisation in practices and higher-quality seaweed products.

This manual has been produced as part of the AgriFutures Emerging Industries Program, which focuses on new and emerging industries with high growth potential. Emerging animal and plant industries play an important part in the Australian agricultural landscape. They contribute to the national economy and are key to meeting changing global food and fibre demands. Most of AgriFutures Australia's publications are available for viewing, free download or purchase online at www.agrifutures.com.au.

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Executive summary

AgriFutures Australia has identified seaweed as one of the most attractive emerging aquaculture industries in Australia because of the role it can play in helping Australian agriculture realise its sustainability goals. The red seaweed *Asparagopsis* spp., when fed to cattle in small quantities, can mitigate methane emissions from livestock by up to 98%. *Asparagopsis* could become the 'wheat' of Australia's coastline, and is the key to a world-first, carbon-neutral meat, grown and processed in Australia.

Asparagopsis cultivation at scale is the single-biggest opportunity for rapid industry growth and optimising social and environmental outcomes (Kelly, 2020). Existing demand from the livestock sector to reduce carbon emissions and current acceleration in investment means *Asparagopsis* production could make a substantial contribution to the Australian seaweed industry's targets of being a $100 million industry by 2025, creating 1,200 direct jobs and reducing domestic greenhouse gas emissions by 3%.

However, there are technical gaps to realising cultivation of *Asparagopsis* at scale. Outcomes of a literature review, discussions with industry and findings from the *Australian Seaweed Industry Blueprint* (Kelly, 2020) indicate that developing the breeding and cultivation techniques for this seaweed at scale requires significant research and development. This includes: (i) publicly available hatchery and nursery procedures; (ii) development of quality seedstock; (iii) contamination management; and (iv) production technologies.

Research aims and objectives

The manual primarily provides guidelines for cultivating *Asparagopsis taxiformis* tetrasporophytes. It serves as a starting platform for industry stakeholders and researchers to cultivate the species by providing practical insights to improve the efficiency of the hatchery-to-nursery production process and generate seaweed juveniles suitable for commercial production.

Research approach and methods

The research approach and methods used in developing the manual were varied. To understand the methodologies previously used to cultivate *Asparagopsis* spp., we undertook desktop research, followed by general dialogue with researchers and private industry groups, both nationally and internationally, to determine knowledge gaps integral to achieving our aims and objectives. We then designed and developed the Seaweed Aquaculture Research and Hatchery (SARaH) laboratories and facilities to accommodate the requisite equipment, instruments and workspaces to facilitate the research activities. SARaH lab personnel developed hatchery, nursery, production and de-contamination methodologies and procedures by running multiple lines of inquiry and fast reiterations of trials to achieve rapid and reproducible outcomes.

Results and key findings

A comprehensive manual for *Asparagopsis* cultivation has been compiled by the SARaH lab group, which could significantly advance the commercial cultivation of this seaweed in Australia. The availability of the manual and the information contained herein may stimulate further emergence of the domestic seaweed industry, particularly the field of *Asparagopsis* cultivation, leading to increased standardisation in practices and higher-quality seaweed products.

The use of synthetic fertilisers can provide necessary macro and micronutrients for *Asparagopsis* growth, while refining cultivation approaches and manipulating environmental conditions (light quantity, light wavelength, temperature, nutrient supplementation, contamination control) to determine optimal cultivation requirements has been found to increase seaweed growth rates. These optimisations can improve the efficiency of the hatchery-to-nursery production process such that growth of seaweed juveniles into mature harvestable product can be achieved in four to six weeks, depending on the early-stage hatchery and nursery techniques.

Industry implications

Advancing *Asparagopsis* cultivation: This comprehensive manual provides valuable insights into successful and unsuccessful cultivation of *A. taxiformis,* which could significantly advance the commercial cultivation of this seaweed species in Australia. With the manual's help, those new to *Asparagopsis* cultivation can gain the necessary knowledge and techniques to start a hatchery and nursery for commercial cultivation.

Improving the emerging Australian seaweed industry: The availability of this manual could further stimulate the emergence of the Australian seaweed industry, especially in the field of *Asparagopsis* cultivation. The information contained herein could serve as a reference guide for other hatcheries and nurseries to follow, leading to more standardised practices and higher-quality seaweed products.

Facilitating research and development: The manual and key findings from this project present an opportunity for those experienced in *Asparagopsis* cultivation to provide feedback to SARaH lab group members for further refinement and improvement of their techniques. Constructive feedback could lead to further research and development, which could, in turn, enhance the cultivation of *Asparagopsis* and its commercialisation.

Facilitating environmental benefits: The commercial cultivation of *Asparagopsis*, a species with great potential to mitigate greenhouse gas emissions, could provide environmental benefits. The availability of the manual and the information provided herein could contribute to the expansion of this seaweed's cultivation, leading to reduced greenhouse gas emissions in the agriculture and livestock sectors.

Facilitating economic benefits: Successful *Asparagopsis* cultivation could provide economic benefits to the hatcheries, nurseries and other stakeholders in the seaweed industry. The availability of this manual could help in standardising production practices, leading to increased industry productivity, profitability and competitiveness.

Recommendations

Advancing *Asparagopsis* cultivation: There is a lack of publicly available knowledge and techniques for successful cultivation of *Asparagopsis taxiformis* in Australia. Recommendations related to changing this are:

- Encourage the dissemination of this manual compiled by the SARaH lab to individuals, organisations and stakeholders interested in *Asparagopsis* cultivation.
- Provide training and support to staff at new hatcheries and nurseries to ensure successful implementation of guidance provided in the manual.
- Conduct further research and development to improve the techniques detailed in the manual and refine the cultivation process.

Improving the emerging Australian seaweed industry: Due to limited knowledge sharing across the industry and institutes, there is a lack of standardised practices within hatcheries and nurseries, leading to inconsistent seaweed quality. Recommendations related to changing this are:

- Encourage adoption of the manual as a reference guide for hatcheries and nurseries in the Australian seaweed industry.
- Establish industry standards for *Asparagopsis* cultivation based on the practices outlined in this manual or another manual developed by existing industry and R&D groups.
- Encourage collaboration and knowledge sharing among hatcheries and nurseries to facilitate industry-wide improvements.

Facilitating research and development: There is limited publicly available information on *Asparagopsis* cultivation techniques and their optimisation. Recommendations related to changing this are:

- Encourage experienced *Asparagopsis* cultivators to provide constructive feedback on the manual to SARaH lab group members to refine and improve their cultivation techniques.
- Conduct further research and development to identify new and innovative techniques for *Asparagopsis* cultivation, with a focus on sustainability and efficiency.
- Encourage collaboration among researchers, hatcheries and nurseries to facilitate knowledge sharing, to enable industry-wide improvements.

Facilitating economic benefits: There is limited productivity, profitability and competitiveness in the Australian seaweed industry. Recommendations related to changing this are:

- Encourage adoption of the manual as a reference guide for hatcheries and nurseries to improve the productivity, profitability and competitiveness of the *Asparagopsis* cultivation industry.
- Provide support and funding to hatcheries and nurseries to ensure successful implementation of guidance provided in the manual.
- Promote the economic benefits of *Asparagopsis* cultivation to stakeholders in the industry and encourage its expansion.

Optimising conditions for dense cultures: There is a lack of knowledge on optimal conditions for *Asparagopsis* tetrasporophytes culture at commercial-level production densities (e.g. 3-7 g L^{-1}). Recommendations related to changing this are:

- Conduct experiments to test the effects of different environmental conditions on the growth and development of *Asparagopsis* tetrasporophytes at different culture densities.
- Consider using outdoor tank culture to test the effects of natural or ambient conditions.

Asparagopsis cultivation at scale is the single-biggest opportunity for rapid seaweed industry growth and optimising social and environmental outcomes

Introduction

This manual is a compilation of procedures, recommendations and considerations developed by the Seaweed Aquaculture Research and Hatchery (SARaH) Lab at the Indian Ocean Marine Research Centre (IOMRC), Watermans Bay, Western Australia. As part of a key series of outputs, the SARaH Lab has developed this hatchery, nursery and production manual for the red seaweed *Asparagopsis taxiformis*.

Although many techniques developed for *A. taxiformis* are transferrable to *A. armata*, optimisations for this cooler-water species need further work and were outside the scope of this manual. Information on method development, hatchery and nursery operations, production of commercial quantities of seaweed juveniles, contamination control and standard operating procedures (SOPs) have been combined and refined in this comprehensive manual for *Asparagopsis* cultivation, and made publicly available to stakeholders in the emerging seaweed industry in Australia.

The manual is presented for *Asparagopsis* hatchery and nursery operations, including: collection, preparation, maintenance and monitoring of carpospores; tetrasporophyte growth and development; maintenance, monitoring and generation of commercial quantities of seaweed juveniles; and biomass production. In addition, descriptions of the different equipment, instruments and resources used have been provided, along with key information on unsuccessful practices. The aim was to provide the information necessary to kickstart a hatchery and nursery for those new to *Asparagopsis* cultivation, across a range of resources. For those familiar with *Asparagopsis* hatchery and nursery cultivation, we anticipate constructive feedback so that we may refine, enhance and improve upon the information provided herein to drive the emerging Australian seaweed industry forward.

Asparagopsis taxiformis ecology and biology

Asparagopsis taxiformis has a wide tolerance for changing environmental conditions. It is endemic to the cool temperate to tropical waters of Australia, and is common in estuaries and bays, often found in subtidal areas less than 50 centimetres to several metres deep. It can be found either attached to rocks and colonising biogenic material, as an epiphyte on other algae and seagrasses, or free-floating. It is often found in high-energy environments and thrives in nutrient-rich waters. The species prefers salinities about 35 parts per thousand (ppt) but has some tolerance for lower and higher salinities; for example, it grows in Shark Bay, Western Australia at salinities greater than 40 ppt. It is distributed from cool temperate to warm tropical waters, with an optimal temperature range of 15-28 °C.

Asparagopsis taxiformis has a triphasic diplohaplontic heteromorphic life cycle (Figure 1), meaning each of the three phases are morphologically distinct. The species' haploid stage was initially described as *Falkenbergia hillebrandii* (Bornet) Falkenberg 1901 because it was thought to be a separate species. The three life cycle phases are a diploid tetrasporophyte stage, a haploid gametophyte phase and a diploid carposporophyte phase that occurs on the female fronds (Figure 1). Fully grown *A. taxiformis* has sparse branches in which long stolons and erect shoots develop in all directions. The branches, stolons and shoots ramify over and over again, which gives *A. taxiformis* its thallus-like appearance. The ultimate branchlets are filamentous and composed of three cell rows, whereas the larger branches consist of a central medullary filament and a gelatinous matrix surrounded by a cortex three to six cells thick. Gametophytes are terete and are about 200 mm in height. They form dense, pink intertwining clumps. Practically all red algae have a life cycle that alternates between a sexual stage and an asexual or spore-plant stage. In the case of an *Asparagopsis* species, the spore stage is either small, unobtrusive and epiphytic, or free-floating, and so different to the sexual stages that meant it was originally thought to be a separate genus – *Falkenbergia*. *Asparagopsis taxiformis* has a thread-like construction, with bands of three pericentral cells ringing narrow central cells.

