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Deliverable 5.3.3
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INTRODUCTION

Within the Interreg Italy-Croatia ARGOS, the Research Center for Marine Ecosystems and Fisheries of IZSAM based in Termoli, as part of the pilot project **"Transfer of knowledge on controlled reproduction and breeding of flat oysters (*Ostrea edulis*) and sea urchins (*Paracentrotus lividus*) for the diversification of shellfish production through integrated aquaculture techniques''**, carried out in the period January 2021 - June 2023, experimental activities related to the adaptation, growth and maturation of the specimens of *Ostrea edulis* and *Paracentrotus lividus* under controlled conditions, described below. Developing an excellent cycle of algal production in succession has allowed the development of diets suitable for the maintenance and larval development of the organisms mentioned above. Good results have been obtained regarding the larval development of the sea urchin *Paracentrotus lividus* of which the phases of metamorphosis and growth are being analysed. It was possible to follow all the developmental stages of the oysters, from the housing of the adults to the emission of the gametes and the final metamorphosis into spat. In addition, tests for the transfer of adult organisms to longlines have been planned to assess the suitability of the selected area for the diversification of shellfish production.

General overview

The new challenges of aquaculture are directed towards the application of "innovative" experimental protocols, which favour a production system capable of reconciling the interests of consumers, the sustainability of production, the environmental impact and the reduction of fishing pressure on natural stocks. The diversification of production towards valuable but currently underexploited species and the use of integrated systems, which allow the breeding of several species at different trophic levels, can provide a valid answer to these needs. The pilot action is focused on the development and promotion of innovative technologies in aquaculture, which facilitate the diversification of production towards species that are still under-exploited and of high commercial value. In Italy, the practice of oyster farming is marginal, and generally associated with other main productions such as mussel farming (*Mytilus galloprovincialis*) and venericoltura (*Tapes philippinarum* and *Tapes decussatus*). The flat oyster (*O. edulis*) is a promising candidate for the diversification of Italian shellfish production. Oyster farming does not foresee structural changes to mussel cultivation plants, rather it requires a lower floating force (buoys) for the least weight to be supported, reducing the wear of long-line facilities. Another species able to combine the expectations of the company and the market with the need for sustainable management of resources is the sea urchin (*P. lividus*).

Despite the high commercial value, also this species is, however, still poorly bred, with considerable repercussions on the consistency of the natural populations. For this species, the feasibility of breeding protocols involving the use of structures that are not very complex, requiring management procedures that are equally simple and not excessively expensive has been demonstrated, allowing therefore to place echinoculture alongside other aqueous activities. One of the main problems of oyster farming is represented by fouling, that is, the plant and animal species that settle on the surface of the structures used weighing them considerably. Sea urchins with their grazing action would favour fouling control by using it as food. This would also reduce the costs of maintaining the sea urchins themselves, which are still poorly bred, because of their slow growth rate. Finally, oyster filtration would reduce the impact of echinoculture, which is not only efficient but also environmentally sustainable.

OSTRICA PIATTA *(Ostrea edulis)*

The flat oyster or European oyster (*O. edulis*), is a bivalve mollusc native to the Mediterranean with dimensions ranging from 7 to 12 cm (max 20cm) in diameter. It is characterized by an unequal shell of rounded shape, a flat profile and a peeled surface. The right valve is flat and peeled while the left one (with which the mollusc adheres to rocks and other substrates) is concave. The hinge between the two valves is toothless. The surface of the shell very often has encrusting sediments, as well as hosting a very diverse community of epibiont organisms such as hydrozoans, bryozoans and serpulids; the inside of the shell is white and characterized by a single impression of the adductor muscle while the outside is grey-dark black. These bivalves, lacking foot and byssus, fix their shell to the submerged bodies by means of cementing substances emitted by the mantle. *Ostrea edulis* is a species amply diffused in deep waters of the world, both in the littoral and in the sub-tidal zone. In the Mediterranean, especially in the Adriatic, it is the most widespread and bred oyster. In the eastern Atlantic, it is present from Morocco to Norway and in the North Sea, we find it on rocky bottoms or on other solid artificial substrates, starting from the tidal zone. Oysters tend to prefer more salty and clear waters. The natural shoals are usually exploited for breeding purposes, and the catches of the subjects living in the natural environment take place using trawls, rakes, and dredges or are carried out by hand. Of high economic value, *O. edulis* is a very valuable species for its meat rich in omega 3, consumed raw, regularly present on all European markets and marketed alive. *O. edulis* is a hermaphrodite larviparous protandry species. The period of reproduction in the natural populations differs in relation to the geographic location and generally occurs in the summer periods between June and July.

