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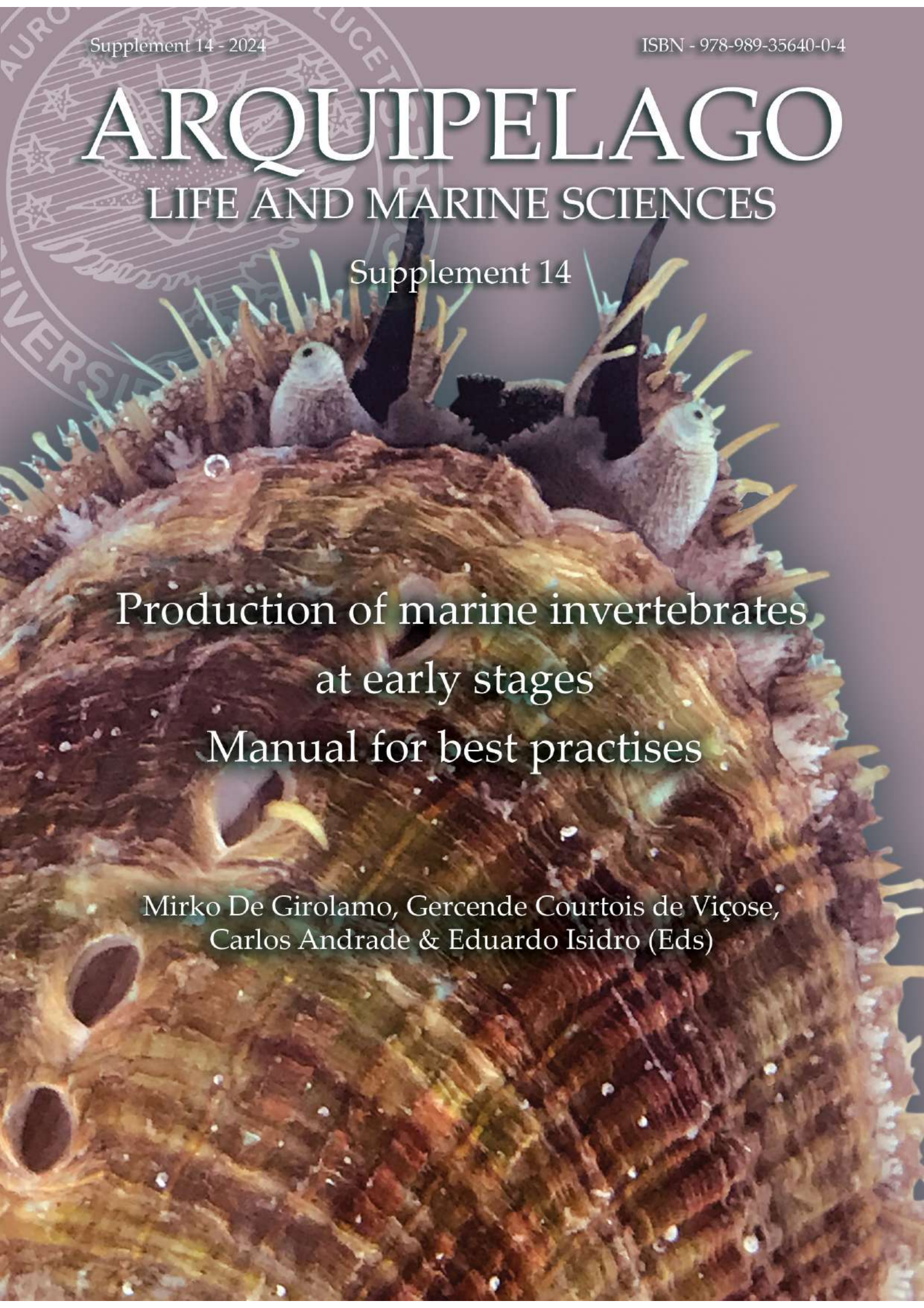
LIFE AND MARINE SCIENCES

Supplement 14

Production of marine invertebrates
at early stages

Manual for best practises

Mirko De Girolamo, Gercende Courtois de Viçose,
Carlos Andrade & Eduardo Isidro (Eds)



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Production of marine invertebrates at early stages – Manual for best practices

Mirko De Girolamo, Gercende Courtois de Viçose, Carlos Andrade & Eduardo Isidro (Eds)

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Production of marine invertebrates at early stages / Manual for best practices

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Production of marine invertebrates at early stages - Manual for best practices

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Courtois de Viçose, G., M.P. Viera, N. Marrero, R. José, R. Luís, D. Castejón, C. Andrade, M. De Girolamo, C. Nunes, A. Patkar, P. Sousa and E. Isidro 2024. Foreword. Pp. 6-6 in: De Girolamo, M., C. Nunes, A. Patkar, E. Isidro, D. Castejón, R. José, R., Luís, P. Sousa, C. Andrade, M. Vieira, N. Sanchez, and G.C. de Viçose 2024. Production of marine invertebrates at early stages - Manual for best practices. *Arquipelago. Life and Marine Sciences*. Supplement 14: 62pp.

FOREWORD

Marine invertebrates offer a plethora of prized products celebrated across countries and cultures. Abalones, limpets, sea cucumbers, and sea urchins, among others, have gained substantial commercial interest not only as culinary delights, but also as genetic resources for biotechnology. Nevertheless, invertebrates play a pivotal role on coastal ecosystem functioning, encompassing essential ecological processes and environmental services. Therefore, commercial exploitation of these wild key invertebrates must be regulated, especially when natural populations are fragile and fragmented.

In an era of global challenges, sustainable food production and marine conservation demand urgent attention to cope with the Agenda 2030 sustainability goals. In this context, low trophic marine invertebrate aquaculture production shines as a beacon of promise. It presents a technology not only to meet the escalating food demand, but also to alleviate the pressure on wild populations, safeguarding our vulnerable marine ecosystems.

The AQUAINVERT project aimed to produce R&D knowledge to promote sustainable and innovative aquaculture of marine invertebrates with commercial interest, to diversify seafood production using environmentally compatible species and methods. Research was mainly focused on the early stages production of two Macaronesian limpet species (*Patella aspera* and *Patella candei*), on the reproduction and larval culture of the sea cucumber (*Holothuria sanctori*) and the sea urchin (*Sphaerechinus granularis*), and on the production of abalone (*Haliotis tuberculata coccinea*). The initiative paved the way for a cross-border exchange platform between Macaronesia Ultra-Peripheral Regions of Europe, to promote applied aquaculture research and development in the participating institutions and showcased the potential of low trophic species production.

This best practices manual synthesizes the advanced outcomes of the project and is a comprehensive guide that pretends to provide knowledge and technical information for aquaculture researchers, students, entrepreneurs and industry professionals.

HOW TO USE THIS MANUAL

This manual is dedicated to detail methods on early-stages culture of the species studied in the AQUAINVERT project, providing both theoretical background and practical guidance on the following:

- Broodstock collection
- Spawning induction
- Fertilization and larval culture
- Use of settlement cues
- Micro and macroalgae production as feeding sources.
- Post-larvae and juveniles' production

To facilitate the implementation of the procedures, illustrated and step-by-step descriptions, together with a list of necessary equipment and materials are provided.

Additional information on sub-procedures, processes and techniques are provided in framed sections.

The manual is designed to complement the scientific knowledge achieved by the works referred to in the text. The readers are strongly encouraged to read those references and refer to them.

Abalone: *Haliotis tuberculata coccinea* (Reeve, 1846)

GERCENDE C. VIÇOSE, M.P. VIERA, N. MARRERO, R. JOSÉ, R. LUÍS, P. SOUSA, C. ANDRADE, D. CASTEJÓN, M. DE GIROLAMO, C. NUNES, A. PATKAR AND E. ISIDRO



Courtois de Viçose, G., M.P. Viera, N. Marrero, R. José, R. Luís, P. Sousa, C. Andrade, D. Castejón, M. De Girolamo, C. Nunes, A. Patkar and E. Isidro 2024. Abalone: *Haliotis tuberculata coccinea* (Reeve, 1846). Pp:7-16 in: Mirko De Girolamo, Gercende Courtois de Viçose, Carlos Andrade & Eduardo Isidro (Eds), 2024 Production of marine invertebrates at early stages - Manual for best practices. *Arquipelago*. Life and Marine Sciences. Supplement 14: 62pp.

GENERAL ASPECTS OF SPECIES - BIOLOGY AND ECOLOGY

The marine gastropod *Haliotis tuberculata coccinea* occurs in rocky areas in the Macaronesian inlands at depths down to 15 metres (Núñez *et al.* 1994; Geiger 2000). This benthic animal has nocturnal habits and feeds mainly on macroalgae. Sex identification is easily determined by the colour of the gonads (light-coloured for males and dark-coloured for females) (Figure 1) (FAO 1995; Horiguchi *et al.* 2000).



Fig. 1. Differences in color of the gonads of *H. tuberculata coccinea*. **A.** Gonad of a female. **B.** Gonad of a male.

The size at first sexual maturity is 25 mm and has been observed to reach a maximum shell length of 80 mm in the wild (Espino and Herrera 2002).

According to Sawatpeera *et al.* (2001), the abalone life cycle is composed of a larval, post-larval, juvenile and adult stage (Figure 2).

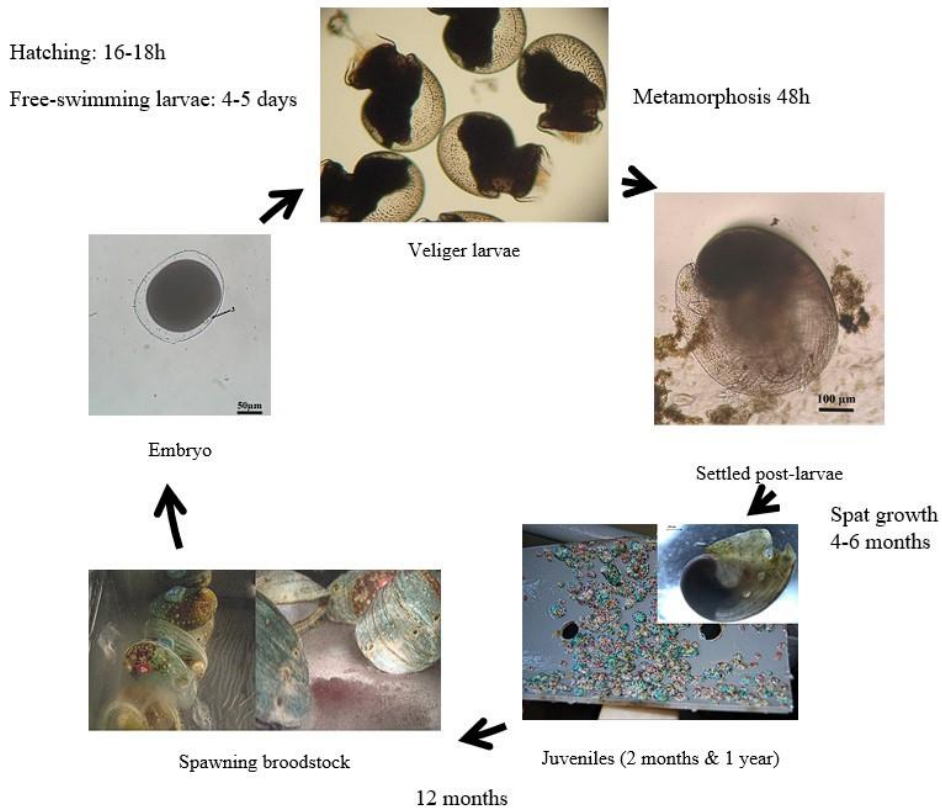


Fig. 2. Life cycle of abalone (Courtois de Viçose 2011).

PRODUCTION OF *HALIOTIS TUBERCULATA* SP. IN THE CANARY ISLANDS

Broodstock maintenance

Broodstock is kept separated by generation and sex in 60 l tanks. Each tank has a maximum of 30 animals. They are fed once a week with a mixed diet of algae, *Ulva rigida* and *Gracilaria cornea*. The tanks are cleaned once a week and replaced every three months for complete disinfection and maintenance.

Larval production

The 39 distinct stages during the embryonic and larval development are completed in 62 hours at 23 °C (Courtois de Viçose *et al.* 2007). During this time, the larvae are maintained with a continuous flow of water and a complete renewal of the water volume every 12 hours. Prior entering the tanks the water is filtered to 1 µm and sterilized by ultraviolet (UV) light (Figure 3).

Materials and tank preparation

- Sieves with 60 µm mesh, for drainage
- Porous hose for air supply
- Egg hatching tray
- Water filtered to 1 µm and sterilized with a UV lamp
- 180 l larval tanks

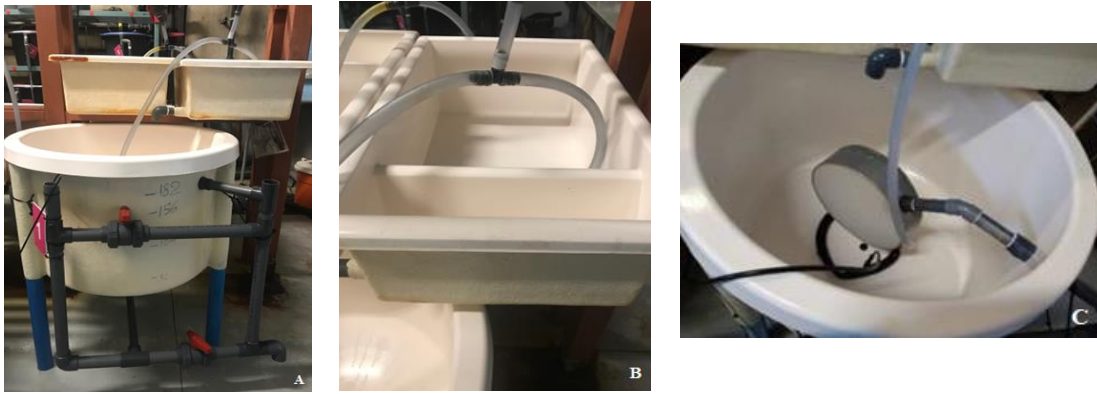


Fig. 3. **A.** Larval culture tank prepared with a hatching tray for fertilized eggs. **B.** Detail of egg hatching tray. **C.** Detail of air and water inlet and outlet pipes.

SPAWNING PROTOCOL

Broodstocks are kept separated by sex to minimize the induction of spontaneous spawning.

In nature, environmental factors such as spring tides temperature, etc., regulate sexual maturation and spawning of the animals (Hooker and Morse 1985). In captivity, spawning is induced by controlled induction methods when the animals are mature. The sexual maturity of the animals is estimated from gonad development using a scale ranging from 0 to 3, where 0 indicates no gonad development and 3 indicates very mature animals (see spawning protocol procedure). The most common induction methods are thermal shock, ultraviolet radiation and hydrogen peroxide (Hahn 1992; Kikuchi & Uki 1974).

Hydrogen peroxide method provides satisfactory results with low cost and easy storage basic reagents. However, these reagents are harmful to the gametes, so once gamete ejection begins, the recipients must be rinsed and refilled with filtered seawater (1 μm). Only gametes released in water free of the reagents are used for fertilization. Ultraviolet radiation is a very effective method but requires a higher initial investment. In this protocol we describe both methods.

Materials

- 40 l white trays
- 50 l black containers
- 500 μm mesh sieve
- 125 μm mesh sieve
- 10 l buckets with lids
- Dissecting microscope
- UV lamp
- Bogorov egg-counting chamber
- Manual counter
- 10 ml glass pipette
- Perforated Secchi disk

Procedure



Broodstock selection

- Mature females from the broodstock are selected with a gonad index of 2 / 3.