The mature diploid tetrasporophyte produces four haploid tetraspores within each tetrasporangium by undergoing meiosis. Tetrasporangia occur in the cortex (the outer edge of cells) of the thallus and can be found anywhere on the blade. The tetraspores appear as red cross-shaped spots and can be observed with a microscope or even a hand lens. When tetraspores are released from the blade, they drift passively in the water column until they settle and adhere to a substratum. The spore will begin to internally divide, then enlarge and develop a multicellular disc. The centre of this disc will then develop a raised dome and the blade will develop from this initial growth. Each disc may produce many upright thalli, each of which may separate from the disc and continue to grow as a free-floating plant.

The plants that develop from tetraspores are either male or female haploid gametophytes (second stage in the life cycle), indistinguishable until maturity. Mature male gametophytes produce white-spotted areas with spermatangia on their thallus, observable under a microscope. Female gametophytes become apparent when their eggs (carpogonia), produced within the cortex of the plant, are fertilised by spermatia (non-motile sperm), and new tissue develops around the zygote. The new diploid, globular structure is the third phase of the life cycle, the carposporophyte, occurring on the haploid female thallus. The small globular structure is a cystocarp, and inside the cystocarp, the original zygote undergoes many cell divisions (mitosis), eventually producing many diploid spores, called carpospores. These non-motile, spherical spores are released into the water column and carried to a suitable substratum by water currents. Once settled on a substratum, the spores will adhere, divide and form a multicellular disc. These multicellular discs will initially produce a protuberance that develops into an upright thallus, which will develop into tetrasporophytes, thus completing the life cycle. Each disc may also produce many upright thalli, each of which may separate from their disc and continue to grow as a free-floating plant.

Besides the relatively complicated three-phase sexual reproductive life cycle, *A. taxiformis* is also able to reproduce asexually, through vegetative propagation. A single individual has the capacity to become hundreds or thousands of individuals, through continual fragmentation. Each fragment produced will grow and develop into an individual, and these blades can be further fragmented into several individuals, and so on. Each fragment has the capacity to then grow and branch into its own blade. This allows for a much simpler means of propagation, and creates consistency in a culture environment. Vegetative propagation is the most common means of culture, as it is quicker, easier and more efficient than starting from spores, and allows for consistency, as all blades in a culture can be genetically identical, all having the same parent (sometimes referred to as a clone). This is very important if the blades are being grown for a specific characteristic, such as bromoform consistency, specific life stage or morphology, or favourable growth rates and biomass yields. However, the vegetative propagation of a blade does not have an attachment stage, so the culture method needs to be adapted to the local needs and situation of the grower.

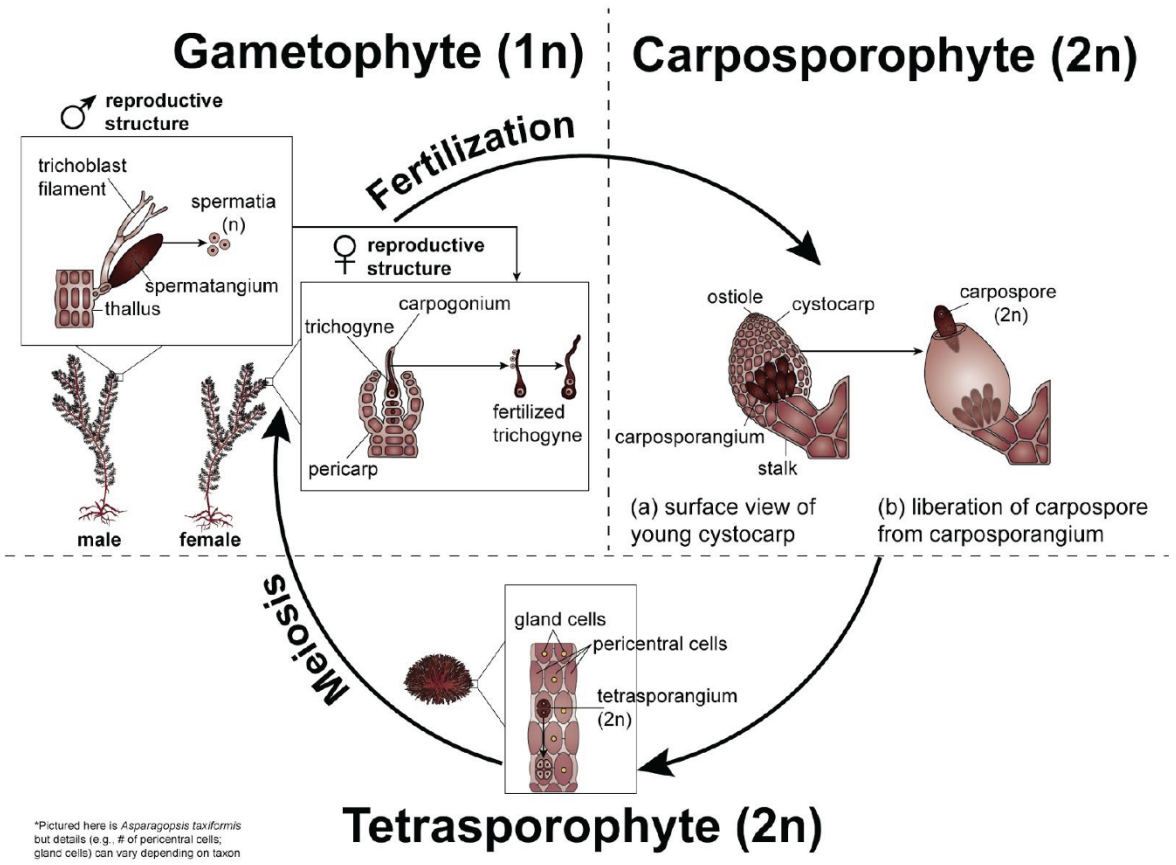

Figure 1. Life cycle in the red alga *Asparagopsis* spp. (phylum Rhodophyta; class Florideophyceae; order Bonnemaisoniales). This is an example of triphasic sporic meiosis (Khen 2020).

Asparagopsis taxiformis has a wide tolerance for changing environmental conditions. It is endemic to the cool temperate to tropical waters of Australia, and is common in estuaries and bays.

Hatchery and nursery set-up

The hatchery is used for liberating *Asparagopsis* carpospores from cystocarps and supporting the germination and early growth of young tetrasporophytes. The nursery is for maintaining stock cultures and for nurturing the growth of tetrasporophytes for biomass production, or to generate sufficient reproductive biomass for tetraspore production ready for seeding onto cultivation string, prior to out-placement to sea.

Regardless of the size or goals of the hatchery and nursery, each is designed to grow tetrasporophytes or young gametophytes by replicating the essential environmental conditions (water temperature, light, salinity, nutrient levels) found in their native habitat.

The function of a hatchery and nursery is to: (1) liberate carpospores; (2) germinate carpospores and support early growth through to sporelings; (3) replicate the natural environmental conditions; and (4) control contamination. Separating the hatchery and nursery conditions for seaweed cultivation is a common practice in seaweed aquaculture, for several practical reasons:

1. **Optimal growth conditions:** The hatchery and nursery phases of *Asparagopsis* production have different environmental and nutritional requirements. The hatchery phase involves the early life stages of seaweed, such as spore germination and initial growth, which often require specific conditions, such as controlled light, temperature and nutrient levels, to maximise survival and growth. The nursery phase, by contrast, involves caring for and growing young seaweed plants that have already developed in the hatchery. Nursery conditions are typically optimised for rapid growth and can differ from hatchery conditions.

2. **Scale and space management:** The separation of hatchery and nursery facilities allows for better management of space and resources. Hatchery operations often require more controlled and sterile conditions, which can be achieved in smaller, specialised set-ups. Once the *Asparagopsis* tetrasporophytes reach the nursery stage and becomes more robust, they can be transferred to larger systems, optimising the use of available space and resources.

3. **Contamination control:** By separating hatchery and nursery facilities, the contamination risk between different life stages is minimised. Hatcheries are susceptible to contamination from various sources, such as unwanted microorganisms or pollutants. Keeping the hatchery and nursery stages separate helps reduce the likelihood of disease or adverse effects on the growing *Asparagopsis*.

4. **Life stage-specific management:** *Asparagopsis* has varying requirements and faces distinct challenges at different life stages. By isolating the hatchery and nursery stages, aquaculturists can tailor their management practices and interventions to address the specific needs of *Asparagopsis* at each stage more effectively. This can lead to improved growth, survival and health of the *Asparagopsis* juveniles.

5. **Research and development:** Separating hatchery and nursery conditions allows researchers and aquaculture practitioners to study and optimise each stage independently. It helps in identifying the critical factors influencing successful cultivation at different life stages and contributes to refining and improving the overall *Asparagopsis* seaweed cultivation process.

By maintaining separate hatchery and nursery conditions, seaweed cultivators can better control the growth environment and achieve more success in producing healthy and commercially viable seaweed juveniles for subsequent deployment in larger-scale production systems.

Operating the hatchery and nursery requires understanding and use of basic laboratory equipment, attention to detail, and the ability to monitor and control the environmental conditions to support *Asparagopsis* growth. A variety of hatchery and nursery designs and procedures are available for private and commercial seaweed growers; however, each is specifically tailored for the goals and capabilities of the nursery. For instance, cost can vary dramatically depending on the nursery design and the equipment purchased.

The definitions, equipment and procedures described in this manual were utilised by SARaH lab group members during two years of experimentation and trials, and were found to be successful. The economic feasibility of this set-up has not been provided since the SARaH lab is a research institute. However, alternative equipment and procedures could reduce costs. Depending on an individual's background – farmer or scientist – these nursery processes may be considered relatively easy or difficult. However, for an interested *Asparagopsis* grower, no matter what background they have, by following these procedures and maintaining the described conditions, spores from reproductive *Asparagopsis* may be liberated, isolated and grown in the nursery. In four to six weeks, the tetrasporophytes will be mature and ready for harvest.