Being a protandric hermaphrodite species, it has both male (which mature before) and female organs; consequently, the fecundation of the eggs happens by means of spermatozoa produced by other individuals and dragged by the respiratory current. Gamete release can be induced by several environmental factors including temperature, chemical and physical stimuli, water currents or a combination of these.

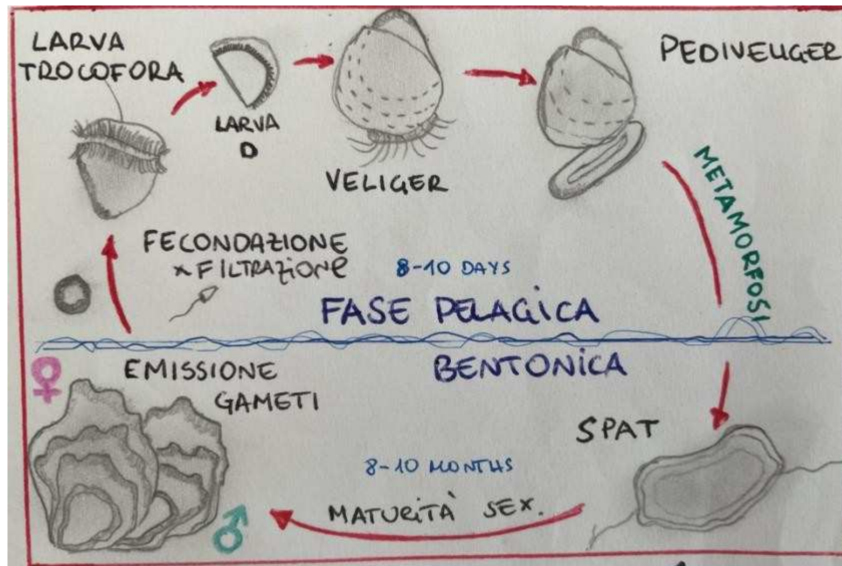


Figure 1: *O. edulis* life cycle summary scheme

The first stages of larval development take place in the inhalation chamber of the shell cavity of the oyster. The fecundated eggs (60µm in diameter), when they are laid, pass through the gills and are retained in the mantle chamber. They then remain in the pallium cavity for all stages of development. Sperm is captured through the opening by inhalation. The trochophore larvae come to life with a diameter of 70-90 µm and have a planktonic phase for 2-3 weeks up to a dimension of 270 µm. The period in which the larvae are retained in the mantle chamber, usually lasts 8-10 days and this period may vary due to environmental and physiological factors, then the larvae are released into the water column. In this pelagic state, two metamorphoses occur. In the first one, the trochophore transforms into Veliger (160 µm) characterized by two ciliate protuberances (Fig.2) and develops a useful foot for probing the substratum on which to take root transforming into pediveliger. In the second metamorphosis, the pediveliger becomes a small bivalve oyster (spat), which in two or three days reaches the substrate on which it will stick. The spat reaches sexual maturity in 8-10 months and becomes definitely adult after 2-3 years. The species' reproductive success depends on several factors, mainly diet and water temperature.