Synchronization of gametes expulsion

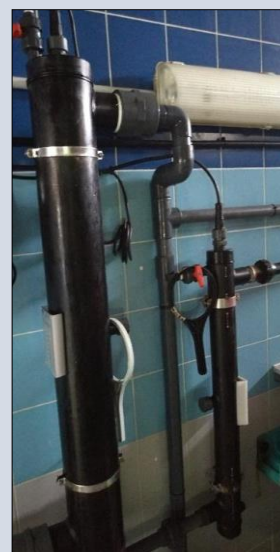
- Males tend to expel gametes earlier than females, so females are induced first and males are induced one hour later, to synchronize gametes expulsion.

Induction methods

Ultraviolet radiation (UV):

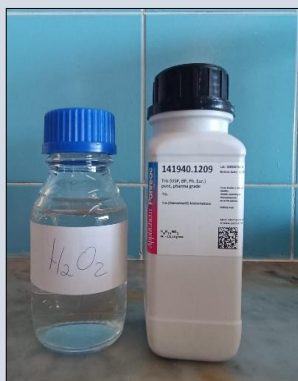
Animals are placed, separated by sex and batch, in induction containers and trays, which are filled with 1 μm filtered seawater and sterilized by a UV lamp. Containers are covered and left in darkness for a few hours until spawning begins.

Animals are left in contact with the UV irradiated seawater during various hours until spawning begins at dusk.



Hydrogen peroxide (H_2O_2):

Males and females are placed in separate containers with water filtered to 1 μm . To this, 6.6 ml of Tris (hydroxymethyl-methylamine) buffer solution per liter of water is added to raise the pH to 9.1 before adding 4 ml l^{-1} of H_2O_2 solution 15 min later. Gamete ejection takes place approximately 3 h after the addition of the reagents, while broodstock remains in the dark.



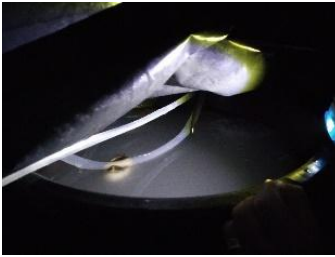
Preparation of hydrogen peroxide and *Tris* buffer:

A 6% hydrogen peroxide solution is prepared by dissolving 20 ml of 33% hydrogen peroxide in 80 ml of distilled water. The preparation is kept at 0 - 4°C. To prepare the Tris buffer, dissolve 24.2 g of Tris in 100 ml distilled water.



Distribution of broodstock

· Once spawning begins, the females are placed in white trays and the males in black containers so that the gametes can be easily observed once they are expelled.



Gamete collection

· Sperm remains in the water column, while the dark oocytes, settle to the bottom of the containers. Eggs are siphoned, through a 500 µm sieve to retain possible feces and debris, into a bucket of known volume. Sperm is collected with a beaker.



Fertilization

· Sperm is introduced into the bucket containing the oocytes and the water is gently stirred with a perforate disc to enable homogeneous distribution and contact between the gametes. To achieve maximum fertilization rates, gametes are left undisturbed by one hour.



Rinsing fertilized eggs

· Fertilized eggs are transferred to a 120 µ sieve and rinsed for 20 minutes with 1 µm filtered and UV sterilized water to remove excess spermatozoa.



Estimation of fertilization rate

· After rinsing, eggs are transferred to a bucket of known volume of water and three samples are taken with a pipette and placed in Bogorov chambers. The total number of eggs and the number of fertilized eggs in the sample are counted and fertilization rate determined:

$$\text{Fertilization rate (\%)} = \frac{\text{Total fertilized eggs}}{\text{Total eggs}} * 100$$



Identification of viable eggs

· Unfertilized and fertilized eggs can be distinguished as the latter show a differentiated chorion and the first polar body. One hour after fertilization the eggs begin their cell division and after a few hours, the early stages of embryonic development can be observed.



Distribution of fertilized eggs

- The number of fertilized eggs introduced into the hatching trays is calculated to have a larvae concentration ranging between 1 to 2 larvae/ml in the larval tank.

Larval production

Larvae hatch around 12 h after fertilization. Hatching trays are designed to allow newly hatched larvae to pass from the tray to the larval tank via water current, while unfertilized or non-viable eggs remain at the bottom of the tray.

Larval tanks are kept in flow through, with 1 μm filtered UV sterilized and aerated seawater. During this period the larvae are not fed as they are lecithotrophic. The trays are removed once all larvae have been transferred to the larval tanks (Figure 4).

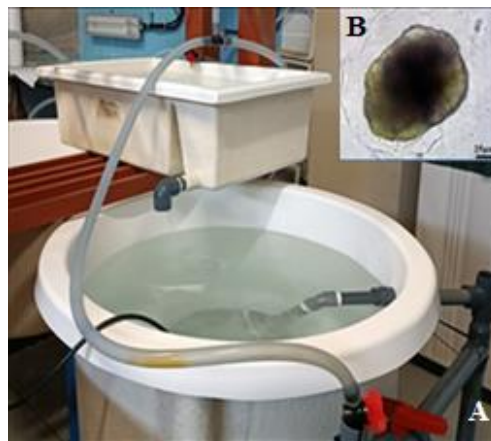


Fig. 4. A. Larval culture system. B. Trochophore larva.

Larval transfer to post-larval production

Larvae are transferred approximately 62 h after spawning when the third tubule can be observed on the cephalic tentacles. At this stage, the larvae begin to search for a suitable substrate to attach for metamorphosis. Once metamorphosed the larvae will lose their crown of cilia, their ability to swim, and the peristomal shell will begin to form. At this stage, exogenous feeding begins with the supply of benthic diatoms (Hahn 1989; Sawatpeera *et al.* 2001; Courtois de Viçose *et al.* 2007).

In nature, the settlement and metamorphosis of abalone are induced by chemical, biological and physical signals associated with substrates such as the presence of crustose coralline algae and other microalgae or macroalgae (Li *et al.* 2006; Courtois de Viçose *et al.* 2012c). In production farms, spores of the green macroalgae *Ulveella lens* are used to induce attachment and subsequent metamorphosis of various abalone species (Courtois *et al.* 2012b).

Ulveella lens sporophytes that colonize the settlement plates located in the post-larval tanks (2500 l) are produced through plates with mature patches of *U. lens* introduced into the containers. Sporulation was induced by addition of nutrients provided by F/2 medium. Once the settlement plates are colonized by *U. lens* germlings, the tanks are considered ready to receive the larvae.

Materials

- 125 µm mesh sieves
- 15 l buckets
- 50 l trays
- Bogorov chamber
- Postlarvae culture tanks (2500 l)
- UV radiation lamp

Procedure



Larval collection

- A 120 µm sieve is placed at the outlet of the larval tank drain to collect the larvae.
- This sieve is placed in a tray with filtered seawater to maintain the larvae immersed.
- The tank is slowly drained to avoid damage the larvae.



Counting competent postlarvae

- Once the tank has been emptied, post-larvae are transferred from the sieve into a 10 l bucket. Three 4 ml samples are taken and observed in the Bogorov chamber to count competent larvae ready to settle.
- The total number of competent larvae gathered in each container is estimated and used to calculate the total number to be transferred to the larval settlement tanks.



Transfer of post-larvae to settlement tanks

- Post-larvae are transferred to the settlement tanks to reach a maximum of 0.5 individuals per cm² of settlement substrate.

Post-larvae and juveniles production

Post-larvae designate the phase between larvae metamorphosis and the appearance of the first respiratory pore (Hooker and Morse 1985). During the first 4-5 months, they are fed exclusively on a mixture of benthic diatom species (see below in diatom production section). After this period, once they reach 5 - 6 mm length, they gradually begin to feed on a mixed diet of macroalgae, *Ulva rigida* (green) and *Hydropuntia cornea* (red), until the diatoms are completely replaced. The animals remain under this feeding regime in the post-larvae tanks until they reach a minimum size of 15 mm, when the juveniles are transferred to the grow-out tanks. The culture time during this phase lasts 6 - 9 months (Figure 5).

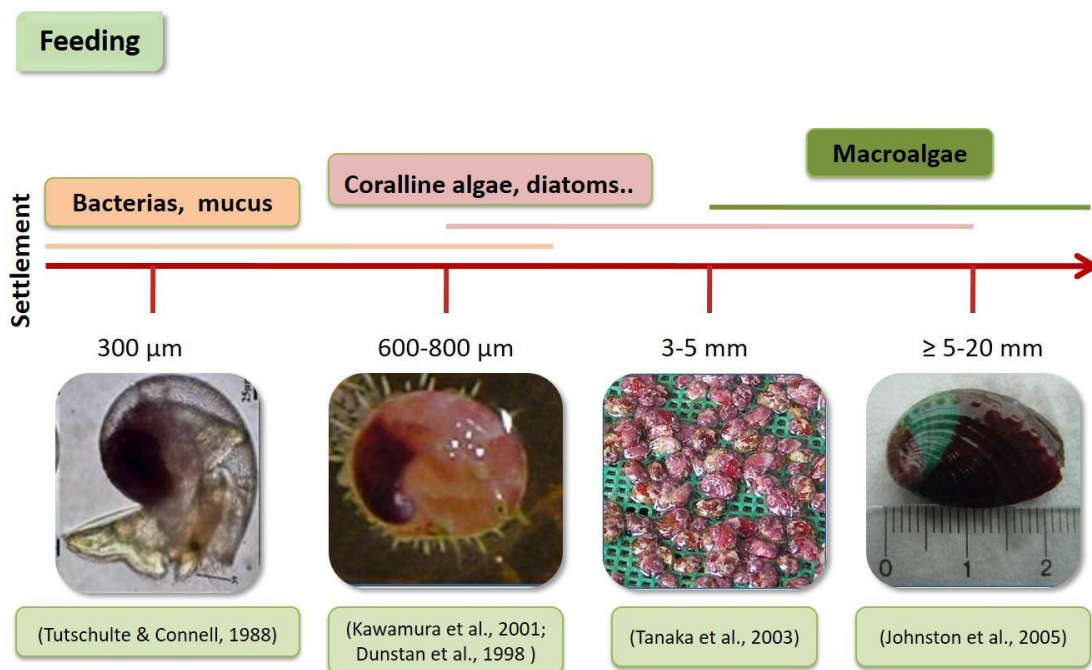


Fig. 5. Feeding pattern of the abalone (Viera 2014).

During the diatom feeding stage, the development of diatoms in the tank is controlled by a shading net that regulates the amount of light penetrating the tank (Figure 6).

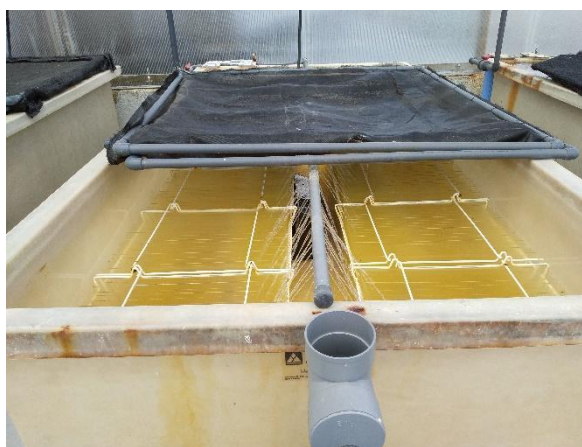


Fig. 6. Settlement tank with shading cover.

Feeding protocol of post-larvae and juveniles

Materials

- 125 µm mesh sieves
- 10 l buckets
- 5 l measuring jug
- Siphon
- 250 ml measuring cup
- 15 l basket
- 1 and 10 µm bag filters
- 1 mm, 125 and 500 µm filters

Procedure



· Water is filtered at 10 and 1 μm by mechanical filters located at the inlet of the post-larvae tanks. These filters are cleaned once a week.
 Before feeding, the water inlet is closed during 24 h to facilitate the adherence of the diatoms to the settlement plates.
 The tank is left uncovered during the diatom feeding period to promote diatom's growth.



· Post-larval tanks outlets are fitted with filters to prevent post-larvae and juveniles' escapes. Filters mesh size varies from 125 μm to 500 μm and 1 mm and are replaced according to post-larvae growth during the 6 to 9 months of this production phase.



· Diatoms are harvested through a siphon, collecting only diatoms adhered to the walls of the culture bags and placed in a 10 l bucket.



· Diatoms in the bucket are stirred with a 250 ml measuring cup so that they are well distributed and do not settle at the bottom.



· Each post-larval tank is supplied, weekly, with the diatom production from 20 l bags. Diatoms are distributed slowly and evenly throughout the tank, through a 125 μm sieve to break up any possible accumulations of algae.



· Once the juveniles reach 4-5mm, macroalgae are added to the diatom diet, initially with *U. rigida*, which has a softer texture, and then with *H. cornea*, once they reach 1 cm in length and have a more developed radula. (Viera *et al.*, 2005).

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Limpets: *Patella aspera* Röding, 1798 and *Patella candei* d'Orbigny, 1840

CARLA N., M. DE GIROLAMO, J. RODEIA, E. ISIDRO, R. JOSÉ, P. SOUSA, C. ANDRADE AND D. CASTEJÓN



Nunes, C., M. De Girolamo, J. Rodeia, E. Isidro, R. José, P. Sousa, C. Andrade and D. Castejón 2024. Limpets: *Patella aspera* (Lamarck, 1819) and *Patella candei* (d'Orbigny, 1840). Pp: 17-38 in: Mirko De Girolamo, Gercende Courtois de Viçose, Carlos Andrade and Eduardo Isidro (Eds) 2024. Production of marine invertebrates at early stages - Manual for best practices. *Arquipelago*. Life and Marine Sciences. Supplement 14: 62pp.

Limpets are marine gastropods whose biological and ecological specializations challenge their aquaculture development (Mau and Jha 2018a). However, important advances have been achieved in recent years with respect to different limpet species, such as the Hawaiian *Cellana sandwicensis* (Mau *et al.* 2018; Mau and Jha 2018b; Nhan and Ako 2019) and the Mediterranean *Patella ferruginea* (Ferranti *et al.* 2022; Guallart *et al.* 2020).

Patella aspera and *P. candei* are endemic species to the Macaronesia Region (except Cabo Verde) (Côrte-Real *et al.* 1996; Faria *et al.* 2017; Faria *et al.* 2018; Weber and Hawkins 2005) (Figure 1). The two species share several eco-biological traits: both colonize and graze on rocky substrates, from the intertidal to the upper subtidal margin (Sousa *et al.* 2020); both are winter breeders, with higher gonadal development occurring between January and March (Henriques *et al.* 2012; Sousa *et al.* 2017; Vasconcelos *et al.* 2023), although in the Azores *P. candei* spawns throughout the year, peaking in summer (Curdia *et al.* 2005). Limpets are broadcast spawners that rely on the production of swimming planktonic larvae to disperse through the marine environment (Rodríguez *et al.* 1993). Hard substrates colonized by crustose coralline algal assemblages (CCA) might be the natural recruitment grounds for both limpet species (Castejón *et al.* 2021b; Castejón *et al.* 2023b, c).