Essential capabilities and equipment

Essential capabilities and equipment required for successful cultivation of *Asparagopsis taxiformis* are shown in Table 1.

Table 1. Capabilities and equipment required for successful cultivation of *Asparagopsis taxiformis*.

Item	Description
Temperature	*Asparagopsis* grows in water between 15 and 28 °C but we have found that 17-21 °C is optimal for *A. taxiformis*. The nursery must be able to control water temperature within this range through a combination of heater/chillers and water baths, or the use of climate-controlled rooms. We used a mix of constant-environment rooms and water baths thermally regulated by a sump tank and heater/chiller unit, and production systems using recirculating water with thermal regulation via a sump tank and heater/chiller unit.
Light	Light intensity, wavelength and light hours per day (photoperiod) must also be controlled. This may be achieved by using environmentally controlled growth chambers or external light fixtures. A photoperiod of either 10:14, 12:12 or 14:10 (light:dark) was successfully used in the SARaH lab. The most suitable light was cool white light, provided either by T8 fluorescent tubes or LED lights with all-white light.
Light-measuring meter	A LiCor LI-1400 data logger with a 2π quantum sensor is an instrument used to measure and monitor the intensity of the light (photosynthetically active radiation; PAR) the seaweed receive. The light wavelengths are measured in micromoles per square metre per second (μmol photons m^{-2} s^{-1}). The measuring probe can be submerged in the culture vessels to gain an accurate reading. It is important to use a meter that is submersible and measures in micromoles.
Fresh water	Distilled or milli-Q water must be readily available for cleaning glassware, equipment, mixing reagents and nutrient media. The volume needed will depend on the scale of the operation. SARaH had access to both distilled water for nutrient media preparation and milli-Q water for cleaning.
Water-filtering device, water filters and ultraviolet (UV) radiation	Any configuration of pumps, filters, tubing and UV sterilisation devices can be used to purify and sterilise the seawater for use in the nursery. Various-sized mesh filter cartridges can be used in line with the pumping system to clean the seawater. Filters can be purchased in a wide range of pore and membrane sizes; 5.0, 1.0, 0.5 and 0.2 micron (μm) filters can be used together to obtain relatively low bacterial counts in the water. A UV sterilisation device exposes the filtered seawater to UV radiation, which is used in many aspects of nursery production.
Culture vessels	Culture vessels are used to grow sporelings after germination and to maintain stock cultures. A great deal of flexibility is possible here. Culture vessels ranging in size from 250 mL to 4 L (or larger) may be used, but the environmental parameters mentioned here must be maintained. At the SARaH lab, we used 1 L glass vessels as our culture vessel of choice, but also 250 mL and 500 mL glass Erlenmeyer vessels.

Seawater	A reliable source of seawater is necessary for isolation and grow-out of the *Asparagopsis* carpospores, tetrasporophytes and gametophytes. Depending on the location, seawater may be piped directly from the ocean or transported in via a water cartage vehicle. Contaminants must be removed from the seawater (i.e., the seawater must be disinfected or sterilised) prior to use. Synthetic seawater could be used but becomes expensive at larger scales and we did not test its use with *Asparagopsis*. Seawater needs to be clean and sterile; Table 2.1 describes and compares various methods for cleaning seawater. The IOMRC facility had access to 25 µm filtered seawater sourced from the Marmion Marine Park in the Indian Ocean off Western Australia. The SARaH lab further treated the water down to 0.5 µm and UV sterilised, then pasteurised.
Production tanks	Production tanks are also referred to as production aquaria or growth tanks. A great deal of flexibility is possible here. Tanks ranging in size from 4 L to 1000 L (or larger) may be used, but the environmental parameters mentioned here must be maintained. Depending on the trial, we had access to 25 L circular and rectangular tanks, and up to 10,000 L outdoor tanks.
pH	The acidity/alkalinity range must be monitored and controlled. *Asparagopsis* carpospores, tetrasporophytes and gametophytes grow best when the pH is between 7.0 and 9.0. The pH must be measured regularly and adjusted with either regular water changes or water exchange, or the addition of carbon dioxide (CO_2) gas as needed. A pH probe and meter are essential for monitoring the general health of the cultures.
Microscope	A dissecting and compound microscope is required. The carpospore cells that are released and captured for culture are 20 µm and can only be seen with the use of a dissecting microscope and/or compound microscope depending on the level of detail that is required and the budget. We used a combination of the two. The dissecting microscope was used for cystocarp and carpospore extraction plus general observations on the tetrasporophytes. The compound microscope was used to observed contamination in the water within our cultures at 400x magnification.
Aeration	Adding air or 'bubbling' air into the culture vessels or production tanks is also essential to maintaining pH and improving the nutrient and light availability to tetrasporophytes and gametophytes. This is typically done using air pumps or air compressors, along with air filters to remove airborne contaminants. Aeration is essential for proper tetrasporophyte growth in the culture and production vessels. Air filters can be placed in line with the air tubing to reduce the risk of introducing airborne contaminants. The SARaH lab recommends using 0.2 µm air filters to eliminate potential contaminants.
Laminar flow unit	Laminar flow units are essential to working within a sterile environment when inoculating cultures or making nutrient media. Laminar flow units are integral to maintaining clean instruments during media preparation and culture inoculation, and maintaining a clean stock culture, and during carpospore liberation.
Sterilisation and contamination control	A process for sterilising or removing contaminants is mandatory. As shown in Table 2, standard sterilisation techniques include autoclaving, tyndallisation, pasteurisation, filtration, and ultraviolet (UV) irradiation. There are numerous considerations (e.g. cost, time, effectiveness) to be aware of when choosing the technique that most effectively meets the needs of the nursery. The positives and negatives of each of these processes are listed in Table 2. After experimenting with most of the procedures described in Table 2, the SARaH lab adopted the protocol filtration to 0.5 µm, ultraviolet (UV) irradiation, then pasteurisation. While autoclaving is great for freshwater, we tended to get salt precipitation in seawater so opted for pasteurisation. This protocol was able to remove almost all contaminants at a cost and time much less than transporting autoclaved seawater prior to use.
Refrigerator	The refrigerator is necessary for storing culture nutrients and vitamins, as well as chilling small quantities of seawater. The SARaH lab recommends storing several gallons of filtered seawater for releases and as emergency back-up water if a water chiller fails. Stored seawater, however, should be used within one week of collection to prevent bacteria growth.
Nutrient media	Guillard's F/2 media or Cell-Hi® F2P (pre-made F2 media), Provasoli's enriched seawater (PES) and vitamins are the components added to the seawater to accelerate *Asparagopsis* growth. Germanium dioxide (GeO_2) is added to suppress diatom growth. See Appendix B for the nutrient media composition and preparation that the SARaH lab found most effective.

Hatchery set-up

The hatchery environment is a growth chamber or incubator with sensitive temperature and light control. In trials undertaken by the SARaH lab, temperature was maintained at 18-19 °C and light was adjusted based on the stage in the hatchery cycle. The incubators were set at a 10:14, 12:12 or 14:10 (light:dark) photoperiod. Choose one photoperiod and maintain it throughout the hatchery-nursery-production cycle. Any shift in photoperiod can trigger production of other life stages. Germination was typically carried out at 5 µmol photons m^{-2} s^{-1}. At four days, light was increased to 10 µmol photons m^{-2} s^{-1}. After the first branch (6-8 days) until 14 days, light intensity was increased to 15-20 µmol photons m^{-2} s^{-1}. The light ramping (5–10–15–20 µmol photons m^{-2} s^{-1}) process improved germination success and subsequent growth over the first two weeks. Within the hatchery, 60 mm petri dishes were the culture vessel of choice because they were sterile, easy to work with and sealable, and tetrasporophytes were easy to dislodge and transfer to the nursery culture vessels. Sterilisation or removal of contaminants (e.g. unwanted algae, protozoa and bacteria) from the seawater is one of the most important and challenging activities in the growth process. As outlined in Table 2, there are a number of ways this may be accomplished. The availability of running seawater, the volume of seawater being used, and the equipment on site all play a role in deciding which process may be utilised. Within the nursery, tetrasporophytes were cultivated in 1 L glass jar culture vessels because they were easy to clean and sterilise due to their large opening, easy to work with, and sealable with a transparent lid, and because tetrasporophytes rarely settled and re-attached.

Nursery and production set-up

The nursery was an illuminated rack system, set up either within a constant-environment room (CER) or a water bath with thermally regulated water. The production system was essentially a larger version of the nursery system. The production system for tetrasporophytes can take many forms (tanks, raceways, photobioreactors, ponds) and will be based on the farmer's preference. Here, we focused on the nursery set-up but the information is transferable to larger production systems. For culture vessels in the water bath, the water height was maintained at one-third to half the height of the culture vessel water volume, which was sufficient to maintain thermal regulation of the seawater within the culture vessel. The temperature of the nursery system was maintained at 18-19 °C. The nursery rack lighting was set at a 10:14, 12:12 or 14:10 (light:dark) photoperiod. However, only one photoperiod was maintained throughout the hatchery-nursery-production cycle. Large changes in photoperiod can trigger alternative life stages, which was not appropriate at this early stage. When tetrasporophytes were transferred to the nursery (from petri dish to tumble or free-floating culture), light intensity was maintained at 20 µmol photons m^{-2} s^{-1} for the first 24-48 hours to reduce stress, then increased to 30 µmol photons m^{-2} s^{-1} for the next 24-48 hours, and finally to 50 µmol photons m^{-2} s^{-1} for the following 4-6 weeks until tetrasporophytes were fully mature.

Asparagopsis production could make a substantial contribution to the seaweed industry's target of being a $100m industry by 2025.

Hatchery and nursery procedures

Liberation and isolation of carpospores from mature cystocarps (borne from Gametophyte thalli; see Figure 1) is the first step in establishing cultures in the hatchery. The carpospores are contained in the cystocarps, which are present for up to nine months each year off the Perth coast, with summer and winter dieback. These carpospores germinate primarily in response to temperature and light.

SARaH lab nursery activities have focused on facilitating liberation of carpospores from cystocarps, germination of carpospores and subsequent growth of tetrasporophytes within the hatchery before transfer to the nursery. Once tetrasporophytes are large enough to be fragmented, large quantities of seaweed juveniles can be generated in a short period for biomass production.

This manual stems from work performed in the SARaH lab's hatchery and nursery, and details procedures followed by lab personnel in starting cultures from carpospores and producing biomass within onshore facilities. The procedures were developed following a review of the literature and testing in the lab, and successful technique development in the lab, complemented by best professional judgment. Together, they represent a practical and successful methodology for culturing *Asparagopsis*.