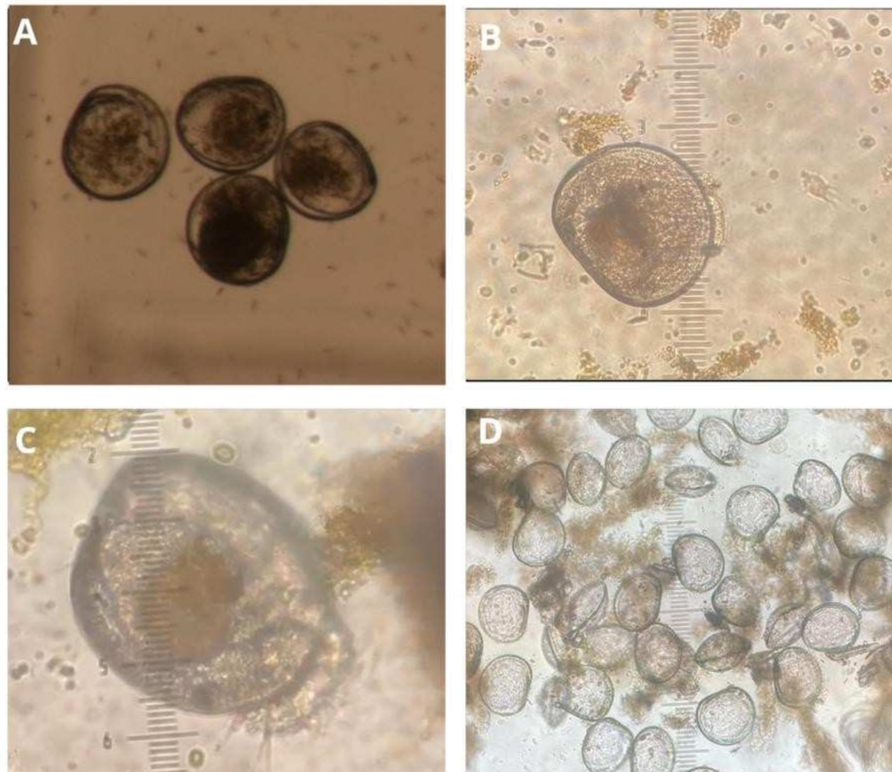


Figure 2: A-D: Photo larvae of *O. edulis* observed under the inverted microscope, magnification 32x B-C: Veliger with evident ciliate protuberances.

RICCIO DI MARE (*Paracentrotus lividus*)

The sea urchin *P. lividus* is an echinoderm characterized by a slightly depressed spheroidal body, with a constant pentaradial symmetry (alternation of 5 radial or ambulatory zones and 5 interradial or interambulacral). It reaches an average diameter of 4 cm, excluding the spines. The organism has a variable colouration with violet, reddish, and brown-green (Fig. 3). The spheroidal body is covered and protected by a rigid calcareous dermal skeleton, formed by embryonic plates. The plates bear numerous tubercles on which the spines are articulated. Other small organelles are present: the ambulatory pedicels connected to the aquatic apparatus and the pedicellariae, which contribute to the capture of the prey and the cleaning of the dermal skeleton. The oral side is facing downwards characterized by a mouth known as the "lantern of Aristotle", and the anal one upwards, where there is the genital pore responsible for the emission of gametes (Fig.3). The whole organism is characterized by a particular water system that allows it to perform many functions such as breathing, digestion and locomotion. In the interambulacral position, there are 5 gonads of variable colouration from light yellow to intense orange.