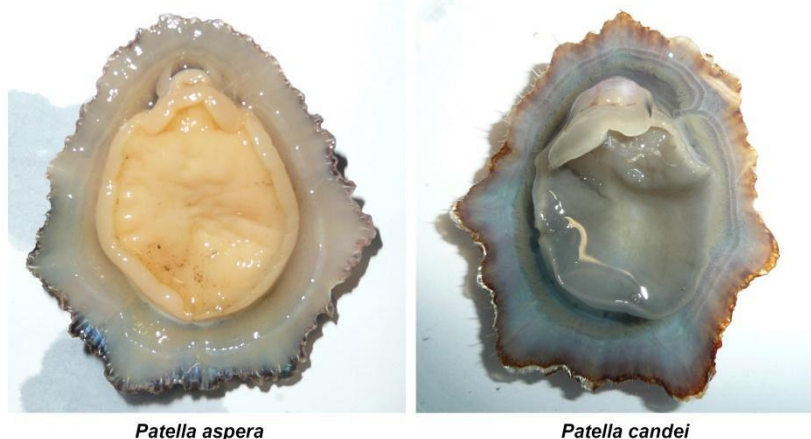


Fig. 1. Ventral view of A. *Patella aspera*. B. *Patella candei* (specimens from Madeira)

Aquaculture production of limpets

The protocol to produce limpets is based mainly on the results of Aquainvert project and data published by Cañizares *et al.* 2021, Castejón *et al.* (2021a, b, 2022, 2023a, b, c) and Nunes *et al.* (2021, 2024).

Broodstock harvest from the wild and transportation

Adult specimens (shell length ≥ 35 mm) are collected in rocky intertidal areas during low tide or in the shallow subtidal areas by snorkeling or scuba-diving. Specimen detachment is done with a blunt-scraping knife (known as lapeira in Azores and Madeira). Limpets have a strong grip, which makes it difficult to detach them without injury which usually leads to their death (Guallart *et al.* 2020; Mau and Jha 2018a). Damage during harvest can be minimized by finding individuals on flatter rock surfaces not exposed to desiccation for long periods of time.

Materials

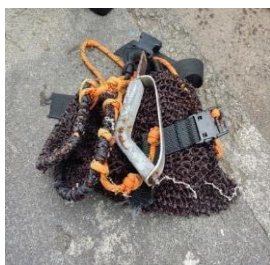
- snorkel or scuba gear, if required
- blunt knife (“lapeira”)
- protection gloves
- insulated / portable cooler
- sea harvest bags (mesh size < 1 cm), or commercial plastic bags

Procedure



Limpet collection

· A blunt knife is placed below the shell, to be used as a lever. The detachment of the limpet should be done in a single, strong and quick movement, with the blade parallel to the foot-base.



· Collected limpets are gathered in a net bag always kept underwater (e.g., inside a tidal pool or inside a cooler or bucket with seawater).



Limpet selection

· Injured (indicated with a white arrow) and dead limpets should not be mixed with healthy limpets. If on site selection is not possible, damaged and dead specimens should be removed as soon as possible at the culture installations.

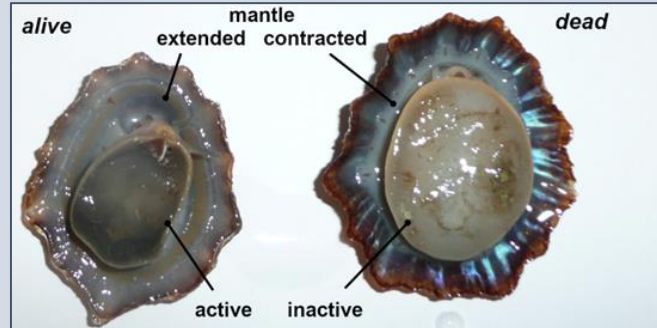


Transportation

· The net bag with limpets is placed inside a portable cooler filled with seawater. Commercial plastic bags can be used as an alternative to net bags. Desiccation of the animals should be avoided and transportation time minimized.

Life and death in limpets

Recently dead specimens can be difficult to distinguish from weakened live specimens. Alive specimens are active, and the extended mantle reacts when touched. Dead specimens are inactive, and the contracted mantle does not react when touched.



Acclimatization and broodstock management

Limpet broodstocks are kept in holding tanks in an open flow-through system (water exchange $\geq 30\% \text{ h}^{-1}$), with oxygen saturation above 90%. Environmental parameters such as temperature ($18 \pm 3 \text{ }^\circ\text{C}$), salinity ($36 \pm 1 \text{ g/l}$) and photoperiod are kept at natural conditions. Tank volume ranged from 75 l to 1000 l; they can be circular or rectangular shaped with flat bottom, made of fiberglass or polyethylene (Figure 2 A-D).

Animal density can vary between 0.1 and 0.3 specimens per litre. Polyethylene sheets are recommended to cover the walls of the culture tanks enabling easy limpet detachment when necessary (Figure 2 D). After collection, limpets are placed on movable substrates, such as the $23 \times 34 \text{ cm}$ corrugated fiberglass plates (Figure 2 E-F), to facilitate handling and density management. Placing the limpets directly in the bottom of the tanks is possible but not advisable, due to the rapid deterioration of water conditions if mortality occurs.

It is highly recommended to have food available for harvested limpets upon arrival at the culture installations. This may consist of natural biofilms or biofilms of *Ulvela lens* and *Ulva sp.* produced as auxiliary cultures (Figure 2 E-F). A few weeks, in outdoor tanks with flow-through systems and under natural conditions, are sufficient to create a natural biofilm on corrugated plates or plastic liners. If limpets are held in captivity for a short stay (less than 1-2 weeks) no food supply is necessary.

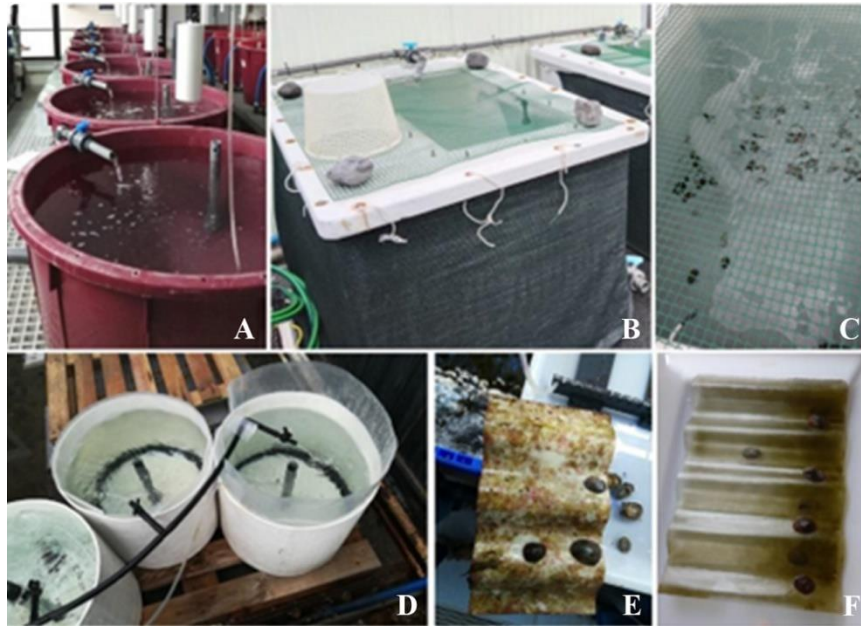
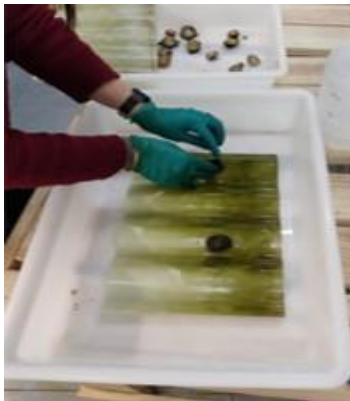


Fig. 2. A. Indoor 200 l tanks; B–C. Outdoor 1,000 l tanks; D. Outdoor 75 l tanks; E. Limpets on a corrugated fiberglass plate covered with natural biofilm, showing grazing marks; F. Limpets on a corrugated fiberglass plate covered with *Ulvella lens* biofilm, produced as auxiliary culture.

Materials

- culture tanks with flat bottom
- 42 cm wide continuous tubular polyethylene bag
- 23 × 34 cm corrugated fiberglass plates
- plastic trays
- blunt knife (lapeira)
- siphon (15mm internal diameter)

Procedure



- Limpets are placed on fully submerged plates which are initially positioned horizontally in the flow-through system.
- After 5–10 minutes, the attachment of the limpets to the plates is checked.
- Plates with limpets securely attached are distributed and positioned vertically inside the holding tanks.
- Limpets that don't reattach, or show light scrapes in the foot, must be placed in quarantine tanks.



- Holding tanks should be checked every day during the first week and every 2–3 days during the second week for cleaning and removal of dead specimens. A hand siphon is used to clean the bottom of the tanks from faeces and other residuals. The procedure is done weekly after the acclimatization.

Artificial reproduction

Artificial reproduction of *P. aspera* and *P. candei* is performed during the peak of the reproductive season (from November to the beginning of May). Secondary sexual characters are not described for limpets, thus sex identification requires invasive techniques (biopsies) or sacrificing the specimens. Nhan and Ako (2019) and our observations of the Macaronesian species reported that it is possible to recognize the sex by observing the colour of the gonads through the integument, if these are in an advanced stage of maturation (stage III or higher of maturation index; Orton *et al.* 1956) (Figure 3).

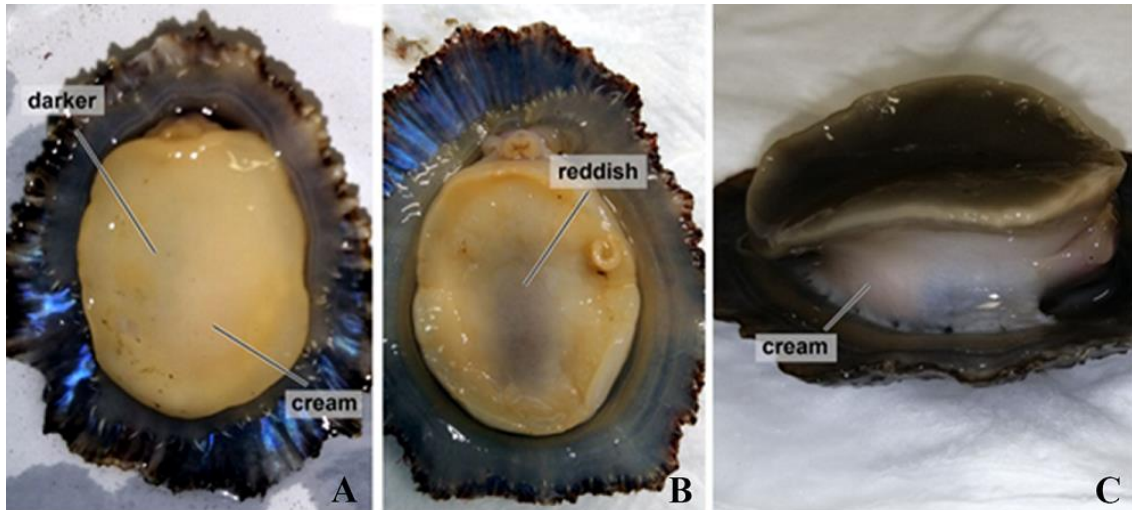
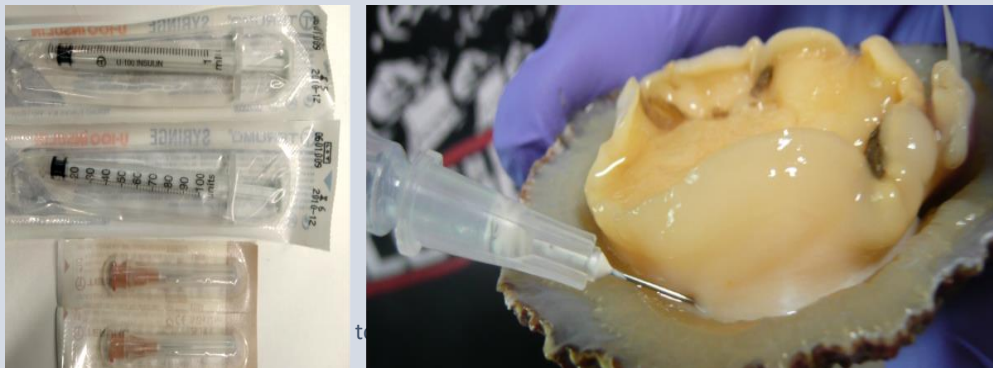


Fig. 3. *P. aspera*: (A) male gonad is observed as a cream patch over a darker background corresponding to the viscera, and (B) the female gonad as a reddish central patch. C. *P. candei*: male gonad may be visible as a cream patch contrasting with the colour of the foot; females are not yet identifiable.

Biopsy as non-lethal method for sex identification in limpets

The biopsy is a skilled, straightforward, non-lethal method for sex identification in limpets, in which small samples of gonad are taken, causing minimal injury to the specimens. The oocytes in females are coloured (reddish in *P. aspera* and purple-brownish in *P. candei*) and a sperm is a milky white fluid. For this purpose, insulin syringes (1 ml) and needles (0.45 × 12 mm) can be used. The biopsy should pierce the lateral integument and foot muscle in the left side of the animal.



Spawning induction, preparation of sperm/oocytes pools, artificial maturation (activation) of the oocytes, fertilization, collection of the larvae and larval rearing are all performed using filtered (cartridge filters of 1 to 5 μm) and UV-sterilized seawater (abbreviated as FSW). All the procedures are done in an acclimatized room temperature (17 ± 2 °C).

Spawning induction: preliminary results

Successful spawning in *P. aspera* and *P. candei* can be achieved using thermal shock, intense bubbling, and their combination. Released gametes are ready for fertilization and no egg activation is needed. Nevertheless, optimized protocols are required since the occurrence of a successful spawning is still unpredictable.

Materials

- blunt knife
- 50 l and 130 l tanks
- plastic colanders
- tray
- paper towel
- Dissecting microscope
- Standard aquarium air stones
- Aquarium heater
- Aquarium Chiller
- Ice packs
- Transfer pipette (1 ml)
- 80 and 335 μm sieves
- 5 l plastic beakers
- Waterproof digital thermometer
- Handheld multiparameter

Procedure

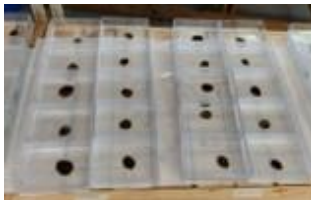
Preparation of the adult specimens

- Adult limpets are gently detached and covered between wet towels on a tray for 1 h.



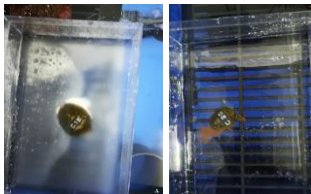
Application of the spawning induction treatment

- Limpets are then placed in a tank where spawning induction treatment is applied for 4h to 4h 30min. See box: Spawning induction methods for limpets.



Post-stimulus application

- All limpets are placed in individual boxes (400 ml of FSW, 18 ± 1 °C) for 2–3 h. Gametes emission should be checked every 30 minutes.



Spawning

- If spawning occurs (A – sperm; B and C - oocytes), the gametes are collected and filtered to remove faeces and other debris. Sperm is filtered through an 80 μm mesh and collected into a clean beaker.



- Oocytes are collected using a 335 μm sieve and washed, before being placed in clean beakers. Gametes are then pooled for *In vitro* fertilization (page 29).

Spawning induction methods for limpets

Limpets are exposed to the spawning induction treatment for 4h – 4h 30min.

A. Thermal shock (temperature rise)

Limpets are placed in a tank filled with FSW heated 3 °C above seawater temperature, with an aquarium heater (e.g., if limpets were conditioned to ≈ 17 °C, FSW is heated to ≈ 20 °C).