The *Hatchery and nursery procedures* section is organised into the following work areas:

1. Collection of reproductive thalli (field collection)
2. Cystocarp extraction (hatchery)
3. Carpospore liberation (hatchery)
4. Carpospore germination (hatchery)
5. Tetrasporophyte growth (nursery)
6. Culture biomass (production)

Collection of reproductive thalli

Whole, healthy and reproductively fecund gametophyte thalli bearing mature cystocarps can be collected from the wild. 'Healthy and reproductively fecund' gametophytes are defined as those with deep red colouration and minimal bleaching, and with each thalli bearing at least 10-20 mature cystocarps. 'Mature' cystocarps are defined as those with deep red coloration and that are approximately 1 mm in diameter. The carposporangium (ball of spores) is clearly visible within the semi-transparent cystocarp. At collection, thalli should be held in catch bags and kept immersed in seawater to avoid temperature shocks. Thalli should then be transferred to an esky or cooler with site seawater for transportation to the marine research facility. In the lab, thalli can be stored within a holding system with a temperature that matches that of the collection site, and held under white light at an intensity of 80-100 µmol photons m^{-2} s^{-1} and 10:14 photoperiod, and with aeration (see Chapter 3).

Cystocarp extraction

Equipment and instruments required for cystocarp extraction are:

- Three plastic trays (30 cm)
- Two containers (1 L)
- Seawater (filtered and UV-treated if possible)
- Petri dishes (90 mm)
- Petri dishes (90 mm) with 1% agar
- Tweezers with serration
- Transfer pipettes
- 200 µL pipette and tips for 100 µL
- 4 L UV-filtered and sterilised seawater (19 °C prepared the day before)
- 1 L Cell-Hi® F2P (stock solution 30 g L^{-1} at 0.1 g L^{-1} basal concentration, 19 °C prepared the day before) or alternative nutrient media (see nutrient protocols)
- 1 L culture vessels

Step 1: Clean bench area and instruments with ethanol 70%.

Step 2: Clean the *Asparagopsis* spp. thalli by physically removing any large epiphytic algae or debris, as well as any small macro-invertebrates. Use filtered seawater (19 °C) to wash off any finer particles and algae lodged within the thalli. This will need to be repeated three or more times until no macro-invertebrates or epiphytic algae are visible. Use a dissecting microscope to assist in visual inspections after each rinse. Heavily contaminated parts of the gametophytes can be removed.

Step 3: Once cleaned, submerge and spread out a handful of *Asparagopsis* spp. thalli in a plastic tray with clean seawater (Figure 2a). Visually inspect the thallus of the gametophytes for fertilised cystocarps. These look like small pink-to-white-coloured balls approximately 1 mm in diameter (Figure 2b). Place fecund thalli (two or three only to reduce contamination) within a clean plastic tray that has ~3 cm of filtered seawater.

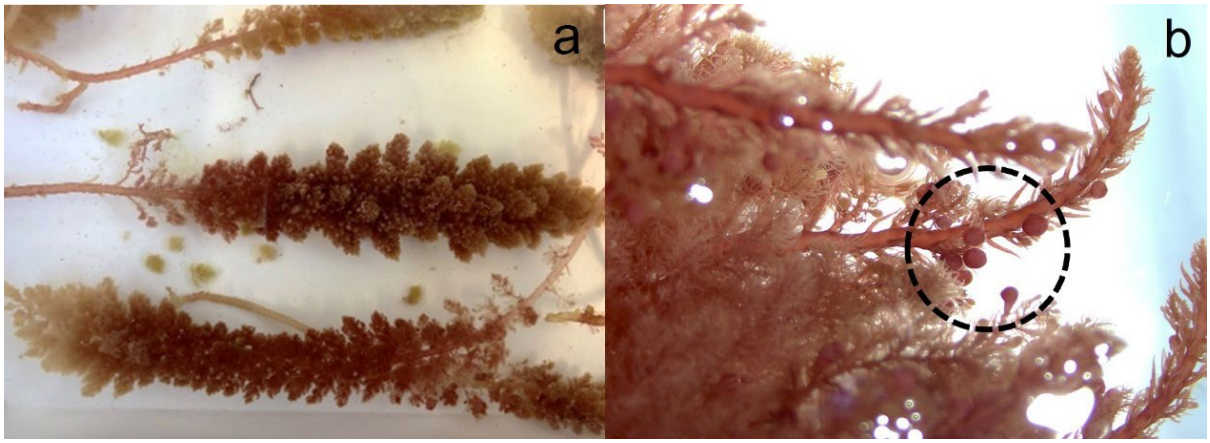

Figure 2. (a) *Asparagopsis taxiformis* thalli spread out in tray for inspection and cleaning; (b) *A. taxiformis* thalli with mature cystocarps (small pinkish balls).

Step 4: Look at the thalli under the microscope to identify cystocarps that appear healthy, prioritising larger cystocarps. These should ideally be free from any visible contamination and epiphytic growth (Figure 3a). Under the microscope, look for fertilised cystocarps. These will be round and swollen, and the carposporangium (ball of spore) will be clearly visible (Figure 3b). Identify healthy cystocarps and transfer these to the petri dish. Discard malformed or dark red cystocarps (Figure 3c) – these have not been fertilised. Avoid all other debris, cystocarp husks and cystocarps that are damaged.

Step 5: Using a pair of fine forceps, remove the individual cystocarps from the thallus and transfer to a petri dish containing filtered, sterilised seawater. Care should be taken to leave a small amount of thallus attached to the cystocarps to allow for agar dragging (see Appendix A). After dislodging the cystocarps, complete further processes quickly as cystocarps will begin releasing carpospores immediately after they have been excised from the thallus.

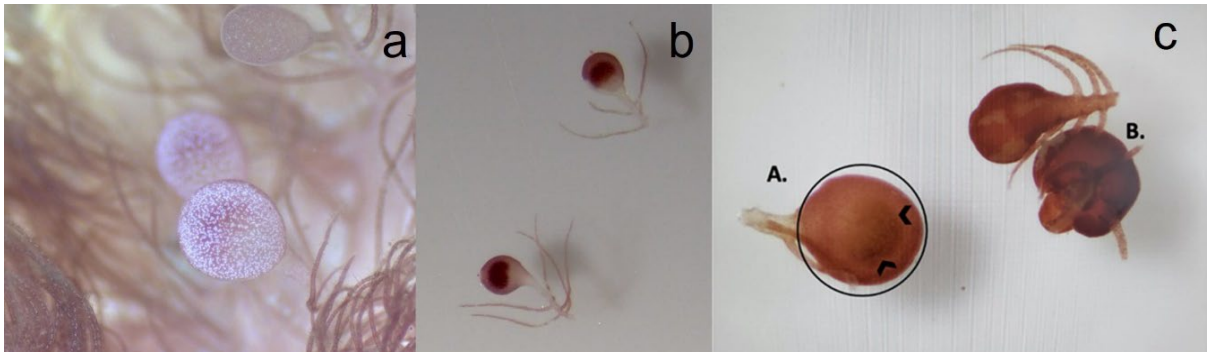

Figure 3. (a) *Asparagopsis taxiformis* cystocarps attached to the gametophyte thallus; (b) *A. taxiformis* cystocarps after excision from the thallus to allow for cleaning using the agar drag method; (c – A) *A. taxiformis* fertilised cystocarp with arrows highlighting sporangium; (c – B) malformed and dark red cystocarps that are unfertilised.

Step 6: Prepare four petri dishes with filtered and sterilised seawater next to a dissecting microscope for disinfection and a three-stage washing of cystocarps after selection. Using a 3 mL pipette, transfer cystocarps across the three petri dishes of filtered and sterilised seawater before placing them in a final petri dish with filtered and sterilised seawater. Cystocarps can be also 'washed' with small jets of filtered and sterilised seawater from a 3 mL pipette.

Step 7: To further clean cystocarps, use forceps and gently drag an individual cystocarp through the agar plate. The aim is to maximise contact of the cystocarp with the agar, so rotate the cystocarp after each drag. Drag each cystocarp three or four times. If the thallus breaks, cystocarps can be 'pushed' through the agar using a pair of fine forceps. Ensure the cystocarp structure does not break as small quantities of agar may get trapped inside.

Step 8: After dragging, clean each cystocarp with a three-stage rinse. Pipette each cystocarp into a clean dish and repeat the process two more times to remove agar and any other contamination.

Step 9: Using a dissecting microscope, ensure all agar and excess thallus is removed (e.g. Figure 4). If necessary, use a scalpel blade to remove any excess thallus (as shown in Figure 3c). If any excess thallus is removed or agar observed, re-wash the cystocarps.

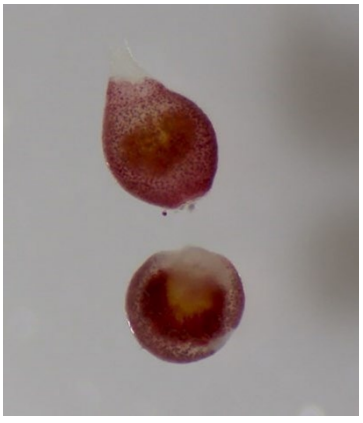

Figure 4. Cleaned and healthy *Asparagopsis taxiformis* cystocarps with excess thallus removed. The top cystocarp has begun releasing spores.

Carpospore liberation

Step 10: The clean cystocarps can now be transferred to a culture petri dish. A culture petri dish contains 30 mL filtered and sterilised seawater and 100 µL Cell-Hi® nutrient solution (0.1 g L^{-1} working concentration) and GeO_2 at a concentration of 0.1 g L^{-1} to prevent diatom growth. Close the petri dish, seal with plastic film and label with a coded batch number.

Step 11: Repeat steps 4-10 until the desired number of petri dish cultures has been reached.

Step 12: Culture petri dishes should be stored in an illuminated incubator held at 19 °C. The initial light intensity should be 5-20 µmol photons m^{-2} s^{-1} and a photoperiod of 10:14 (light:dark). Ensure the minimum light intensity is not below 5 µmol photons m^{-2} s^{-1} otherwise germination, growth and survival may be impacted. Ensure the maximum light intensity does not exceed 20 µmol photons m^{-2} s^{-1} to minimise stress to the carpospore.

Step 13: After excision from the thallus, cystocarps begin releasing carpospores almost immediately. Cystocarps can continue to release carpospores for several days, however 80% of the carpospore release occurs with the first 48 hours. Each cystocarp can release between 50 and 400 carpospores. To reduce overcrowding in cultures, transfer cystocarps to a new dish after 24 hours. This process will need to be systematic, a few dishes at a time to minimise the exposure of carpospores to different light and temperature. That is, for each batch of cystocarps, you will have 'A' and 'B' isolates.