It is a hermaphrodite species with separate sexes, does not have sexual dimorphism and the fecundation of the eggs is of external type. Widely distributed throughout the Mediterranean, it is one of the most important macro-herbivores from an ecological point of view since, through its intense grazing activity (grazing), is able to greatly influence the operation and the dynamic structure of the benthic communities' mesolitoral hard bottom. The reproductive season, depending on the various geographical areas and the relative environmental conditions, goes from April to July; the second reproductive event can take place in the autumn months. After the release of the gametes in the marine environment, the spermatozoa will quickly fertilize the egg cell, determining the formation of the fertilization membrane. The embryo thus generated encounters numerous cellular divisions that within 48/72h lead it to become a young larva (pluteus) characterized by a well-formed digestive tract and 4 arms. From the eleventh to the twentieth day, the organism undergoes several changes that lead it to develop first 6, then 8 arms. Finally, after about 30 days, the organism is ready to metamorphose and become a young adult: it reaches a size of 2 cm in 2 years, the commercial one in 4 years. The gonads of the organism are highly appreciated at the culinary level, and an excessive fishing effort has led to a depletion of the natural banks. The species is therefore widely exploited in spite of the numerous activities and the regulations that control the harvesting (biological stop fishing that, depending on the different regions, goes from 1 May to 30 June).

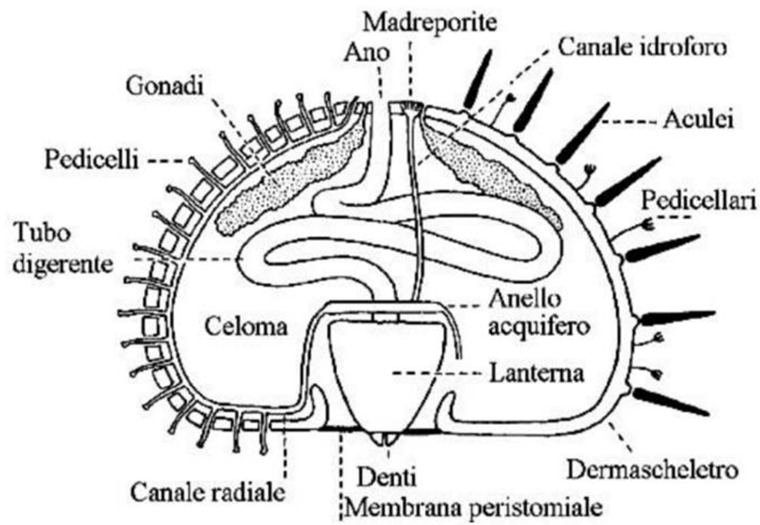


Figure 3: Anatomy of the sea urchin *Paracentrotus lividus* - adult organisms of purple and brownish colouring.

Goals of the Pilot Action

The overall goal of the pilot project is to promote oyster farming integrated with echinoculture as a viable economic and ecological alternative to mussel farming.

The specific targets concern:

- Optimization and transfer to stakeholders of new controlled reproduction techniques for flat oysters (*O. edulis*) and sea urchins (*P. lividus*) for seed and juvenile production.
- Transfer to stakeholders of innovative technologies for growth and maturation in structures normally used for mussels (long-line) in the open sea, through integrated aquaculture techniques.

Activities carried out

In the period January 2021 - June 2023 the activities reported in the introduction were carried out, divided into the following operational phases:

1. Adaptation, growth and maturation of specimens of *Ostrea edulis* and *Paracentrotus lividus* under controlled conditions.

2. Larval development and metamorphosis from embryos obtained from breeders of *Ostrea edulis* and *Paracentrotus lividus* matured under controlled conditions.

3. Growth of juveniles of *Ostrea edulis* and *Paracentrotus lividus* in long-line structures for mollusc farming.

4. Technology transfer to productive enterprises.

MATERIALS AND METHODS

Breeders' collection

Fifty adult specimens of *Ostrea edulis* were collected in June 2022 in local areas. The organisms were transported to the laboratory in containers with an oxygen aerator and subsequently, the chemical-physical parameters of the maintenance water (temperature, pH, dissolved oxygen and salinity) were measured. The specimens have been freed with an abrasive retina from the encrusting organisms present on the valves such as live and dead specimens of some polychaete taxa, barnacles, encrusting bryozoans and red coral algae. Biometric parameters, such as length, width, thickness and weight (Fig. 4) were detected and finally, the organisms were acclimatised before being transferred to relaying aquariums (Fig. 5). Of these 20 individuals, subsequently, 10 were selected as reproducers and transferred to a tank used for collecting the larvae emitted. As for the species *Paracentrotus lividus*, fifty specimens were collected in June 2022 in local areas at a depth varying between 3 and 6 m, transported to the laboratory and subjected to the same adaptation protocol used for oysters (Fig.5) after taking biometric measurements (Fig.4).