B. Thermal shock (temperature drop)

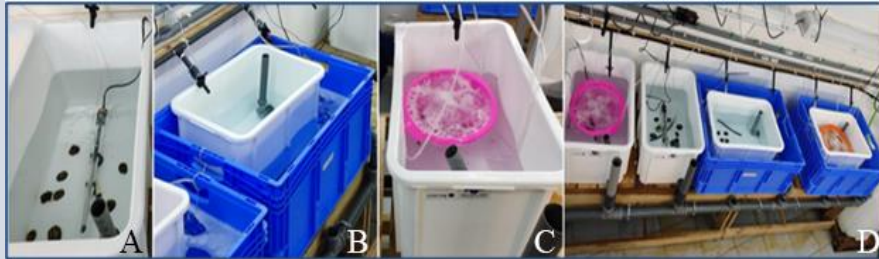
Limpets are placed in a tank with FSW 3 °C below sea water temperature (e.g., if limpets were conditioned to ≈ 17 °C, FSW is cooled to ≈ 14 °C). A water bath, connected to an aquarium chiller, is used to help maintain the FSW inside the spawning container cooled down. Ice packs are used if necessary.

C. Intense bubbling

Limpets are placed inside a plastic colander, with standard aquarium air stones to provide adequate (strong) aeration. Initial inductions were done at natural sea water temperature (≈ 17 °C).

D. Combining thermal shock and bubbling

Limpets are placed inside a plastic colander with standard aquarium air stones attached in the bottom of it. The colanders are placed in tanks where FSW temperature is adjusted (to ≈ 14 °C or 20 °C) as described previously.



Spawning induction for *Patella aspera* and *Patella candei*: (A) thermal shock – temperature rise, (B) thermal shock – temperature drop, (C) bubbling and (D) combined.

Post-spawning induction recovery

Limpets are placed in holding tanks with food at low density (0.1 individuals/l). Monitoring is required in the following days to remove any dead specimens (Figure 4).

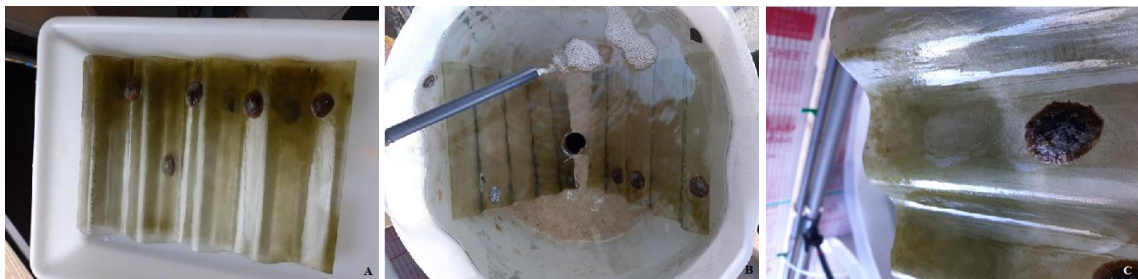


Fig. 4 A. Limpets placed on plates covered with *Ulvella lens*. B. Holding tank (75 l) with limpets recovering from spawning induction treatments. C. Limpet showing a normal grazing behaviour a few days after spawning induction.

Gonad extraction and gamete maturation

Dissection is the most widely used method for obtaining gametes from mature limpets. In this case, the oocytes need to be artificially matured via alkalized seawater baths to enhance fertilization success. The sex can be identified by gonad colouration (Figure 5). It is recommended to use gonads at stage III or higher (Orton *et al.* 1956) for fertilization purposes.

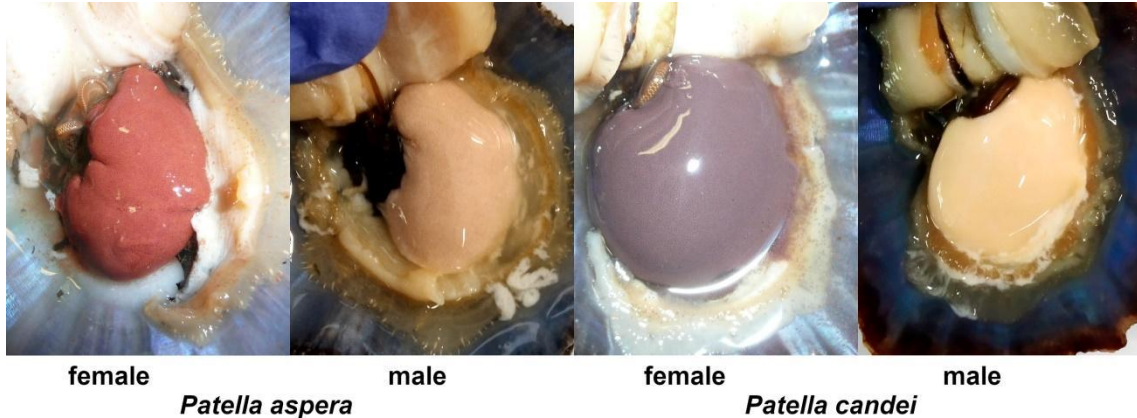


Fig. 5. Sex identification by gonad examination in limpets.

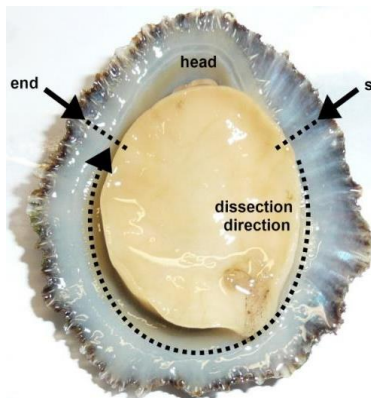
Dissection procedure

Dissection must be performed carefully to avoid breaking the inner organs. Scalpel blades with rounded tips are better suited for the limpet anatomy resulting in easier manipulation and use.

Materials

- scalpels with rounded-edge blades
- plastic fish trays (5–8 l)
- stainless electronic caliper
- absorbent paper
- aluminum foil
- petri dishes
- precision balance

Procedure



- The surface to be dissected is covered with absorbent paper and aluminum foil.
- Pre-selected specimens for dissection are placed, facing upward, in tray.
- The scalpel incision is done as shown in the figure, passing through the mantle and foot musculature. After incision the foot can be turned to show the gonads. Gametes will be released if gonad is mature.

Preparation of the sperm pool

Dissection of males should be performed before females as the life expectancy of sperm is longer than that of the oocytes. It is also recommended to gather sperm from at least four specimens to minimize the risk of collecting poor quality sperm. The sperm pools can be kept in the fridge for several hours before being used for fertilization.

Materials

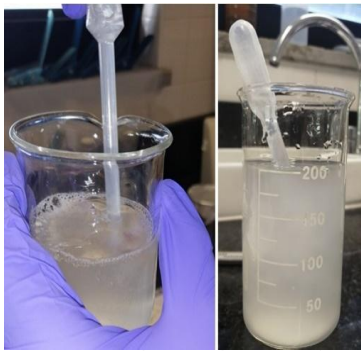
- beakers 100–600 ml volume
- blunt tip laboratory tweezers
- Sieves with 55 – 80 μm
- fridge
- transfer pipette (3 ml)

Procedure



· After dissection, small pieces of the gonad are collected with a transfer pipette and dropped inside a small beaker with FSW. Rupture of underlying organs should be avoided.

· Gonad pieces are picked and released repeatedly inside the beaker with the transfer pipette. The flow pressure releases sperm from the gonad turning the FSW into a white solution.



· Once the sperm has been collected from at least four specimens, the sperm solution is passed through a mesh (55 to 80 μm) into a new clean beaker to remove major debris.

· The beaker with the clean sperm solution is then conserved in the fridge.

Preparation of the oocyte pool

The number of specimens to be sampled for oocytes depends of the desired larval production. But in any case, several females should be pooled to minimize the risk of collecting poor quality oocytes. Transfer pipettes with wide opening are required to generate enough flow pressure to release the oocytes without damaging them; eventually, the pipettes tip can be cut to adjust the size.

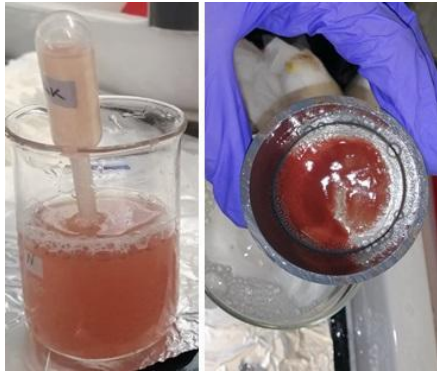
Materials

- beakers 100–600 ml volume
- transfer pipettes (3 ml)
- 55 and 200 μm nylon sieves
- scissors
- transfer pipettes (wide mouth 9 ml)
- blunt tip laboratory tweezers

Procedure



- After dissection, the entire female gonad is carefully sampled and dropped into a glass beaker (50 to 200 ml) using a scalpel, avoiding the rupture of the digestive system.
- Gonads are broken down by the flow pressure generated with the wide mouth transfer pipette, by repetitively pulling and releasing the gonads.
- The solution of oocytes is passed simultaneously through two sieves: a 200 μm mesh to trap large debris (such as connective tissue) and a 55 μm mesh to collect the oocytes.



- The oocytes obtained in the 55 μm sieve are washed repeatedly with FSW to remove the hemolymph and other residuals. The washed oocytes are dropped into a beaker with clean FSW.

Artificial maturation (activation) of the oocytes

Low fertilization rate of the oocytes obtained by dissection can be enhanced by artificially maturing (or activating) them in an alkaline bath (Guallart *et al.* 2020; Hodgson *et al.* 2007; Pérez *et al.* 2016). The optimal alkaline bath for *P. aspera* and *P. candei* consists of ammonium hydroxide (NH_4OH) at pH 9.0 – 9.5 during 10 – 20 minutes.

Materials

- NH_4OH (28–30 %)
- 55 - 80 μm mesh sieve
- pH meter
- dissecting microscope
- optical microscope
- 500 ml wash bottles
- measuring cylinder
- plastic beakers (2 - 5 l)
- transfer pipettes (1 ml)
- thermometer
- Bogorov Counting Chamber, or a petri dish with a transparent grid
- tally counter

Preparation of the alkaline bath

The pH of a FSW solution is raised to 9.0 – 9.5 by addition of a few drops of NH_4OH , with a transfer pipette. The needed volume of this alkaline solution may be prepared in plastic (polypropylene) beakers. The solution should be well mixed after adding the base; the pH must controlled during and after its preparation.



Procedure



- Oocyte density in the alkaline bath ($100 - 350$ oocytes \times ml⁻¹; 2 l beakers) should be sufficient to form a thin layer of oocytes in the bottom. Oocytes are collected in a sieve (55 or 80 μm) to remove excess FSW and transferred into a NH_4OH alkaline bath (pH 9.0 – 9.5) for 10–20 min (17 ± 2 °C).
- Once the alkaline bath has finished, the oocytes are recollected using a 55–80 μm sieve and gently washed with FSW until the pH is normalized. At the end of the washing process a pH control is done with a pH meter.
- Oocytes are pooled into plastic beakers (2–5 l) with clean FSW at room temperature until fertilization.

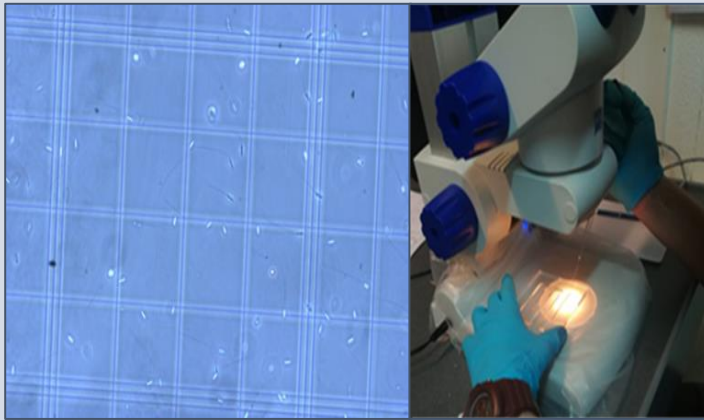
Counting limpet gametes

Sperm

To facilitate this procedure, a sperm sample is diluted in FSW and Lugol is added to immobilize the sperm cells. The dilution is loaded into the Neubauer chamber with a micropipette. Cell count is done under a light microscope with help of a tally counter. The dark-field provides an easier visualization. Cell count methodology in the Neubauer chamber is described in figure 1 (page 51).

Oocytes

Oocyte pool is gently stirred to homogenize cell distribution inside the container. Four to six 1 ml samples are collected with a transfer pipette or a micropipette and placed in a Bogorov Counting Chamber (or a petri dish above a transparent grid). Counting is done under a dissecting microscope and helped with a tally counter.

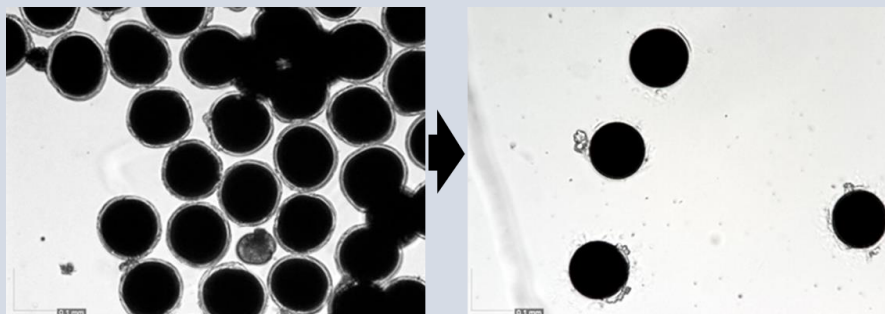


Left. Sperm cells in a Neubauer chamber. Right. Counting oocytes using a Bogorov counting chamber

Oocyte activation control

This step calculates the percentage of oocytes activated by the maturation treatment and suitable for fertilization. At least six samples of 50–100 oocytes are taken and observed under a light microscope. The percentage of active oocytes (with spherical shape and chorion partially or totally removed) is calculated.

$$\text{active oocytes (\%)} = (\text{total active oocytes}) \times (\text{total oocytes})^{-1} \times 100$$



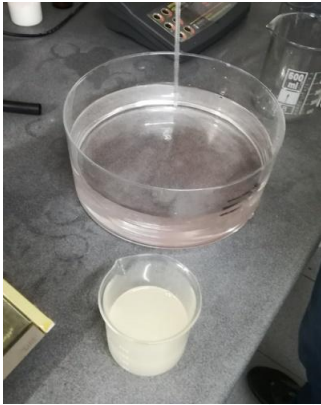
In vitro Fertilization

This protocol applies to gametes obtained both by spawning induction and by gonad dissection. Recommended gamete density is 50–100 oocytes/ml and 10^5 – 10^6 sperm cells/ml. The sperm can be kept in contact with the oocytes during the entire incubation or removed after fertilization is confirmed (2–3 hours). Fertilization is performed on static condition, with no aeration or agitation. Since oocytes sink in FSW, they should be left to deposit in the bottom of the containers in a thin layer. Thermal limit for fertilization, incubation and hatching is unknown, so they should be carried out below 20 °C as a preventive measure. Successful fertilization and incubation were obtained using a variety of containers: 3 l glass crystallizers, and 0.6–5 l glass and polypropylene beakers. Light regime may have no consequence on hatching success; effective hatching occur both under continuous darkness as well as in continuous light.