Step 14: To transfer cystocarps from isolate 'A' and inoculate isolate 'B', prepare a petri dish with 30 mL of 18-19 °C filtered and sterilised seawater and nutrient solution (100 µL Cell-Hi® 30 g L^{-1} at 0.1 g L^{-1} working concentration) and GeO_2 at a concentration of 10 mg L^{-1} to prevent diatom growth.

Step 15: Under a dissecting microscope, check the health of the cystocarps, removing and disposing of broken and/or empty cystocarps (husks). Only transfer cystocarps with carposporangium visible inside (red ball of carpospores; see Figure 3) to a new dish with 30 mL filtered and sterilised seawater and Cell-Hi® nutrient solution at a concentration of 0.1 g L^{-1} and GeO_2 at a concentration of 10 mg L^{-1} to prevent diatom growth. Close both dishes ('A' and 'B'), seal with plastic film and label with a batch number; e.g. 221102A or B – the date plus the isolation.

Step 16: Repeat process for all dishes from isolate 'A'.

Step 17: Return 'A' and 'B' isolates to the incubator, keeping the light, temperature and photoperiod the same.

Step 18: After the 'B' isolate has been liberating carpospores for 24 hours, it is time to discard all cystocarps from 'B'. Select all isolate 'B' petri dishes from the incubator and remove and discard old cystocarps.

Carpospore germination

Step 19: After 24 hours, the carpospores will begin to swell and germination occurs between 24-48 hours (observing a small transparent rhizoid and a bunch of dividing cells or early-stage filament protruding from the carpospore (Figure 5a).

Step 20: After germination, a rhizoid (transparent finger-like projections from the basal end of the tetrasporophyte) begins to develop, which anchors the carpospore (now tetrasporophyte) to the petri dish (Figure 5b). Once the rhizoid has been observed (48 hours), on day three the culture dishes can have a water change to flush out cystocarp debris and metabolites released into the petri dish water, and to replenish nutrients.

To change the water in a petri dish change, use a pipette to remove the old petri dish water. Choose a location on the petri dish where tetrasporophytes are not visible to prevent damage to tetrasporophytes.

Alternatively, tetrasporophytes should be anchored to the petri dish by the holdfast. If so, prepare new culture medium for each culture (i.e. 30 mL of 19 °C with Cell-Hi® nutrient solution at 0.1 g L^{-1} working concentration and GeO_2) and pour the petri dish out into an empty container for later disposal. Quickly refill the petri dish with the prepared 30 mL culture medium.

Replace with 30 mL of 19 °C filtered and sterilised seawater and 100 µL 19 °C Cell-Hi® nutrient solution (0.1 g L^{-1} working concentration) and 10 mL of GeO_2 to prevent diatom growth (see Appendix A). Wrap petri dishes and return to the incubator.

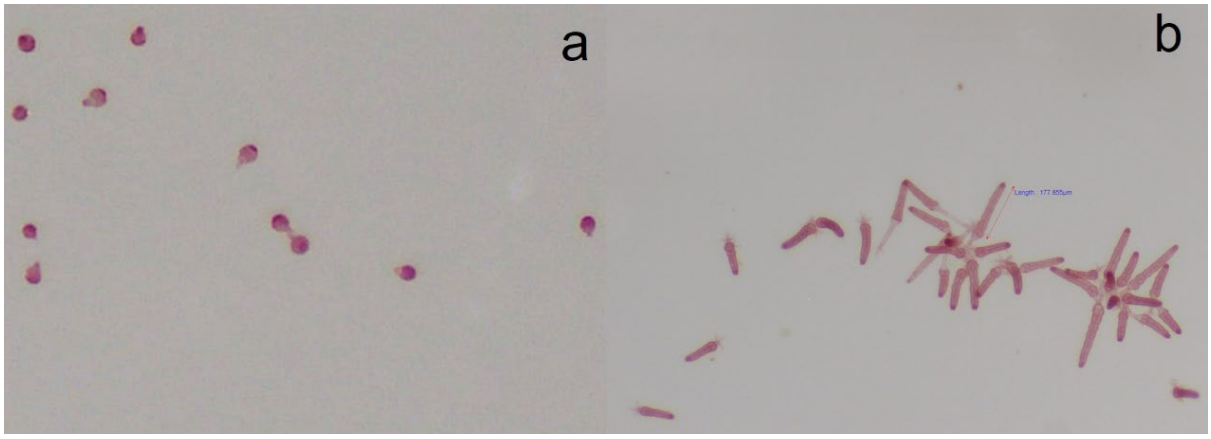

Figure 5. (a) Petri dish cultures of *Asparagopsis taxiformis* after 24 hours with germinated carpospores (30 µm diameter); (b) 48-to-72-hour-old carpospores developing a rhizoid (light pink tail) and filament (vivid red filament 60-200 µm long).

Step 21: Monitor isolates daily to check for tetrasporophyte survival, health, growth and contamination. If there are signs of contamination, discard the petri dish. For the first four days, there should be lateral elongation of the sporeling filament and it should be a red-to-pinkish-red colour.

Step 22: After five or six days, tetrasporophytes begin to produce their first branch (Figure 6). When this happens, the light intensity can be increased to 10 µmol photons m^{-2} s^{-1} and a photoperiod of 10:14 (light:dark).

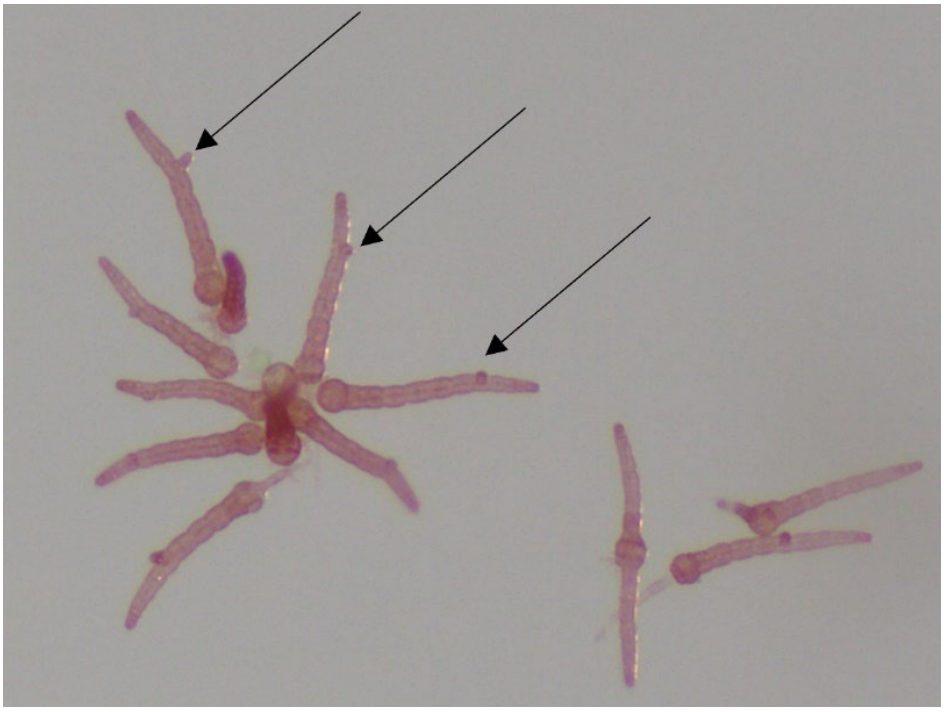

Figure 6. *Asparagopsis taxiformis* tetrasporophytes showing branching after six days in petri dish culture.

Nursery procedures

Step 23: Once you have observed >90% of the petri dish showing branching (about day 8-10), the tetrasporophytes can be scraped from the petri dish and transferred to tumble (aerated) culture. To transfer tetrasporophytes in petri dish to tumble culture (Figure 7), tetrasporophytes need to be scraped and suspended in the petri dish using a bacterial loop and a 3 mL pipette, and transferred to a 1 L vessel containing 19 °C filtered and sterilised seawater and 10 mL Cell-Hi® at 0.3 g L^{-1} production concentration (if alternative production nutrient media are to be used, consult the nutrient media standard operating procedures for those media and relevant working concentrations) and 10 mg L^{-1} of GeO_2 to prevent diatom growth.

Transfer the young tetrasporophytes in the 1 L culture vessel to a constant environment room at 19 °C with approximately 10-15 µmol photons m^{-2} s^{-1} and a photoperiod of 10:14 (light:dark). Then, add gentle aeration to circulate the tetrasporophytes and prevent the freshly liberated holdfasts from sticking to the culture vessel. Cap the culture vessels to minimise the risk of contamination and reduce evaporative loss of water (Figure 7).

Figure 7. *Asparagopsis taxiformis* and *A. armata* tetrasporophytes in 1 L culture stock culture vessels with filtered aeration lines.

Step 24: Over the next six days, incrementally increase the light each 48-hour period to 15, 30 and 50 µmol photons m^{-2} s^{-1}, respectively. The photoperiod should remain at 10:14 (light:dark).

Step 25: Monitor tumble cultures daily to check for tetrasporophyte survival, health, growth and contamination. If there are signs of contamination, discard the culture.

Step 26: Water changes occur weekly using the following procedure. Strain the culture vessel water and tetrasporophytes through a 25 µm micro-filter and then suspend the tetrasporophytes in a new, sterile 1 L vessel containing 19 °C UVSW and 10 mL Cell-Hi® nutrient solution at 0.3 g L^{-1} production concentration (if alternative production nutrient media are to be used, consult the nutrient media standard operating procedures for those media) and 10 mg L^{-1} of GeO_2 to prevent diatom growth. Transfer the young tetrasporophytes in the 1 L culture vessel back to the constant environment room at 19 °C, 50 µmol photons m^{-2} s^{-1} and a photoperiod of 10:14 (light:dark), and add gentle aeration to circulate the tetrasporophytes. Cap the culture vessels to minimise the risk of contamination and reduce evaporative loss of water.

Step 27: The production life of a tetrasporophyte is four weeks from the time it is growing under 50 µmol photons m^{-2} s^{-1} in tumble culture. Harvest size at maturity was 8-10 mm for our strains in Perth.

Production procedures

Step 28: To generate commercial quantities of seaweed juveniles to build culture biomass for production, mature tetrasporophytes can be rapidly fragmented. Fragments from 0.1-1 mm in length are viable options for generating new tetrasporophytes, and are essentially at the same stage as tetrasporophyte sporelings that are transferred from a petri dish to tumble culture (8-14 days old). To fragment one or many mature tetrasporophytes, place the tetrasporophytes in a 50-100 mL beaker with 10-20 mL filtered and sterilised seawater at 19 °C.