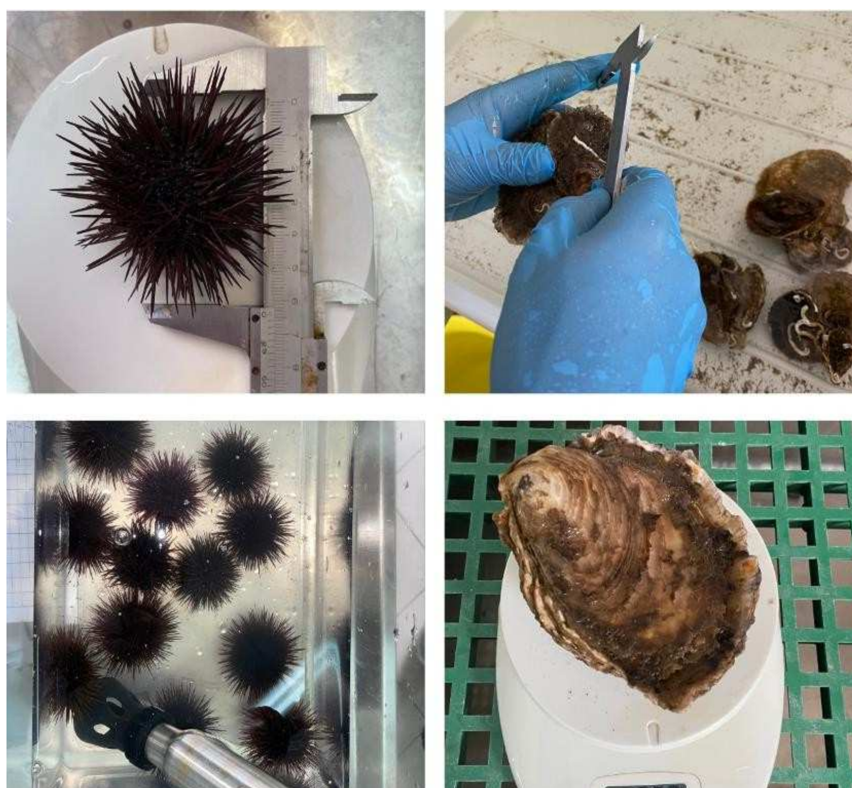


Figure 4: Collection of biometric parameters of *O. edulis* and *P. lividus* organisms

Optimisation of housing conditions

A closed-loop aquarium, with a volume of about 100 litres, was used for the housing of the organisms, equipped with a wool/perlon pre-filter and a hyperactive carbon filter, an active filter biological filter with *Lithothamnium calcareum*, ultraviolet sterilization plant and refrigeration unit with titanium evaporator complete with electronic thermostat with adjustable Δ T 0.1 C. Improved housing conditions in synthetic seawater with temperatures between 18 and 25°C, salinity between 32-36%o, pH between 7.5-8.5, dissolved oxygen values between 8 - 9.5 ppm and lighting conditions between 0L/24B and 16L/8B.

Two tanks have been set up. The first (Tank A) has been used for the relaying of the breeders of the species *P. lividus*. Inside it was created with particular attention to a shady area, as much as possible similar to those present in nature, to allow the curls to maintain their normal physiological behaviour. In addition, oyster shells and shell fragments have been introduced as elements of environmental enrichment in orderto allow organisms to "cover" with the latter as they would in a natural environment. A second tank (Tank B) has been suitably equipped for the relaying of the *O. edulis* reproducers; inside it has been inserted several collectors for the collection of the larvae born after the emission of the reproducers. During an initial testing phase, some combined housing tests were carried out between specimens of *O. edulis* and *P. lividus* (Fig. 5), in order to assess the compatibility of the two species with polyculture.