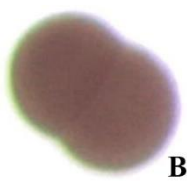
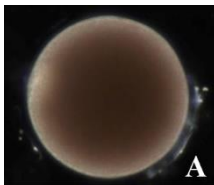
Materials

- oocyte pools
- sperm pools
- 600 ml to 5 l plastic beakers, or 3 l glass crystallizers
- Bogorov Counting Chamber, or
- petri dishes with transparent grid
- 80 µm sieves
- transfer pipette (1 ml)
- dissecting microscope
- cavity microscope slides
- Neubauer improved chamber
- micropipettes P100 & P1000 and tips
- measuring cylinder
- light microscope
- Lugol
- tally counter

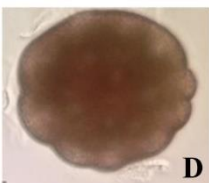
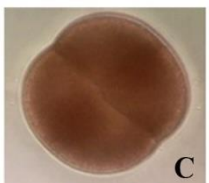
Procedure



- Oocytes are placed in the selected vessel (50–100 oocytes/ml).
- Sperm is added (10^5 – 10^6 sperm cells/ml) to the oocytes, using a micropipette or transfer pipette, and gently mixed for a few seconds.



- Fertilization rate is usually estimated 2–3 hours after the start of the fertilization: at least six samples of 50–100 eggs are collected with a micropipette, placed in cavity microscope slides, and observed using a light microscope. They are then classified as oocytes (unfertilized, A) or as embryos (B–D).



- Fertilization rate is then calculated as:

$$\text{Fertilization (\%)} = \frac{N \text{ embryos}}{N \text{ oocytes} + N \text{ embryos}} \times 100$$



- (Optional) Once fertilization is confirmed, the embryos and eggs are washed with FSW in a 55–80 μm sieve to remove sperm. The eggs are then placed in clean containers with FSW (crystallizers or beakers).
- Incubation is done under static condition (no water renewal, stirring or aeration). Containers are covered and kept at room temperature (17 ± 2 °C) for 24h.

Larvae: harvest, handling and counting

Harvest

Limpet larvae need to be picked up twice: after hatching (24 h post-fertilization) and by the end of larval rearing phase (usually 72 h post-fertilization). The swimming larvae with the highest quality are recovered by careful syphoning the upper $\frac{2}{3}$ to $\frac{4}{5}$ of the water column of the rearing tank over a sieve (55 to 80 μm). It is recommended to use a glass bowl with FSW to keep the base of the sieve underwater, minimizing air exposure and desiccation of the larvae.

Handling

Limpet larvae are resilient to handling procedures such as syphoning, filtering, and brief agitation. Stirring or agitation is required to take homogeneous samples for counting. In homogeneous water samples trochophores are easier to collect than pediveligers, as the latter tend to retract into their shells, sinking quickly.

Counting

Larval density is estimated by gently stirring the water sample with a transfer pipette, to homogenize larvae distribution in the water column. Four to six 1 ml samples are collected with a transfer pipette and loaded into Bogorov counting chambers (or Petri dishes with a transparent grid). Larvae are counted under a dissecting microscope and their morphology can be used to estimate the percentage of viable specimens.



Larval rearing

Similarly to what has been described in other limpets (*Ferranti et al.* 2018; *Mau et al.* 2018; *Nakano et al.* 2020), larval development in *P. aspera* and *P. candei* can be described by the following swimming planktonic stages (Figure 6): trochophore, veliger and pediveliger.

Larvae of both limpet species are lecithotrophic, relying on their yolk reserves to complete their larval development (Figure 7).

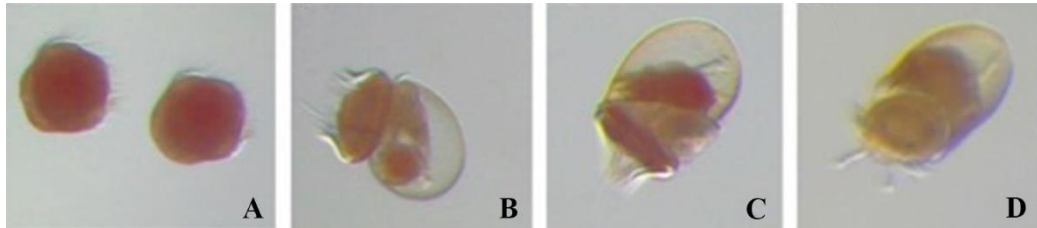


Fig. 6 *P. aspera*: stages of larval development. **A.** Trochophores (24 h post-fertilization). **B.** Veliger (48 h post-fertilization). **C.** Young pediveliger (72 h post-fertilization). **D.** Mature pediveliger (9 days post-fertilization).

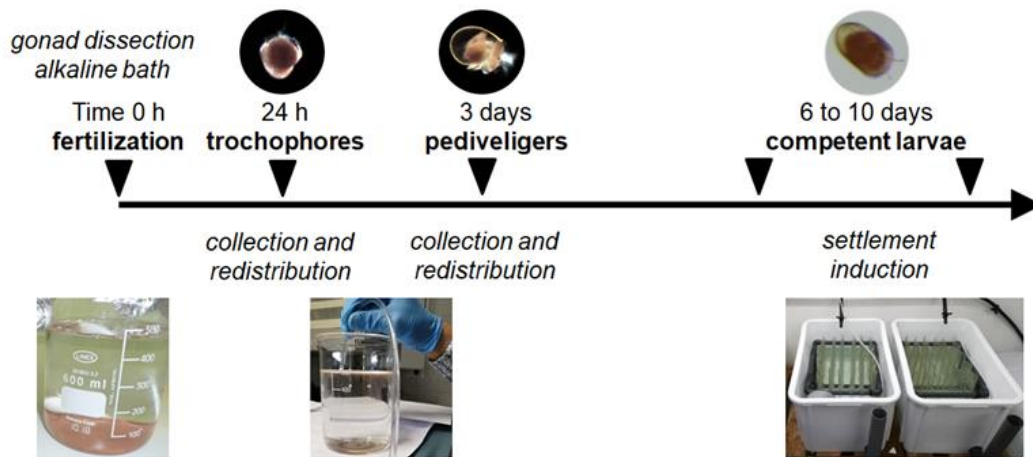
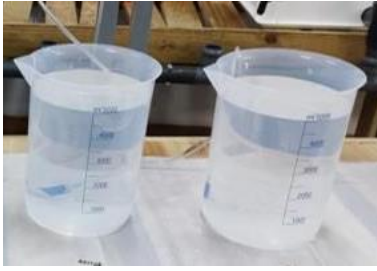


Fig. 7. Resume of the larval production and larval culture processes in limpets.

Materials

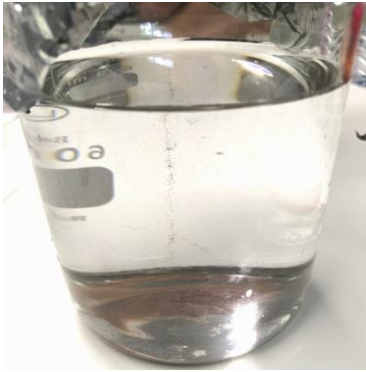
- Larval culture vessels: glass or plastic beakers (0.5 – 5 l)
- siphon tube (4-7 mm internal diameter)
- measuring cylinder
- Bogorov counting chamber or, petri dishes with a transparent grid
- tally counter
- dissecting microscope
- Aquarium air stone

Procedure



- Trochophores are harvested from the incubation vessels and placed on clean beakers (0.6 to 5 l, glass or plastic) with FSW, at densities of 5–10 larvae/ml. Volume adjustment and larval transference is done with measuring cylinders.

- Light aeration is optional. Temperature is kept at 17 ± 2 °C in a controlled-temperature room. Photoperiod can vary from 12 h light: 12 h dark, to continuous light, using fluorescent or LED tubes. Water renovation is not required (Figure 9).



- Larval culture lasts until the pediveliger stage is reached (72 h post-fertilization). Swimming pediveliger larvae are collected from the culture vessels and placed in a clean beaker with FSW, to be counted and then used in the next production phase.

Larval settlement and post-larvae production

Similarly to other limpet species, settlement in *P. aspera* and *P. candei* occurs when the larvae lose their velum and the development of the head, digestive system and teleoconch starts (Figure 8). Successful settlement can be achieved using biofilms dominated by *Ulvella lens* and encrusting coralline algal assemblages. Larvae should be collected at 72 h post-fertilization, otherwise crawling behavior and mucus production will challenge the collection of the larvae. Settlement - competence has been reported to be achieved 5 – 6 days post-fertilization, but the settlement occurs at different times depending on the types of biofilms available.

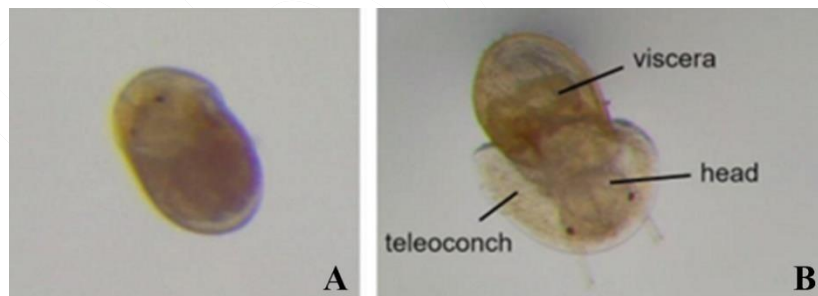


Fig. 8. *Patella candei* post-larval stages. **A.** Metamorphic without velum. **B.** Post-larvae showing head, viscera and teleoconch.

The production of post larvae is done in an acclimatized room (18 ± 1 °C). Oxygen saturation is kept above 90%. Cultures are maintained under artificial light (LED lights, at 11.3 ± 3.0 $\mu\text{mol}/\text{m}^2/\text{sec}$) with a photoperiod of 12 h light: 12 h dark. Settlement is done in 50 l white polyethylene culture tanks, with rectangular shape and flat bottoms (Figure 9A). Corrugated plates covered by algae biofilms are used as substrate (Page 49). This culture design requires a flow-through water system with 125 μm sieves fitted to the water outlets to prevent larval escaping during the settlement phase (Figure 9B).



Fig. 9. Culture system used for limpet settlement and post-larval production. A. Tanks with 23 × 34 cm corrugated fiberglass plates, held vertically and at equidistant distances by the PVC supports. B. Water outlet with a 125 µm mesh sieve.

Materials

- 50 l polyethylene tanks
- PVC plate supports
- 125 µm mesh sieve (tank outlet)
- 23 × 34 cm corrugated plates covered with an algae substrate (settlement trigger)
- plastic beakers (1 - 5 l)
- measuring cylinder
- 10 l bucket
- Bogorov Counting Chamber
- dissecting microscope

Procedure



Settlement inducer

- *Ulveella lens*, CCA, diatoms or a mix between these algae are selected as settlement inducers.

Larval settlement system

- Selected plates are placed vertically inside 50 l tanks in a flow-through system (water exchange 4.8%/h).



Larval density

- Pediveliger larvae are introduced into the settlement tanks at a density of 0.1 larvae/ml.

Water renewal

- The water exchange is gradually increased over the first three days, from 4.8 %/h to 20 %/h.

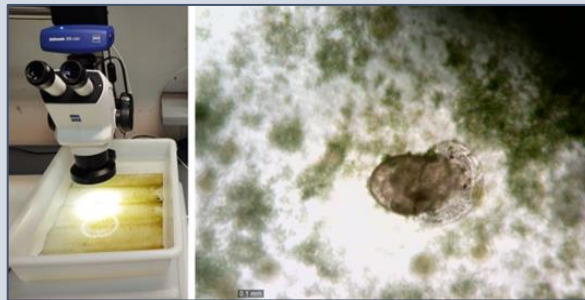
Settlement control

It is recommended to monitor settlement at least two weeks post-fertilization. The plates are observed under a dissecting microscope, preferably with an articulated arm, or with a digital microscope (e.g. Levenhuk DTX TV Digital Microscope). Each plate is placed in a tray filled with FSW to avoid desiccation of the specimens and biofilm during observation. Plates are completely examined to count the total number of post-larvae. Line transects can also be used for settlement estimation, although this method is not precise because the distribution of post-larvae on the surface of the plates is usually highly heterogeneous.

The percentage of settlement is calculated as:

$$\text{Recruitment (\%)} = (N_{\text{post-larvae}}) \times (N_{\text{initial}})^{-1} \times 100$$

where $N_{\text{post-larvae}}$ is the number of post-larvae observed and N_{initial} is the number of pediveliger larvae placed initially in the settlement tanks.



Left: Plate observation under a dissecting microscope with an articulated arm; **Right:** *P. aspera* post-larvae on a plate covered with *U. lens*.

Juvenile grow-out

Supply of adequate diet for post-larvae and juveniles is essential to ensure their survival and growth. The experimental nursery system is designed to allow limpet post-larvae to grow up to 20 mm in shell length in 7–8 months.

Post-larvae and early juveniles are reared in the 50 l tank system described previously (page 33). Later, animals are moved to tanks with higher capacity (130 l), which helps in rearing routines. Feeding regime consists of weekly supply of a mixture of diatoms *N. salinicola* and *Nitzschia* sp. (1:1) at a concentration of 10^4 cells/ml. Plates covered with *U. lens* are supplied whenever the algae is almost grazed out (Figure 10).

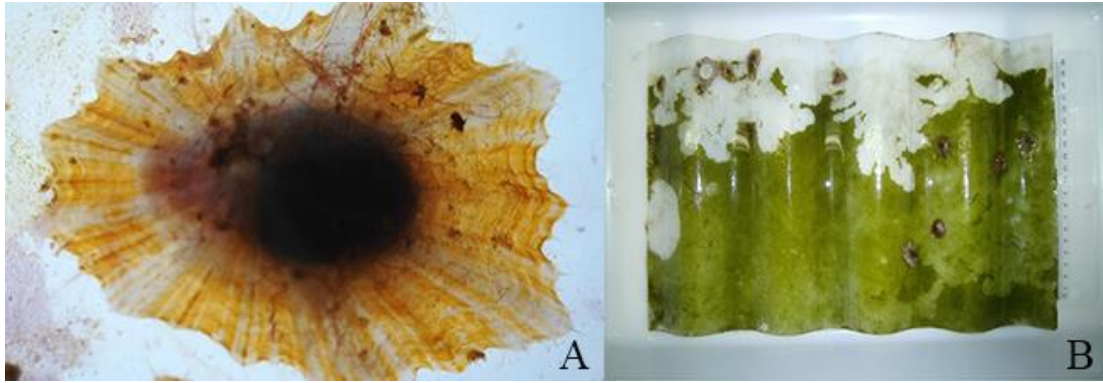
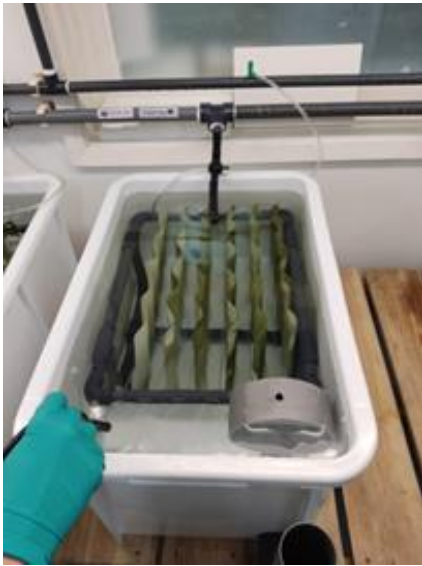


Fig. 10. **A.** *P. aspera* juvenile 181 days old, created from artificial fertilization and reared in an experimental nursery on Aqualab's research facility. **B.** Corrugated plate covered by a biofilm of *Ulvella lens*. Numerous juveniles and grazing marks can be observed.