Step 29: Using a hand-held milk frother or another device that doesn't result in the tetrasporophytes being emulsified, agitate the tetrasporophytes for 20-30 seconds or until the tetrasporophytes are completely fragmented and no large (>1 mm) fragments are observed.

Step 30: One mature tetrasporophyte (8-10 mm in diameter) can generate 500-700 viable juveniles; 500-700 tetrasporophytes are required to populate a 1 L vessel at a final density of 5 g L^{-1} (Figure 8a). A 1 L vessel with fragments generated from a 5 g L^{-1} mature tetrasporophyte culture (Figure 8b) could then populate a >100 L production system. After four to six weeks, a density of 5 g L^{-1} could be achieved in the >100 L production system, which in turn can populate tens of thousands of litres of production systems. Production systems can be wide and varied, with simple production systems (e.g. as shown in Figure 9) generating biomass.

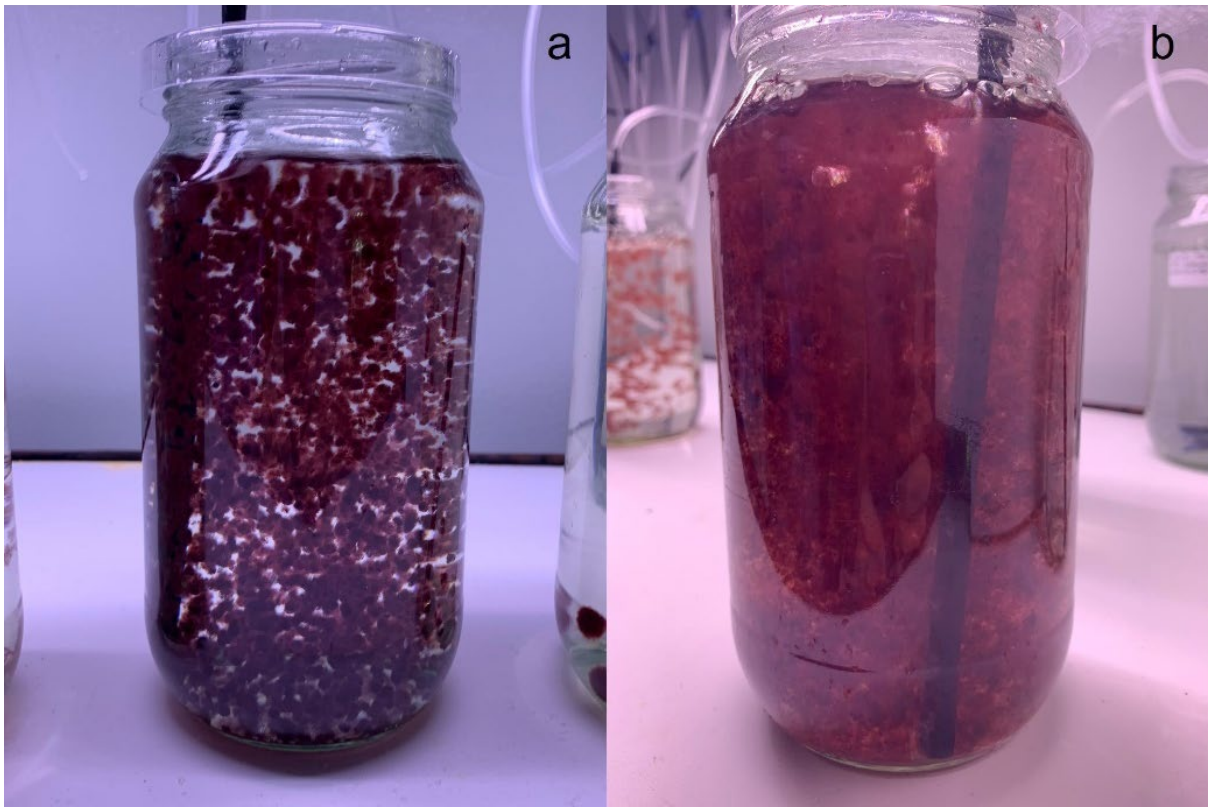

Figure 8. (a) *Asparagopsis taxiformis* mature tetrasporophytes (8-10 mm in diameter) with a density of 5 g L^{-1} in 1 L culture vessel; (b) using our rapid fragmenting technique, 5 g L^{-1} is fragmented to generate tens of thousands of seaweed juveniles with >95% viability.

Figure 9. (a) A simple recirculating system with four 30 L vessels, downwelling lights (50 µmol m^{-2} s^{-1}), thermally regulated water (19 °C) and adequate nutrient supply (0.1 g L^{-1} Cell-Hi®); (b) tetrasporophyte fragments inoculating the culture.

Growth of seaweed juveniles into mature harvestable product can be achieved in four to six weeks, depending on the early-stage hatchery and nursery techniques.

Contamination control procedures

Cultures can be contaminated at many stages of the culturing process (Figure 10). Contamination of *Asparagopsis* spp. cultures can lead to reduced growth rates, lower bromoform concentrations, challenges associated with re-fragmenting or collapse of the entire culture.

There are a considerable number of steps that should be taken to minimise and control the growth of any biological contaminants to ensure healthy growth of *Asparagopsis* cultures. Developing good handling practices and protocols when working with cultures is important to prevent contamination introduction. While completely sterile cultures are not necessarily optimal for *Asparagopsis* growth due to findings suggesting there may be good bacterial and fungal associations (Paul *et al*. 2006), healthy and consistent tetrasporophyte growth can be achieved with low rates of contamination. As such, identification of bad versus good contamination is an ongoing process. Within cultures, identifying the type of contaminant is vitally important (e.g. green/brown/red algae, diatoms, bacterial, fungal), utilising salvage techniques where applicable (see section on harvesting tetrasporophyte tips), and eliminating or implementing effective controls of potential contamination sources is an important component in establishing healthy, stable cultures. For an in-depth summary and explanation of sterile techniques in macroalgae cultivation, see Kawachi and Noël (2005).

Due to the numerous avenues for contamination at various stages of culture, particular care and extra precautions should be taken when working with stock cultures. The primary contaminants identified in the work conducted here appear to be of fungal and algal origins; however, further work is needed to better identify biological contaminants.

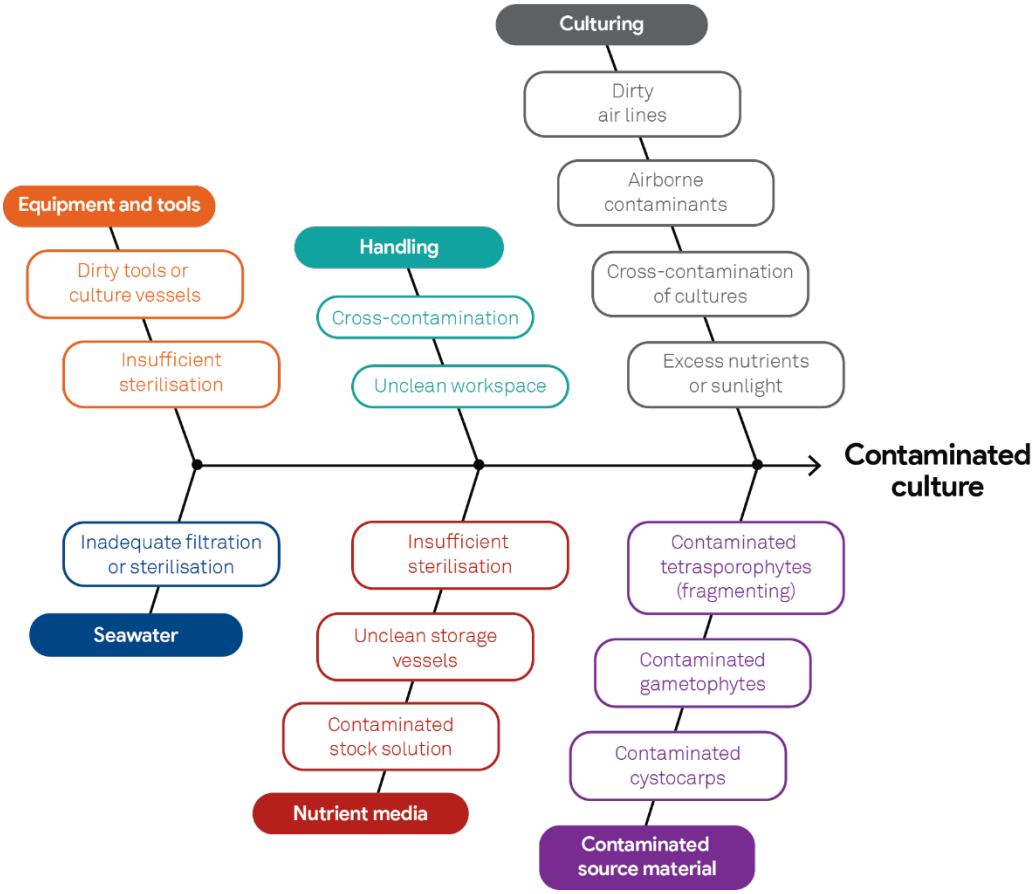

Figure 10. Sources of major contamination vectors when culturing *Asparagopsis* spp.

Contamination types and treatments

Diatoms

Diatoms have the potential to seriously disrupt cultures and can reproduce and grow quickly. The use of GeO_2 has been shown to be inhibitory to diatoms at concentrations of 1 mg L^{-1} and potentially lethal at 10 mg L^{-1} (Lewin, 1966). Other research has shown that most diatoms are strongly inhibited at GeO_2 concentrations of 0.134 mg L^{-1} (Markham and Hagmeier 1982), while staff at CSIRO's Australian National Algae Culture Collection use GeO_2 concentrations of 0.4-1 mg L^{-1} in algae cultures. In early trials with GeO_2, there was no evidence of growth inhibition of red seaweed species by GeO_2 (Markham and Hagmeier, 1982).

The current dose of GeO_2 used for *Asparagopsis* cultures is 10 mg L^{-1}. Early trials with GeO_2 suggest no harmful effects on *Asparagopsis* at a concentration of 10 mg L^{-1} GeO_2 in the growing media.

Biofilms

Contaminants that grow in a biofilm or covering appear to be particularly stressful to tetrasporophytes, often resulting in pigment loss or cell death. Early observations indicate that these contamination types (e.g. Figure 11) can be associated with contaminated stock solutions. Cultures contaminated with these biofilms are generally discarded upon identification of such contaminants. If contamination is still at a very early stage, salvage attempts (e.g. tip harvesting) may be viable, although this is yet to be demonstrated.