Figure 5: *System of tanks for the housing of adult individuals.*

Feeding

During the first 5 days of housing the oysters were not fed. The chemical and physical parameters of the water were checked daily (temperature 20, salinity 32-36‰, dissolved oxygen 8-9.5 ppm and pH 7.5- 8.5). In addition, the production cultures with the Burker chamber were counted to calculate the volume of algae to be administered to the specimens of *Ostrea edulis* necessary for the maturation of the gonads. During the feeding phase (about 7 hours), the water flow was blocked and an aeration system with porous stone was activated (Fig. 8). Immediately after the administration of the algae, water was taken to calculate the initial concentration of the algae. After about 7 hours, before reactivating the water flow, a second sample was taken to evaluate the filtration rate of the individuals. The specimens have been fed daily with massive cultures of *Isochrysis galbana* and *Diacronema lutheri* (Fig. 6). The technique used to obtain the massive cultures of microalgae is that of succession (Tab. 1). The original certified strains from the CCAP-Culture Collection of Algae and Protozoa - Scotland have been sub-cultured and the primary cultures thus obtained have been kept under light and temperature-controlled conditions (thermostatic chamber at 20 ° C, exposure to artificial light for 24h and luminous intensity 3000 Lux) (Fig. 9) until reaching an algal density of about 2,8 x 10^6 cells/ml. Then we moved on to the multiplication phase that includes n.4 steps from volumes of 250ml up to volumes of 10L (Fig. 10) and finally to the production culture in 100L necessary for the feeding of adult individuals (Fig. 11). Before each transition from one culture to the next and throughout the growth phase of the production cultures, algal concentration counts were carried out using a Bürker chamber(Fig. 7). A diet of 1.4x10^{10} algal cells/day per oyster (7x10^9 algal cells of *Isochrysis galbana* and 7x10^9 algal cells of *Diacronema lutheri*) was provided for optimal conditioning of the reproducers and gamete release. The specimens of *P. lividus* instead were fed ad libitum; based on previous experiences (*Fabbrocini et al.,* 2010 - 2011 -2012) a quantity of protein feed equal to 2% of the biomass was administered twice a week. In addition, weekly feeding tests were carried out with carrots (*Daucus carota*) and maize (*Zea mays*) in quantities equal to 2% biomass.

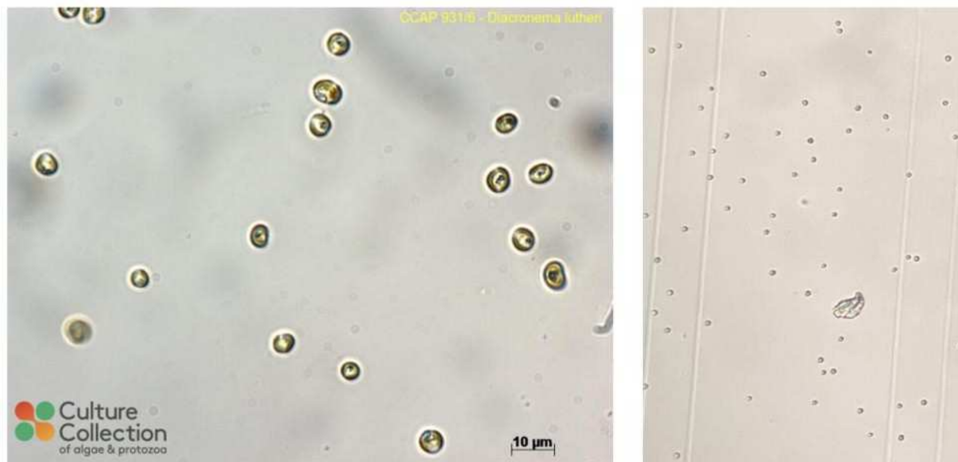


Figure 6: Microscopic observation of *Diacronema lutheri* and *Isochrysis galbana*

Figure 7: Bürker chamber for algal cell count.