Materials

- rectangular tanks (50 and 130 l)
- crystal PVC tubes
- PVC plates support
- plastic beakers (1–5 l)
- siphon (7 to 15mm internal diameter)
- sieves (80 μ m)
- bucket (10 l)
- dissecting microscope
- imageJ software

Procedure



Nursery rearing conditions

- Water renewal is fixed at 20 %/h. Temperature is fixed at 18 ± 1 °C, salinity at 35.3 ± 0.1 g/l, dissolved oxygen at 7.3 ± 0.6 mg/l and pH 8.0 ± 0.0 . Photoperiod is kept at 12 h light: 12 h dark, with light intensity of 11.3 ± 3.0 $\mu\text{mol}/\text{m}^2/\text{sec}$, supplied by LED tubes.
- Nursery tanks are cleaned for the first time 1-2 months after settlement, to avoid disturbing the substrates.
- Bottom of the tanks are siphoned weekly, to remove accumulated faeces and other residues. The support with plates can be removed for a few minutes onto a tray without negatively impacting the limpets.

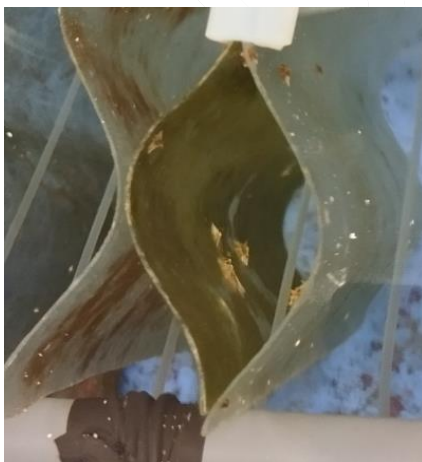


Diatom supply

- The diatoms, *N. salinicola* and *Nitzschia* sp., are placed in 10 l buckets. An immersion blender is used to break down algae agglomerates.
- Each diatom strain is then filtered through an 80 μm sieve, to retain any debris and to further disaggregate the microalgae.

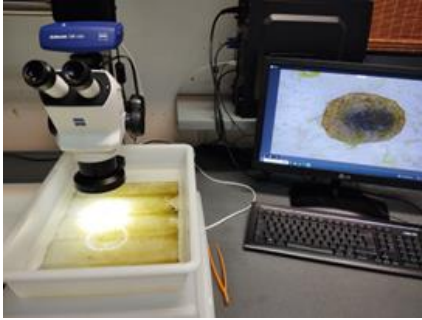


- The required diatom volume is measured and distributed by the limpet nursery tanks with measuring cups. Water flow is stopped for approximately 4 h to allow diatoms to settle on tank surfaces.



Ulvella lens supply

- The timing at which it is necessary to start adding more *U. lens* substrates depends on limpets density and size.
- When algae coverage of a grazed plate drops below 30 - 20%, a plate with fresh feed is added next to it. Plates must be in contact with each other to allow the limpets to move between them.
- Crystal PVC tubes, cut into small pieces, are used as clamps to keep the plates in place.



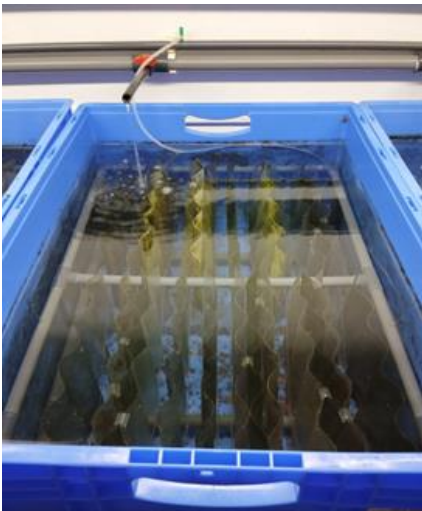
Survival and growth controls

- The system used during early growth and survival control, is similar to what is described above to control settlement (page 33)
- Limpets are counted and photos of a random sample of individuals are taken.



Shell measurements

- Photographs and ImageJ software are used to take shell measurements (length and width).
- Growth is measured with a caliper when animals are larger than 2 mm; clove oil is used as anesthetic for weighing procedures.



Nursery culture scale-up

- Once limpets reach 3 months of age (approx. 3.3 ± 1.4 mm shell length), they can be moved into 130 l rearing tanks, under the same rearing conditions (described for the 50 l tanks), with a water exchange rate of 30 - 40 % /h.
- Empty plates (with no limpets and no food) are removed when detected.

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Sea cucumbers: *Holothuria sanctori* (Delle Chiaje, 1823)

GERCENDE C. VIÇOSE, M. VIERA AND N. SANCHEZ



Courtois de Viçose, G., M. Viera and N. Sanchez 2024. Sea cucumbers: *Holothuria sanctori* (Delle Chiaje, 1823). Pp: 39-42 in: Mirko De Girolamo, Gercende Courtois de Viçose, Carlos Andrade & Eduardo Isidro (Eds), 2024. Production of marine invertebrates at early stages - Manual for best practices. *Arquipelago*. Life and Marine Sciences. Supplement 14: 62pp.

Holothuria spp., commonly known as sea cucumbers, are important species for marine coastal ecosystems functioning and also have a high market value, requiring a sustainable harvest. The demand for these echinoderms has led to the development of applied research for their cultivation, but so far, the studies on the production of *Holothuria sanctori* (Figure 1) are scarce.

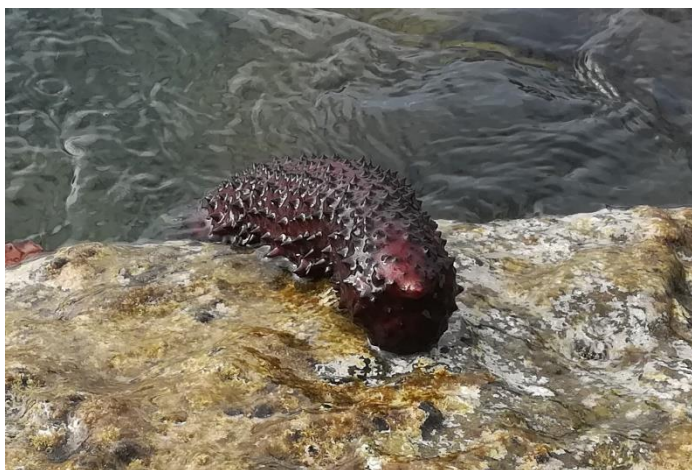


Fig. 1. *Holothuria sanctori* (specimen from Azores)

Holothuria sanctori occurs in the Mediterranean Sea and in the eastern Atlantic Ocean from the Bay of Biscay and Portugal to Azores, Madeira, Canary Islands. These sea cucumbers can be found from intertidal pools to 70 m depth and are associated with rocky substrates. During the day the animals remain hidden in cracks and holes in the rocks and during the night they come out of these shelters to feed (Navarro 2012). Holothurians are detritivores feeding on organic matter contained in sediments. They have separate sexes and no sexual dimorphism was detected (Cherbonnier 1952, Yingst 1976; Moriarty 1982; Amon and Herndl 1991).

***Holothuria sanctori* early stages production protocol**

The protocols for this species described below are based on experimental scale trials.

Spawning induction protocol

Holothuria sanctori has a single annual breeding cycle, with a breeding peak activity in the summer (Navarro 2012). Reproductive patterns are generally related to water temperature (Smiley *et al.* 1991), although they can also be influenced by other factors such as photoperiod, salinity and availability of food (Krishnaswamy and Krishnan 1967; Himmelman 1980; Hamel *et al.* 1993; Morgan 2000). However, in culture farms, several methods are used to induce reproduction such as: mechanical shock (keeping animals dry for 90 min and then subjecting them to a strong current of water for a few minutes); thermal shock (raising the water temperature in the tanks by 6°C using heaters and maintaining it for 90 min); and algal

stimulation (adding microalgae at a concentration of 0.1 g/l for 90 min). Those methods can be used separately or combined to ensure spawning induction (Battaglione *et al* 2002; Renbo and Yuan 2004; Rakaj *et al.* 2018).

The spawning induction and fertilization protocol using mechanical shock and algal stimulation is described below (Magdy *et al.* 2021).

Materials

- 180 l tanks
- Spirulina sp. strain
- 500 µm mesh sieves
- 40 µm mesh sieves
- 10 l buckets with lids
- Dissecting microscope
- Bogorov egg-counting chamber
- Manual counter
- 10 ml pipette
- 250 ml measuring cup

Procedure



Broodstock selection

- Animals weighing more than 150 g are selected.
- Sex is not visually distinguishable, so they are randomly distributed in the tanks at a density of 7 individuals/m². The animals are cleaned with seawater before being placed in the tanks to remove debris and sediment that may have adhered to their skin.



Induction by mechanical shock

- Animals are left to dry for 90 min in tanks and then subjected to a strong stream of water for a few minutes.
- Subsequently, the tanks are refilled with seawater.



Induction by algal stimulation

- Once the tanks are full, *Spirulina* spp. is added at a concentration of 0.1 g/l; for 1 hour the water is not renewed. After 1h water is renewed until the algae are eliminated.



Gamete ejection

- Gametes are expelled from a single gonopore located on the upper dorsal anterior surface of the body. The sperm remain in the water column while the oocytes, which are orange in colour, are deposited on the bottom.



Gamete harvesting and fertilization

- Eggs are siphoned from the bottom of the spawning tank into a 10l bucket; the sperm is collected using a beaker and is added to the bucket. The mix stand for 1 h for fertilization.
- The eggs are transferred to a 40 µm sieve and rinsed with seawater (1 µm filtered and UV sterilized) to remove the excess of sperm. They are then transferred to a bucket with a known volume.



Egg counting and transfer

- Samples of 4 ml are taken with a pipette and placed in a Bogorov counting chamber. The total number of eggs and the number of fertilized eggs in the sample are counted and the fertilization rate of the bucket is found:

$$\text{Fertilization rate (\%)} = \frac{\text{Total fertilized eggs}}{\text{Total eggs}} * 100$$

Once the fertilization rate has been calculated, the eggs are transferred to the tanks at a density of 0.3 eggs/ml.

Larval production

Most sea cucumber species have five planktonic larval stages: early auricularia, middle auricularia, late auricularia, doliolaria and pentactula. Duration of each stage depends on the species and of external factors such as feeding, population density, etc. (Ramofafia *et al.* 1995; Domínguez-Godino *et al.* 2015; Rakaj *et al.* 2018).

Holothurians present a larval phase with different stages (Figure 2) that, depending on the species, can go through a larval stage called "auricularia" during which they feed, or through a stage called "doliolaria", in which they do not feed, followed by the "pentaculata" stage, which is the last stage before their attachment and subsequent metamorphosis and development as juveniles (Raff & Byrne 2006; Pawson *et al.* 2010).

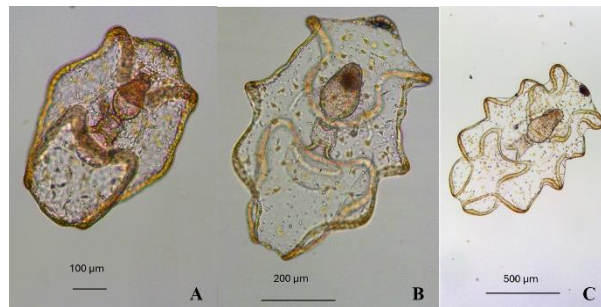


Fig. 2. Larval stages of *H. sanctori*. **A.** Early auricularia. **B.** Middle auricularia. **C.** Late auricularia.

Materials and larval tanks

- 60 µm mesh sieves for drainage
- Porous hose for air supply
- Water filtered to 1 µm and sterilized with a UV lamp
- 180 l tanks

In the *H. sanctori* trials, fertilized eggs are kept in 180 l larval tanks (Fig. 3) until they reach the late auricularia stage. During this period, larvae are maintained in flow through, 1 µm filtered and UV sterilized seawater. The flow rate of the tanks is maintained at a water turnover rate of approximately 2 vol/day. Tanks are subjected to natural light conditions. The early auricularia stage was observed 78 h after fertilization. The middle and late auricularia stages were observed at 150 h and 342 h after fertilization, respectively. When reaching the auricularia stage larvae were fed with two microalgae

(*Nannochloropsis sp.* and *Amphora sp.*), at a density of 5000 cells/ml in equal proportions. The animals were fed 3 times/day with algae concentrations gradually increasing.



Fig. 3 Larval tanks.

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Sea urchins: *Paracentrotus lividus* (Lamarck, 1816) and *Sphaerechinus granularis* (Lamarck, 1816)

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Luís R., R. José, D. Castejón and C. Andrade 2024. Sea urchins: *Paracentrotus lividus* and *Sphaerechinus granularis*. Pp: 43-48 in: Mirko De Girolamo, Gercende Courtois de Viçose, Carlos Andrade & Eduardo Isidro (Eds), 2024. Production of marine invertebrates at early stages - Manual for best practices. *Arquipelago*. Life and Marine Sciences. Supplement 14: 62pp.

Paracentrotus lividus and *Sphaerechinus granularis* (Figure 1) are two major echinoderm species exploited in Europe for their gonads (designated as roe or uni) (Boudouresque & Verlaque 2007; Brundo *et al.* 2016). Both species are intertidal and *S. granularis* can be found down to 130 m depth (Boudouresque & Verlaque 2007; Saldanha & Sacarrão 2003).

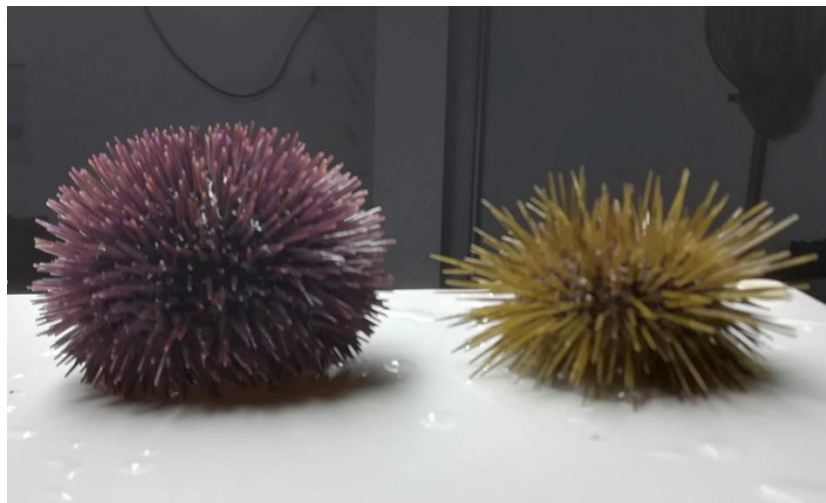


Fig. 1. *Paracentrotus lividus* and *Sphaerechinus granularis* left and right respectively (specimens from Madeira).

Their life cycles are divided in two stages, planktonic larvae and benthic adults. During the planktonic stage the swimming echinopluteus larvae feed on phytoplankton. In the benthic stage sea urchins feed mostly on macroalgae. Sea urchins have separate sexes, although some cases of hermaphroditism have been observed; no sexual dimorphism was detected (Boudouresque & Verlaque 2007; Brundo *et al.* 2016).

Production of sea urchins

Larval production

Sea urchin larval development is completed in 30 days at 19 – 20 °C and presents 5 distinct stages (Figure 2). The larvae are reared in cylindrical-conical incubators under a fixed amount of food (adjusted to each larval stage) and seawater exchange (Kelly *et al.* 2000; Brundo *et al.* 2016, González-Suárez and Hernández 2022). (Figure 3).