Algae

Contaminants from other algae species (i.e. brown, green and red) can also be present in cultures. Since *Asparagopsis* will likely be affected by methods to control other algae species, if other algae are identified within the culture, harvesting tips away from contamination sites is recommended if contamination levels are low and you wish to retain the genetically distinct culture. Depending on the severity of contamination, disposal of the culture may be necessary.

Microscope and imaging software

The use of a microscope is integral for early identification of contamination in cultures. In addition, imaging software (such as Mosaic Image Capture and Analysis) can greatly assist with identifying contaminants through digital imaging that would otherwise not be evident through an optical-only microscope.

Seawater

Seawater can contain a variety of microorganisms, such as diatoms, viruses and algal spores. It is imperative that these are not present when seawater is enriched due to their potential to compete with *Asparagopsis* spp. A process for sterilisation or removing contaminants is mandatory. As shown in Table 2, standard sterilisation techniques include autoclaving, tyndallisation, pasteurisation, filtration and ultraviolet (UV) irradiation. There are numerous considerations (e.g. cost, time, effectiveness) to be aware of when choosing the technique that most effectively meets the needs of the nursery. The positives and negatives of each of these processes are listed in Table 2.

Table 2. Seawater treatment methods to control contaminants.

Treatment method	Definition	Duration	Equipment needed	Positive	Negative	Comments
Autoclaving	Sterilisation using steam (water) under high pressure.	15 minutes at 12 °C	Autoclave	100% effective, Minimum manpower	Expensive, time consuming with large volumes	Autoclaving is very expensive for large volumes of seawater and may alter the chemistry of the water. All living organisms are destroyed. It takes only a few hours with little supervision.
Pasteurisation	Partial decontamination of a substance at a specific temperature and a duration that kills most organisms without major chemical alteration of the substance.	30 minutes at 61.7 °C	Standard stove	80% effective	Labour intensive	This is relatively inexpensive and can be done quite quickly, but only partially destroys living organisms in the liquid. Pasteurisation has been used effectively to control contaminants in some algal nurseries.
Tyndallisation	Sterilisation by heating a substance (seawater) for several minutes on three or four successive occasions.	Five minutes at 90-100 °C; once per day for three days (24 hours between heating).	Standard stove	>99% effective	Moderate manpower, Labour intensive with large volumes	This is effective in destroying most living organisms but requires 24-72 hours and is more labour intensive.
Filtration	The process of passing the seawater through filters.	Varies depending on the filter pore or membrane size; also depends on the number of uses.	Polypropylene and membrane filters sized 5.0, 1.0 and 0.2 µm	80% effective, can filter large amounts of water in a short time	Filtration begins fast, but increased amounts of debris will cause the filter to clog and water flow will decrease.	Relatively inexpensive, takes little time, and removes most living organisms in the seawater. Effectiveness and cost may vary extensively depending on the quality and type of filter used. For example, 0.2 µm membrane filter cartridges cost more and take more time to filter than a 5.0 µm filter.
Ultra-violet (UV) sterilisation	The process of passing the seawater past a closed UV light system.	Tens of litres per minute	UV apparatus	>99% effective	Moderately expensive	Relatively inexpensive and takes little time, but requires management of flow rates and exposure times to maximise effectiveness.
Chlorine dioxide (ClO_2)	Sterilisation by adding ClO_2 into the seawater.	3-20 parts per million and 18-24 hours for ClO_2 to dissipate into the air.	ClO_2, personal protective equipment (respirator), refrigerator and approved containers (storage)	>99% effective, inexpensive, minimum effort required	Toxic substance, protective equipment required	Relatively inexpensive but requires contact time in the range of 18-24 hours for complete dissipation into the air. Also requires safe handling and storage practices, and measurement equipment to monitor concentrations and residuals.

Nutrient media

Due to the high concentrations of nutrients, working media and stock solutions can be particularly prone to contamination. These should be handled carefully and sterilised, using aseptic handling techniques (see Kawachi and Noël 2005). Even a very minor amount of contamination of working media or stock solution can result in excessive and abundant growth of unwanted organisms that can then be transferred to cultures. Observations suggest contamination of nutrient stock often results in biofilm contaminants (Figure 11), often requiring disposal of culture.

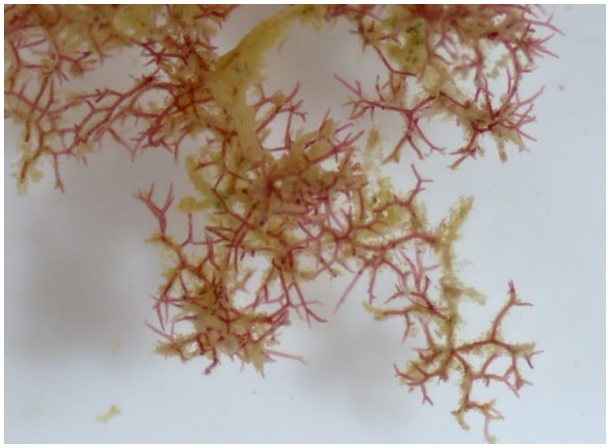

Figure 11. Contamination (yellow/brown) on *Asparagopsis taxiformis* tetrasporophyte from contaminated stock solution.

Guidelines to prevent contamination of working media and stock solutions are:

- Sterilise stock solutions and store in a refrigerator with minimal light to minimise suitable growth conditions of any contaminants.
- Wash hands with 70% ethanol prior to opening to reduce the transfer of bacteria to the lid.
- Where possible, work in a laminar flow when handling stock and working solutions. This will minimise the chance of airborne contaminants entering the solution while containers or vessels are open.
- Pour nutrients aseptically – do not let the lip of the bottle come into contact with anything.
- Do not put non-sterile items into stock solutions.
- Grow media and stock solutions should be sterilised, autoclaving where possible. Not all media is autoclavable; temperature and pressure may result in precipitates or denature heat-sensitive compounds such as vitamins. In these cases, heating or pasteurisation may be sufficient, or filter sterilisation for when adding heat-sensitive compounds after the rest of the stock solution has been sterilised.
- The shelf life of stock solutions should be minimised (within reason) to minimise the likelihood of stocks becoming contaminated.
- Visually inspect nutrient solutions prior to use. Check the bottom of the jar for any growth or cloudiness.
- If possible, transfer the desired nutrient dosage into separate vials prior to sterilisation. This will enable the addition of sterilised nutrients without any handling, preventing the risk of contamination.

If a nutrient solution is thought to be a contamination source, nutrient media can be cultured under both tumble culture and petri dish incubation, and investigated for organism growth. Such trials are recommended to be conducted using an additional media source for comparison alongside filtered seawater for control to distinguish between contaminants from nutrients and from seawater.

For example, in experiments as part of producing this manual, contamination was observed in many tumble cultures. Initial investigation into the contamination source was undertaken by investigating

the nutrient media and sterilised seawater; a trial was set up with UV-treated seawater plus Cell-Hi® nutrients and UV-treated seawater on tumble culture. Substantial contamination was observed in the UV-treated seawater with nutrients. Further investigation was undertaken by running the same treatments in petri dish cultures with the addition of a separate, autoclaved nutrient source, Provasoli's enriched solution. Contaminants grew in the Cell-Hi® treatment only, with nothing observed in UV-treated seawater or UV-treated seawater with PES, indicating contamination of the Cell-Hi stock.

Contaminated source material – field collection

Wild harvested *Asparagopsis* will likely be heavily contaminated with a wide range of organisms, such as fungi, algae and diatoms (Figure 12). Gametophytes are often heavily contaminated and when harvesting cystocarps, the contaminants on the gametophyte (and attached cystocarps) can travel through to cultures. Methods to reduce contamination are:

- Wash gametophytes in filtered water prior to extracting cystocarps.
- Select gametophytes strands or parts that are less contaminated for cystocarp culture.

Cystocarps may be externally contaminated, which can result in unwanted organisms or spores entering petri dish cultures. Epiphytic algae species and other microscopic organisms have been observed growing on cystocarps (Figure 12) and can then be transferred to cultures during isolation periods. Contamination of cystocarps may not always be clearly evident (Figure 12). There are several ways contamination from wild-harvested material can be controlled or reduced, including:

- Manually select clean-looking cystocarps using a high-powered microscope.
- Perform an agar drag on cystocarps – extensively wash cystocarps after dragging.
- Reduce the time cystocarps are left in isolation. This will reduce the time petri dishes are exposed to potential contamination sources.
- Remove excess thallus material with a sterile blade as the thallus can contain microscopic growth of unwanted organisms (Figure 12).

Contaminated source material – culture inoculation

Tetrasporophytes in cultures may also be contaminated. When these are fragmented, the contaminants will likely be transferred into subsequent cultures. To date, efforts to remove contamination from cultures have been unsuccessful. Methods to reduce contamination are:

- Harvest and clean the freshly grown (and less likely to be contaminated) tips from healthy-looking tetrasporophytes.
- Perform an agar drag of the tetrasporophyte tip.
- Undertake three-stage washes when harvesting tips.
- Use the minimum volume of water required when transferring tips. Early trials have used a 2.5 μL pipette for transferring tips.