Fig. 2. *Sphaerechinus granularis* larval development. **A.** Two-arm pluteus (2 b). **B.** Four-arm pluteus (4 b). **C.** Six-arm pluteus (6 b); **D.** Eight-arm pluteus (8 b). **E.** Competent larvae/pre-metamorphic.

Materials and tank preparation

- 50 µm mesh sieves for drainage
- Air supply: soft and centered column of 3 to 4 bubbles per second
- Seawater filtered to 1 - 20 µm and sterilized with a UV lamp
- Cylindrical-conical tanks
- Temperature control (1)

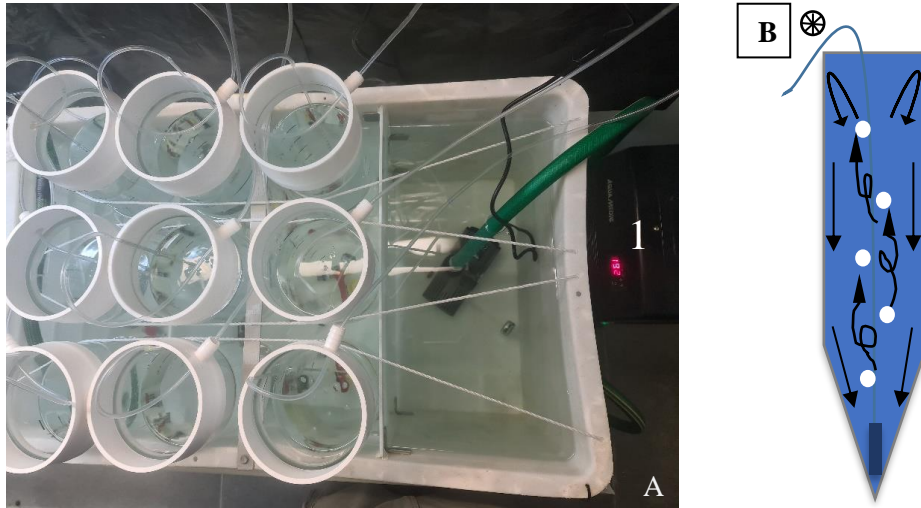


Fig. 3 **A.** *Sphaerechinus granularis* rearing system. **B.** Section of cylindrical-conical incubators.

Spawning protocol

Urchins are kept separated by sex (gamete identification) to facilitate individual selection for spawning ratio and genetic diversity of larval batches.

Sea urchins have one or two natural spawning peaks, but mature individuals are available all year round (Boudouresque & Verlaque 2007). In captivity, reproduction is commonly induced by potassium chloride (KCl) injection. This method can be advantageous by its effectiveness, but it produces a very high broodstock mortality (90 to 100% of induced individuals). As an alternative, Gago & Luís (2011) proposed the agitation method. This method consists of handshaking semi-vigorously the adult urchins for no more than 60 seconds. This method proved to have results as fast as KCl method, but with no mortality associated. Here, only the agitation method is described.

Materials

- 20 µm filtered and autoclaved seawater (enough volume for urchin and egg containers)
- Containers to place individually and fully submerged induced urchins
- 500 ml autoclaved glass beakers for gamete pooling
- Seggewick Rafter counting chamber
- Malassez counting chamber
- microscope
- manual counter and pipettes
- pipettes
- 6 l laboratory glass balloons autoclaved

Procedure



Broodstock selection

- Adult individuals are selected with a 3 males: 2 females ratio
- *P. lividus* test size > 35 mm
- *S. granularis* test size > 50 mm



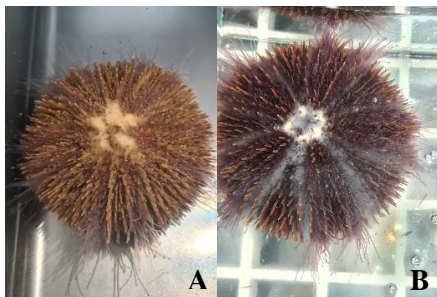
Broodstock preparation

- Adults should be carefully cleaned for debris/algae, washed with filtered and sterilized seawater and placed totally submerged in individual containers.



Spawn induction

- Adult individuals should be agitated with sharp wrist circular movements in all vectors for no longer than 60 seconds. Agitation should be vigorous but not too strong to avoid damage of internal organs.



- Successfully induced females (**A**) will release orange type color eggs; males (**B**) will release white sperm.



Fertilization/egg incubation

- Eggs and sperm are mixed in glass balloons at a ratio of 500 spermatozoa : 1 oocyte.
- Fertilization rate should be estimated 2 h after the fertilization procedure.
- Aeration should be medium to strong and close to balloons bottom glass to avoid egg sedimentation. *P. lividus* incubation takes about 48 h (until prism/early 4 arm stage) and *S. granularis* incubation takes 72 h (until reaching 2 arm stage).

LARVAL PRODUCTION

Once reaching an adequate stage (4-arms for *P. lividus* and 2-arms for *S. granularis*), larvae are transferred to cylindrical-conical tanks with a stocking density of 2 to 5 larvae/ml (Figure 4). Water quality parameters (O₂, pH, salinity) should be monitored and water changes (10% volume, 1-20 µm filtered and UV sterilized) should be done three times a week. Ammonia concentration is monitored on intermediate days (two times a week). Larvae are fed after maintenance of the cylindrical-conical tanks.

Microalgae density should be adjusted to each larval stage: 500 cells/ml for stages 2 and 4 arm pluteus; 1500 cells/ml for 6 arm stage; and 3000 cells/ml for 8 arm stage. Larval development will take approximately 30 days (Luís *et al.* 2023).



Fig. 4. Experimental system with cylindrical-conical vessels used for sea urchin larval rearing.

Materials

- 20 µm filtered and autoclaved seawater
- 500 ml autoclaved glass beakers
- Seggewick Rafter counting chamber
- Microscope
- Manual counter
- Pipettes

Larval transfer to post-larval production (settlement)

About 2 days after rudiment length exceeds the stomach length, the larvae become competent for metamorphosis and settlement. Eight-armed plutei with fully developed rudiments are transferred to the post-larval rearing tanks (Figure 5). These are larger tanks containing settlement plates packed into holders, which are used for both settlement and post-larval rearing. The settlement plates consist of corrugated PVC plates with surfaces preconditioned with diatoms biofilm. To avoid loss of larvae, there is no water exchange until most of the larvae have completed settlement (Brown & Eddy 2015), i.e., three to eight days after the larvae have been transferred.



Fig. 5. Post larval tanks.

Materials

- 50 µm mesh sieves for drainage
- 20 µm filtered and autoclaved seawater
- 500 ml autoclaved glass beakers
- Seggewick Rafter counting chamber
- Microscope
- Manual counter
- Pipettes

Settled juveniles are reared on corrugated plates until they are 5-10 mm test diameter (Figure 6), at temperatures between 16 and 23 °C. Water exchange rate is around seven tank turnovers a day. Aeration should be provided to keep the rearing water mixed and oxygenated. Green algae and/or naturally occurring attached diatoms are cultivated on the corrugated plates and used to induce larval settlement, as well as food for the post-larval stages. The temperature range for *Ulvella lens* propagation should be between 15 to 20 °C. Propagation takes about one month to reach a suitable density to be used as food for the juveniles (Brown & Eddy 2015).



Fig. 6. Juvenile sea urchins growing on a corrugated plate.

Juvenile grow-out production

After reaching 5 to 10 mm, juveniles are collected from the corrugated plates and sorted in various size classes, if necessary. They can be reared in tanks or sea-based cages (intermediate culture - Figure 7). They are usually fed with *Saccharina* spp., but dry grains of maize (*Zea mays*) or specially formulated rations can also be used (Brown & Eddy 2015; Gago & Luís 2011).

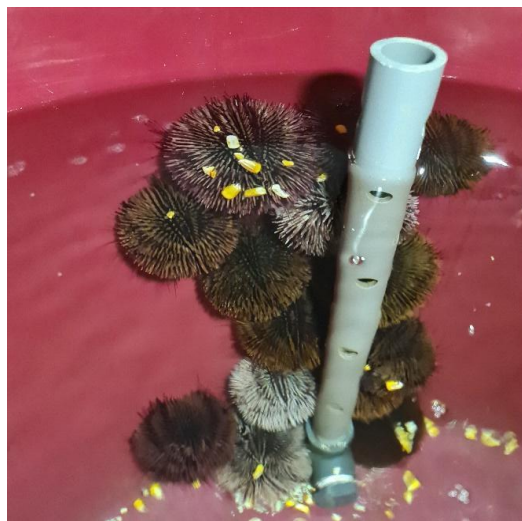


Fig. 7. Juveniles fed with maize.

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DRAFT

Food production for post-larval and juveniles' stages of marine invertebrates

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Courtois de Viçose, G., M.P. Viera, N. Marrero, A. Patkar, M. De Girolamo, C. Nunes and E. Isidro 2024. Food production for post-larval and juveniles' stages of marine invertebrates. Pp: 49-58 in: Mirko De Girolamo, Gercende Courtois de Viçose, Carlos Andrade and Eduardo Isidro (Eds), 2024. Production of marine invertebrates at early stages - Manual for best practices. *Arquipelago*. Life and Marine Sciences. Supplement 14: 62pp.

Cultivation of micro and macroalgae holds a pivotal role in marine organism farming overall. The combination of various species of benthic algae serves as an inducer for planktonic larvae settlement and as a vital food source for newly metamorphosed larvae, juveniles, and adults.

Different benthic diatoms, such as those belonging to the genera *Amphora*, *Nitzschia* and *Navicula*, can positively influence the growth of abalone, limpets, and sea urchins, yet their three-dimensional structure may also act as a deterrent for larval settlement. Other green algae like *Ulvella lens* and red crustose coralline algae (CCA) serve as excellent inducers for larval settlement. Particularly, the encrusting green algae *U. lens* finds extensive use in many abalone and sea urchin hatcheries, demonstrating equal efficiency in limpet recruitment and growth.

In the following paragraphs we present the techniques we have used and developed to produce diatoms in bags, *Ulvella lens* and *Ulva lactuca* on specific substrates and finally, to produce *Ulva rigida* and *Hydropuntia cornea* in tanks.

Diatom production protocol

It is important to use diatoms that meet the nutritional requirements of post-larvae and juveniles of the invertebrate species to be fed. A mixture of different species ensures that these requirements are met to improve growth.

Most used diatoms for *Haliotis tuberculata coccinea* culture are *Navicula incerta*, *Amphora sp.* and *Cylindrotheca fusiformis*, whose biochemical composition meets the nutritional requirements of this species (Courtois de Viçose *et al.* 2012a, 2012c).

Preparation of F/2 culture medium

Diatoms need different nutrients to grow: nitrate (NaNO_3), phosphate (NaH_2PO_4), silicates (Na_2SiO_3), trace metals (FeCl_3 , Na_2EDTA , CuSO_4 , ZnSO_4 , CoCl_2 , MnCl_2 and Na_2MoO_4) and vitamins (Biotin, B12 and Thiamine).

The culture medium F/2 contains these nutrients and shall be used for small volumes production of diatoms in test tubes and 250 ml bottles and flasks. The solutions of each nutrient are prepared separately, and 1 ml of each nutrient is then added to each litre of salt water in the diatom culture.

Materials

- Distilled water (5 l)
- 1 l bottles (4)
- 100 ml bottle (1)
- Analytical scale
- Spoon
- Macronutrients (NaNO_3 and NaH_2PO_4)
- Metasilicate (Na_2SiO_3)
- Trace metals (FeCl_3 , Na_2EDTA , CuSO_4 , ZnSO_4 , CoCl_2 , MnCl_2 and Na_2MoO_4)
- Vitamins (Biotin, B12 and Thiamine)

Preparation of F/2 culture medium

Procedure for macronutrient preparation:

- Weigh 75 g of Nitrate (NaNO_3) and 5 g of Phosphate $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$.
- Dissolve in 1 l of distilled water in 1 l bottle.

Procedure for preparation of silicates:

- Weigh 30 g of sodium metasilicate (NaNO_3).
- Dissolve in 1 l of distilled water in 1 l bottle.

Procedure for the preparation of trace metals:

This procedure is done in three steps:

- First step:
 - Weigh 3.15 g of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 4.36 g of Na_2EDTA .
 - Dissolve in 900 ml of distilled water in 1-litre bottle.
- Second step: the following metals are weighed and dissolved in 100 ml of distilled water in a 100 ml bottle:
 - 0,10 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$
 - 0,22 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$
 - 0,10 g of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$
 - 1,8 g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$
 - 0,06 g of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$
- Third step: 1 ml of the solution prepared in the second step is added to the solution of the first step. Then the final volume of the first solution is adjusted to 1 litre with distilled water.

Procedure for the preparation of vitamins:

- Weigh 0,5 mg of Biotin, 0,5 mg of B12 and 100 mg of Thiamine HCl.
- Dissolve in 1 l of distilled water in 1 l bottle.

Scale-up production of diatoms

Production of diatoms is carried out in a stepwise manner starting from test tubes where the strains are kept. The test tubes will then be transferred to 250 ml bottles and these will be transferred to 4-liter flasks, then up to 20-liter bags (Figure 1).

Diatoms shall be inoculated at a concentration of $1 \cdot 10^5$ cells/ml, a density at which high growth rates are achieved (Courtois de Viçose *et al.* 2012a).

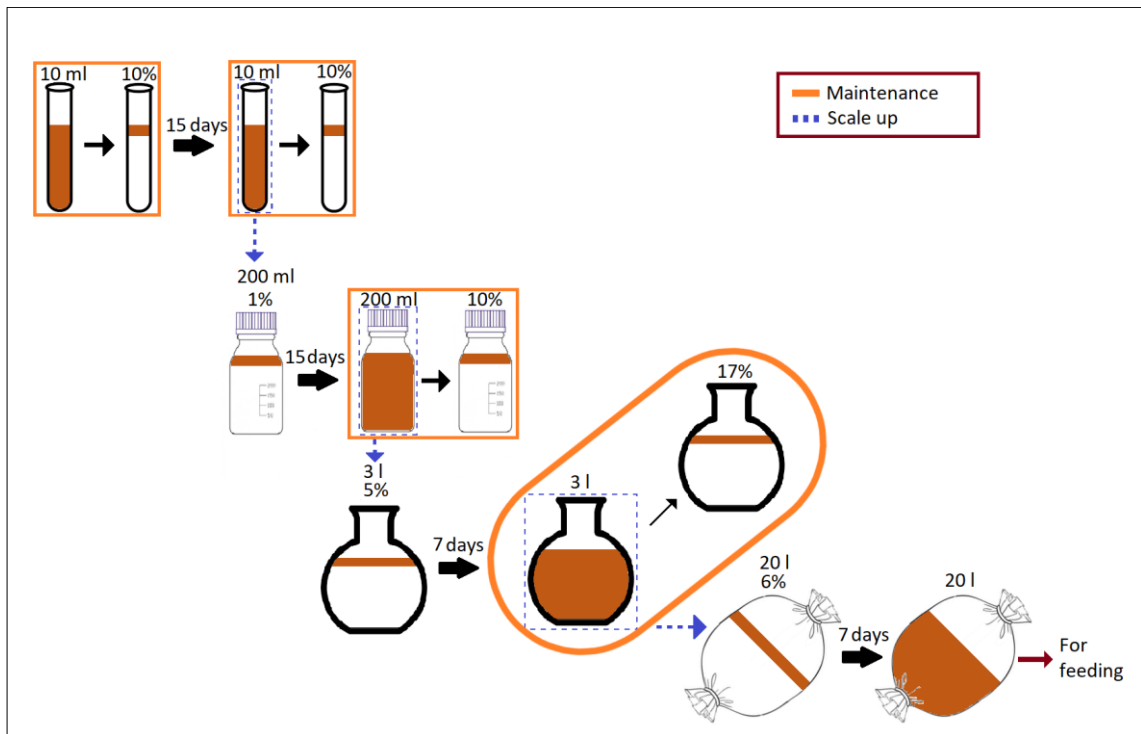


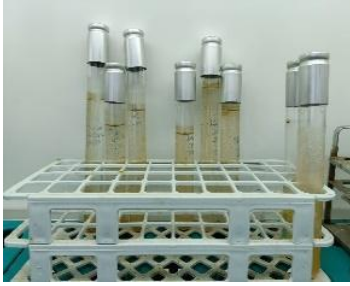
Fig. 1. Diatom cultivation scheme: maintenance phase and scale-up phase.

Materials

- Diatom strains
- F/2 microalgae culture medium
- Bunsen burner
- Test tube
- Metal rack
- 250 ml bottles
- 4 l flat bottom flask
- 1200 m long, 50 cm wide continuous tubular polyethylene bag
- Cable tie
- 50 ml measuring cylinders
- 5 ml pipettes
- 50 l white tray

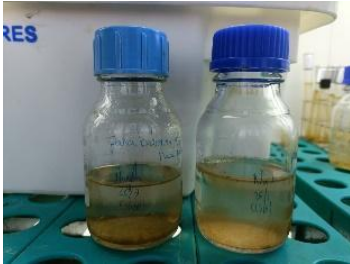
Procedure

- All materials are autoclaved at 121°C for 30 minutes.
- Add 1 ml of each solution of F/2 medium (macronutrients, silicates, metals and vitamins) per liter of salt water to the diatom culture.
- Diatoms are inoculated at 100,000 cells/ml in each step.



Inoculation of test tubes

- Six replicates of each strain are maintained in 10 ml of sterile, 1 μm filtered water in two separate racks.
- At each inoculation, cells are transferred from one test tube to another and scaled up into a 250 ml bottle. The tubes are inoculated every two weeks.



Inoculation of 250 ml bottles

- The 250 ml bottles are inoculated from the test tubes (as detailed in the scheme) and 1 μm filtered and sterilized seawater and nutrients are added to reach 250 ml.
- The bottles are inoculated every two weeks.



Inoculation of the 4-liter flasks

- Flasks are autoclaved and sealed with cotton wool and silver foil.
- The first flask inoculation is performed using diatoms from the 250 ml bottle. Subsequent inoculations use diatoms from the flasks. Re-inoculation from the 250 ml bottles only takes place in case of contamination of the flasks.
- 1 μm filtered and sterilized sea water with F/2 medium, are added to the flasks to reach 3 liters.



Inoculation of 20 l bags

- Bags are used for the final step of diatom production scaling; they are obtained from 1200 m long, 50 cm wide continuous tubular polyethylene sleeve, which is cut into 140 cm fragments.



- The polyethylene bags are closed at the ends with a band and placed on a tray.
- They are filled with 20 l of 1 μm filtered and UV sterilized seawater and inoculated with 1250 ml of diatoms from the 4-liter flasks. Each flask allows the inoculation of two bags.
- F/2 medium is added to the 20 l bags in the same proportions as previously described.
- The bag is closed with another band at the opposite end and is shaken to allow a homogeneous repartition of the nutrients and diatoms.

Cell counts of diatoms

To estimate the density of diatoms to inoculate them at the required concentration, request the use of a microscope, pipette, eppendorf, Neubauer chamber and a sonicator.

To prior the counting, cells must first be disaggregated by taking a sample in an eppendorf and placing it in a sonicator (Figure 2) for 15 s, at a power of 100 W.



Fig. 2. Sonicator.

A sample is then taken with a pipette and placed in the Neubauer chamber to perform the count. The counting method is shown in the figure below (Figure 3).

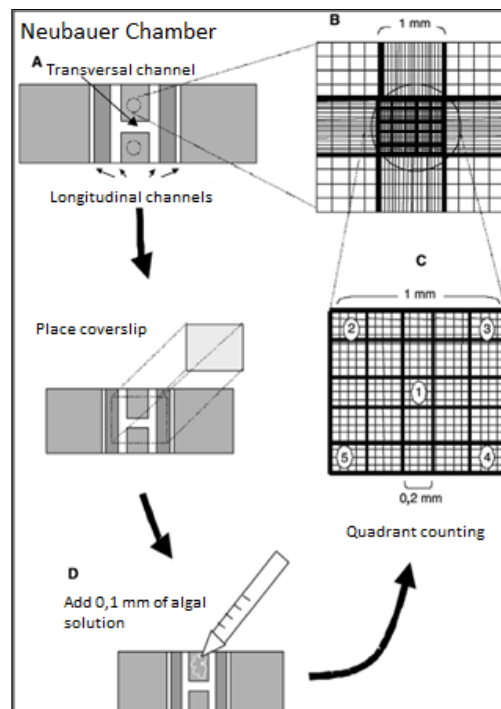


Fig. 3. Counting method in the Neubauer chamber.

Protocol for the substrate preparation of *Ulveella lens*

This protocol is adapted from a study of Hannon *et al.* (2014). Macroalgae cultivation is carried out in 130 l, rectangular polypropylene tanks with a surface area of 0.42 m². Water inlet and outlet as well as the aeration system are independent for each tank. The tanks are maintained under a photoperiod of 12 h L: 12 h D (Figure 1). The water used for each tank is filtered (1 µm) and UV sterilized before starting the induction experiment.

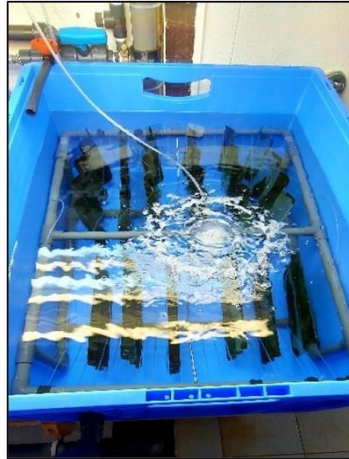


Fig. 1 Inoculant plates of *Ulveella lens*.

Corrugated fiberglass plates of 23 × 34 cm covered with *Ulveella lens*, called inoculant plates, are maintained in a tank of 130 l, with a light intensity of 3.5 Klux and a water temperature of 18 - 20° C. Usually, the water system is opened every two weeks, for two consecutive days, and then closed again. During this period water flow is maintained at 3 l/hr. Nutrients (1 ml/l) and the germanium oxide (2 ml/l) are added when the water system is closed.

Materials

- *U. lens* inoculant plates
- 130 l tanks (80 x 60 x 42 cm)
- Corrugated fiberglass plates (23 × 34 cm)
- Aeration tube with an air stone
- Electric aquarium heater with temperature regulator system
- PVC rack to keep plates in a standing position
- Nutribloom plus
- Germanium oxide
- Bleach (NaOCl) (4 %)
- Sodium thiosulfate (Na₂S₂O₃)

What is Nutribloom plus?

Nutribloom plus is a ready-to-use microalgae culture medium, rich in essential vitamins, manufactured by Necton S.A. Portugal.

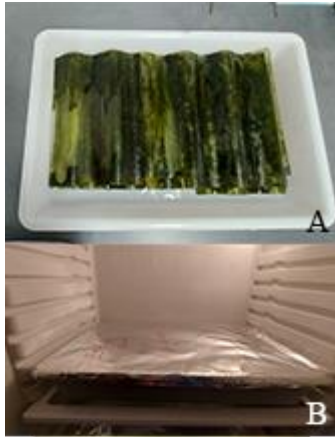
Composition (%): N (2.800), P (0.310), Fe (0.112), Zn (0.014), Mo (0.010), Mn (0.006), Mg (0.005), Co (0.001), Cu (0.001), Thiamine (0.0035), Biotin (0.0005), B12 (0.0003)

Preparation of Germanium oxide

Dissolve 250 mg of germanium oxide powder into one litre of distilled water with the help of a magnetic stirrer. Keep the solution in a fridge for further use.



Procedure



· A tray with autoclaved seawater and *Ulvella. lens* inoculant plates is covered with silver foil and placed in the fridge at 10 °C, in the dark, for 10 days (A, B).

After 10 days, plates are transferred to a 130 l tank. The culture tank, new cleaned plates to be colonized and seawater were previously sterilized with 130 ml of 4% bleach solution for 24 h. The bleach is neutralized with 130 ml of sodium thiosulfate, Na₂S₂O₃, before the tank is used.



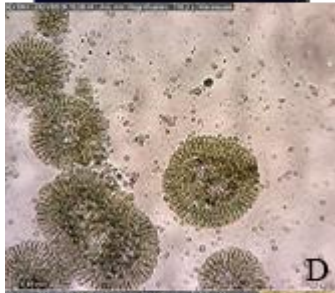
· Inoculant plates are then placed into the induction tank (water from the tray is also added). Culture is kept under continuous light (3.5 Klx) with water temperature at 20 °C. 2ml/l Germanium oxide (GeO₂) and 1 ml/l NutriBloom plus are added.

· The water system is kept closed with continuous photoperiod and slow aeration (C)

· From this step forward, the plates are inverted once every 3 days.

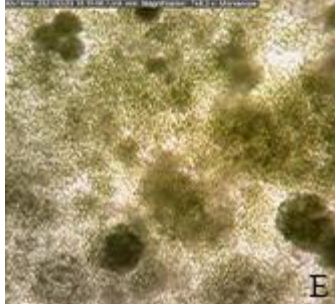
· After 7 more days under sporulation induction conditions, the photoperiod is changed to 12 h L: 12 h D light and seawater is kept at room temperature. Inoculant plates are removed at this stage.

· The water system is opened for a few hours (approx. 7-8 h), with a water flow of 3 l/hr. Nutrients and GeO₂ are added with previously mentioned concentrations after the water system is closed.



· After this, on a weekly basis, the water system is opened for a few hours with a water flow rate of 3 l/hr. Nutrients and GeO₂ are added after the water system is closed.

· Usually, very small green spots of the *Ulvella lens* start to appear at the end of the third week of induction (D).



· After 4–5-week plates appear greenish and covered with *Ulvella lens* (E).

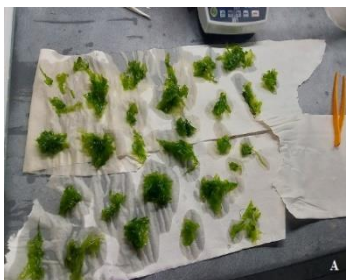
Protocol for the substrate preparation of *Ulva lactuca*

The algae used in this study are collected in the intertidal zone during low tide, with the help of a knife, scissors or by hand. The collected *U. lactuca* thalli are placed in a thermal box and brought to the laboratory, where they are thoroughly washed in freshwater, 2-3 times, to remove dirt and invertebrates like amphipods or isopods to avoid possible grazing effects. The algae are then kept in a tank (650 l) with vigorous aeration and continuous seawater supply (De Girolamo *et al.* 2019).

Materials

- Freshly caught *U. lactuca*
- 130 l tanks (80 x 60 x 42 cm)
- Corrugated fiberglass plates (23 × 34 cm)
- Aeration tube with air stone
- Electric aquarium heater with temperature regulator system
- PVC/plastic rack to keep plates in a standing position
- Nutribloom plus
- Germanium oxide
- Bleach (NaOCl) (4 %)
- Sodium thiosulfate (Na₂S₂O₃)

Procedure



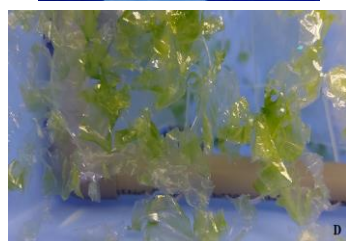
· *Ulva lactuca* is cleaned, and the basal part is removed. Then completely dried with a paper towel, made completely dry (**A**), and weighed for the induction procedure (approx. 65g).



· The weighted *U. lactuca* thalli are cut into small pieces (**B**) and kept in a fridge for 24 hours at 4°C, in the dark.



· Meanwhile a 130L tank is prepared, with newly cleaned corrugated fiberglass plates and seawater, previously sterilized with 130 ml of 4 % bleach solution and then neutralized with 130 ml of sodium thiosulfate, Na₂S₂O₃. The *U. lactuca* thalli kept in the fridge is then transferred randomly over the water surface of the tank (**C**). Water temperature is set to 25 °C with continuous light at 4 Klx (Day 0).



· After two days (Day 3) the air system is opened.
 · On Day 4, plates are rotated, and the tank is covered to produce a complete dark condition for 24 h. Usually, sporulation (part or all thalli is whitish) is visible at this point (**D**).



· On day 5, the tank cover is removed, and continuous light condition is maintained for the next two days. 2 ml/l of germanium oxide (GeO₂) and 1 ml/l of NutriBloom plus are added.

· A new tank is prepared as mentioned in step 3, and plates from the induction tank are transferred to. Water temperature is maintained at 20 °C. Culture is now kept under 12 h L: 12 h D photoperiod with 4 Klx light intensity.

· After this step, on a weekly basis, the water system is opened for a few hours with a water flow of 3 l/h. Nutrients and GeO₂ are added after the water system is closed. The plates are rotated every three days to get uniform growth coverage on the plate. In the third week, the water heater is removed, and water is maintained at room temperature. Light intensity is maintained the same as before.

· The growth of *U. lactuca* after two weeks of induction (**E**).

MACROALGAE PRODUCTION

From late juvenile stages and during growth, animals are fed with a mixed diet of green algae (*Ulva rigida*) and red algae (*Hydropuntia cornea*), whose nutritional characteristics complement each other and fit the abalone nutritional requirements. (Viera *et al.* 2011).

Macroalgae cultivation is performed in 2.5 m³ elliptical fiberglass tanks with a surface area of 2.3 m². Water inlet and outlet as well as aeration system are independent for each tank. The flow rate in each tank is maintained at around 1250 l/h, which is equivalent to a water renewal rate of approximately 12 vol/day. Tanks are maintained under natural photoperiod receiving 84 $\mu\text{M photon m}^2 \text{s}^{-1}$ on average during daylight hours on cloudy days and 1950 $\mu\text{M photon m}^2 \text{s}^{-1}$ on sunny days. The red algae tanks are kept partially covered to limit the irradiation reaching the red algae.

Materials

- *Ulva rigida* and *Hydropuntia cornea* macroalgae
- Analytical scale
- 2500 l tanks
- 50 l baskets
- 50 l harvesting baskets
- 200 l baskets
- 80 l vegetables wringer

Procedure



Inoculation of algae

- *U. rigida* is inoculated at 1 or 2 g/l while *Hydropuntia cornea* is inoculated at 6-8 g/l.
- The seaweeds are harvested fortnightly, alternating the harvest weeks for each seaweed.



Seaweed harvesting

- The tanks are entirely harvested using baskets and automated harvesting systems, emptied, cleaned with 25 % bleach and rinsed to start a new production cycle.



Seaweed draining

- To obtain consistent production data of the harvested biomass throughout the year, the excess water is removed by means of a manual wringer.



Weighing of seaweed

· Harvested algae are weighed for estimation of culture performance: Production (g PS m²/d) and growth (%/d). The amount needed to inoculate each tank is separated, while the rest of the production is used to feed the abalone.

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