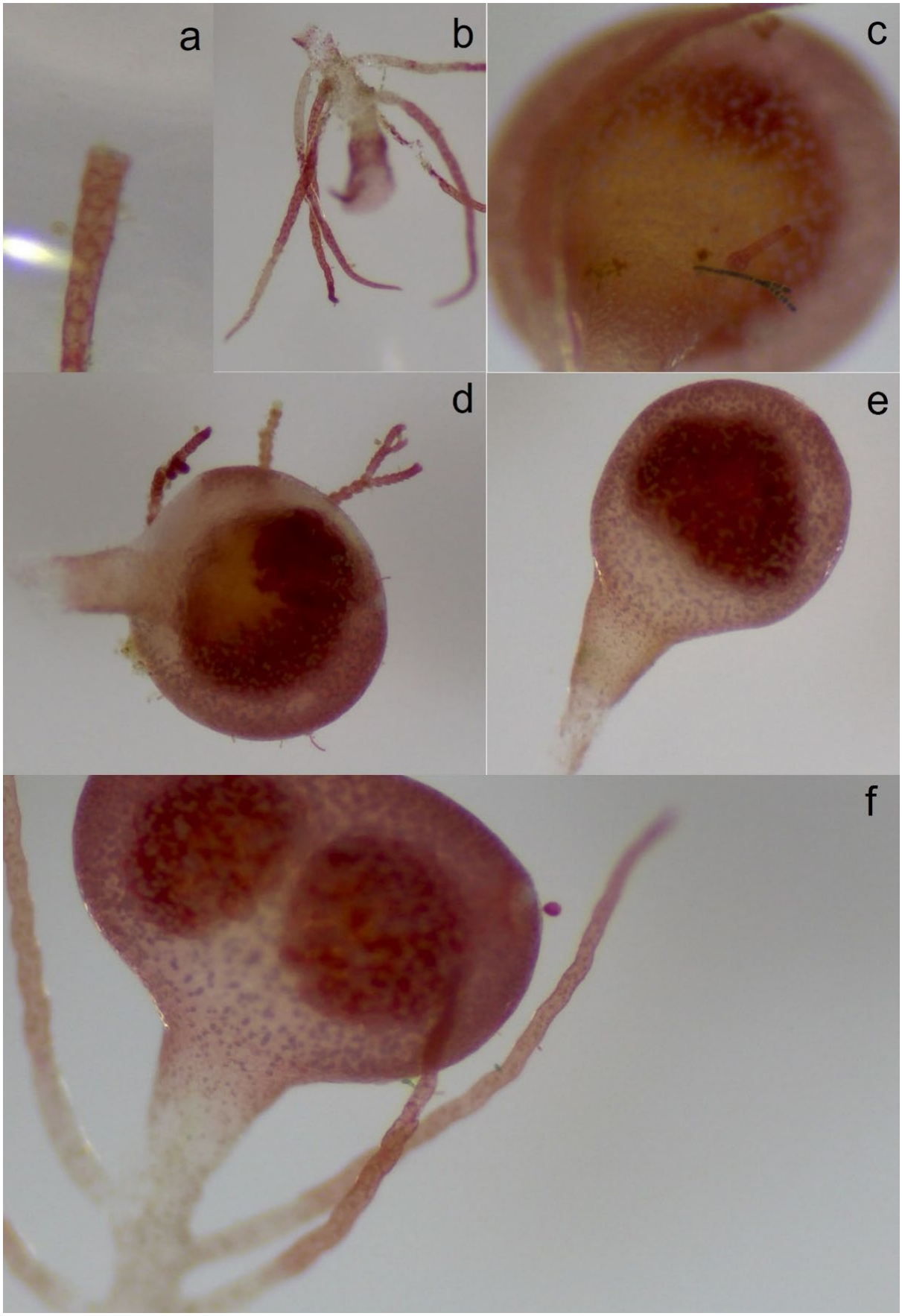

Figure 12. (a, b) Contaminated thallus attached to cystocarp; (c, d) cystocarp contaminated with green algae; (d) cystocarp contaminated with red algae; (e) cystocarp contaminated at the basal portion (green colour at base); (f) cystocarp with green filamentous algae contaminating the thallus.

Hatchery procedures

Clean tools should be used to minimise transfer of any contaminants from gametophytes to the cleaned and rinsed cystocarp cultures. Closely inspect the attached thallus of cystocarps, which can have microscopic organisms attached. While contamination of the cystocarp can be clearly evident, sometimes contamination can be difficult to see. Hatchery procedures to minimise contamination are:

- If possible, avoid cystocarps with external contamination. If part of the thallus is contaminated, the contaminated area can be excised from the cystocarp.
- When isolating and selecting cystocarps, minimise transfer of seawater between dishes.
- If possible, reduce the time that cystocarps are left in petri dishes. If cystocarps are releasing quickly, 30 minutes may be sufficient for a 90 mm petri dish.

Nursery procedures

When working with tumble cultures and in wet lab areas there are multiple avenues for contamination that should be considered. Nursery procedures to minimise contamination are:

- Use filters on air lines to ensure aeration does not introduce contaminants into culture.
- Use lids on culture vessels to minimise contamination from any airborne particles, aerosolised water and water droplets.
- Maintain positive air pressure inside culture flasks to further reduce the risk of contamination from aerosolised water and airborne particles.
- Use low light and nutrients in low-density cultures as this can help *Asparagopsis* achieve higher density, after which it can better compete with contaminants.
- Never transfer water between cultures; for example, when conducting water testing, perform the required tests on water samples rather than the cultures directly.

Handling procedures to minimise contamination

Handle cultures in a way that minimises introducing contaminants. Kawai *et al.* (2005) and Kawachi and Noël (2005) contain extensive guidance on handling techniques and potential contamination pathways to be aware of. Handling procedures to minimise contamination are:

- Use clean or sterilised tools when working with cultures.
- Avoid transferring anything between cultures, e.g. water-monitoring instruments should be used on a sample from the culture water, not placed directly into culture vessels.
- Keep the workspace area clean and regularly sanitise it with 70% ethanol.
- When preparing clean media and nutrients, work in a laminar flow cabinet if possible.

Procedures relating to tools and equipment to minimise contamination are:

- Sterilise or autoclave tools and culture vessels between uses, particularly if working with uncontaminated material.
- Use sterile equipment when handling clean cultures, as unsterile equipment may host bacteria.
- Ensure tools and equipment are properly washed; items may host contaminants or unwanted species if washed inadequately.

Removing contaminated material

Although the methods documented above may assist in controlling already contaminated tetrasporophytes, preliminary observations indicate that once a culture becomes contaminated, returning the culture to an uncontaminated state is extremely challenging, if not impossible.

To date, no cultures with contamination have been successfully ‘cleaned’ using a variety of techniques, such as removal of visible contaminants or dilute peroxide or freshwater treatments. Despite this, general maintenance (i.e. water and culture vessel changes) and the removal of visibly contaminated specimens using a high-powered microscope seems to reduce the growth of contaminants and prolong the life and health of tetrasporophytes that are already contaminated. See Appendix A for observation on the types of contamination the SARaH lab has experienced.

Steps undertaken by the SARaH lab staff to control or remove contamination were:

- Contaminated cultures were examined under a high-powered microscope.
- Tetrasporophyte (fragment and pompom stages) were checked until contamination was identified or all tetrasporophytes were checked.
- Any contaminants visible at up to 50x magnification were manually removed.

Despite removal of all visible contaminants at up to 50x magnification, contamination remained present after four weeks. However, despite still being present in cultures, the severity of contamination remained relatively low after four weeks.

Researchers, hatcheries and nurseries are encouraged to collaborate to facilitate knowledge sharing, to enable industry-wide improvements.

Implications

This manual primarily provides guidelines for cultivating *A. taxiformis* tetrasporophytes. The implications on the emerging Australian seaweed industry as a result of its availability are detailed below.

Advancing *Asparagopsis* cultivation: This comprehensive manual provides valuable insights into successful and unsuccessful cultivation of *A. taxiformis,* which could significantly advance the commercial cultivation of this seaweed species in Australia. With the manual's help, those new to *Asparagopsis* cultivation can gain the necessary knowledge and techniques to start a hatchery and nursery for commercial cultivation.

Improving the emerging Australian seaweed industry: The availability of this manual could further stimulate the emergence of the Australian seaweed industry, especially in the field of *Asparagopsis* cultivation. The information contained herein could serve as a reference guide for other hatcheries and nurseries to follow, leading to more standardised practices and higher-quality seaweed products.

Facilitating research and development: The manual and key findings from this project present an opportunity for those experienced in *Asparagopsis* cultivation to provide feedback to SARaH lab group members for further refinement and improvement of their techniques. Constructive feedback could lead to further research and development, which could, in turn, enhance the cultivation of *Asparagopsis* and its commercialisation.

Facilitating environmental benefits: The commercial cultivation of *Asparagopsis*, a species with great potential to mitigate greenhouse gas emissions, could provide environmental benefits. The availability of the manual and the information provided herein could contribute to the expansion of this seaweed's cultivation, leading to reduced greenhouse gas emissions in the agriculture and livestock sectors.

Facilitating economic benefits: Successful *Asparagopsis* cultivation could provide economic benefits to the hatcheries, nurseries and other stakeholders in the seaweed industry. The availability of this manual could help in standardising production practices, leading to increased industry productivity, profitability and competitiveness.

Recommendations

Recommendations stemming from work undertaken by SARaH lab group members in developing this manual are detailed below.

Advancing *Asparagopsis* cultivation: There is a lack of publicly available knowledge and techniques for successful cultivation of *Asparagopsis taxiformis* in Australia. Recommendations related to changing this are:

- Encourage the dissemination of this manual compiled by the SARaH lab to individuals, organisations and stakeholders interested in *Asparagopsis* cultivation.
- Provide training and support to staff at new hatcheries and nurseries to ensure successful implementation of guidance provided in the manual.
- Conduct further research and development to improve the techniques detailed in the manual and refine the cultivation process.

Improving the emerging Australian seaweed industry: Due to limited knowledge sharing across the industry and institutes, there is a lack of standardised practices within hatcheries and nurseries, leading to inconsistent seaweed quality. Recommendations related to changing this are:

- Encourage adoption of the manual as a reference guide for hatcheries and nurseries in the Australian seaweed industry.
- Establish industry standards for *Asparagopsis* cultivation based on the practices outlined in this manual or another manual developed by existing industry and R&D groups.
- Encourage collaboration and knowledge sharing among hatcheries and nurseries to facilitate industry-wide improvements.

Facilitating research and development: There is limited publicly available information on *Asparagopsis* cultivation techniques and their optimisation. Recommendations related to changing this are:

- Encourage experienced *Asparagopsis* cultivators to provide constructive feedback on the manual to SARaH lab group members to refine and improve their cultivation techniques.
- Conduct further research and development to identify new and innovative techniques for *Asparagopsis* cultivation, with a focus on sustainability and efficiency.
- Encourage collaboration among researchers, hatcheries and nurseries to facilitate knowledge sharing, to enable industry-wide improvements.

Facilitating economic benefits: There is limited productivity, profitability and competitiveness in the Australian seaweed industry. Recommendations related to changing this are:

- Encourage adoption of the manual as a reference guide for hatcheries and nurseries to improve the productivity, profitability and competitiveness of the *Asparagopsis* cultivation industry.
- Provide support and funding to hatcheries and nurseries to ensure successful implementation of guidance provided in the manual.
- Promote the economic benefits of *Asparagopsis* cultivation to stakeholders in the industry and encourage its expansion.

Optimising conditions for dense cultures: There is a lack of knowledge on optimal conditions for *Asparagopsis* tetrasporophytes culture at commercial-level production densities (e.g. 3-7 g L^{-1}). Recommendations related to changing this are:

- Conduct experiments to test the effects of different environmental conditions on the growth and development of *Asparagopsis* tetrasporophytes at different culture densities.

- Consider using outdoor tank culture to test the effects of natural or ambient conditions.

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