Figure 8: Organisms in the feeding phase with the off flow and active oxygenator.

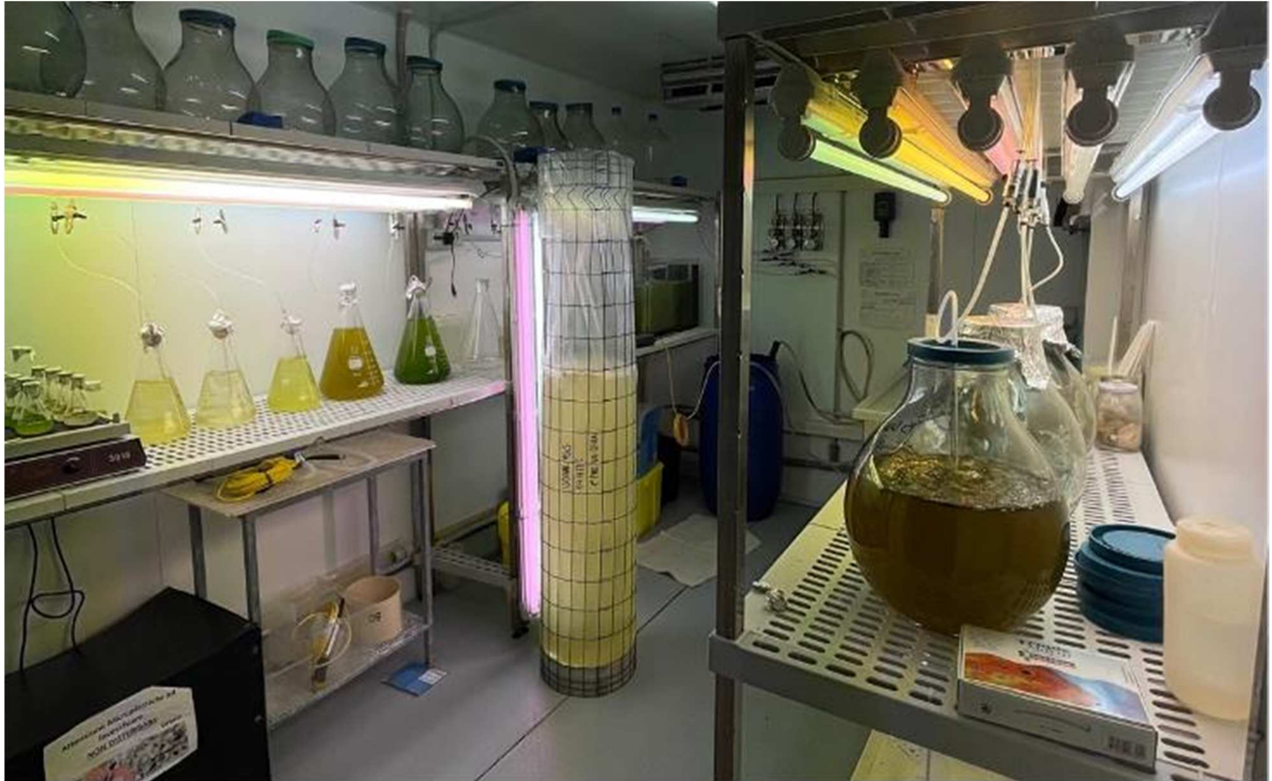


Figure 9: Thermostatic chamber with algal cultures in succession

Figure 10: A Multiplication cultures in 50ml, 250ml, 2l, 5l flasks - B Multiplication cultures in 5l - 10l flasks

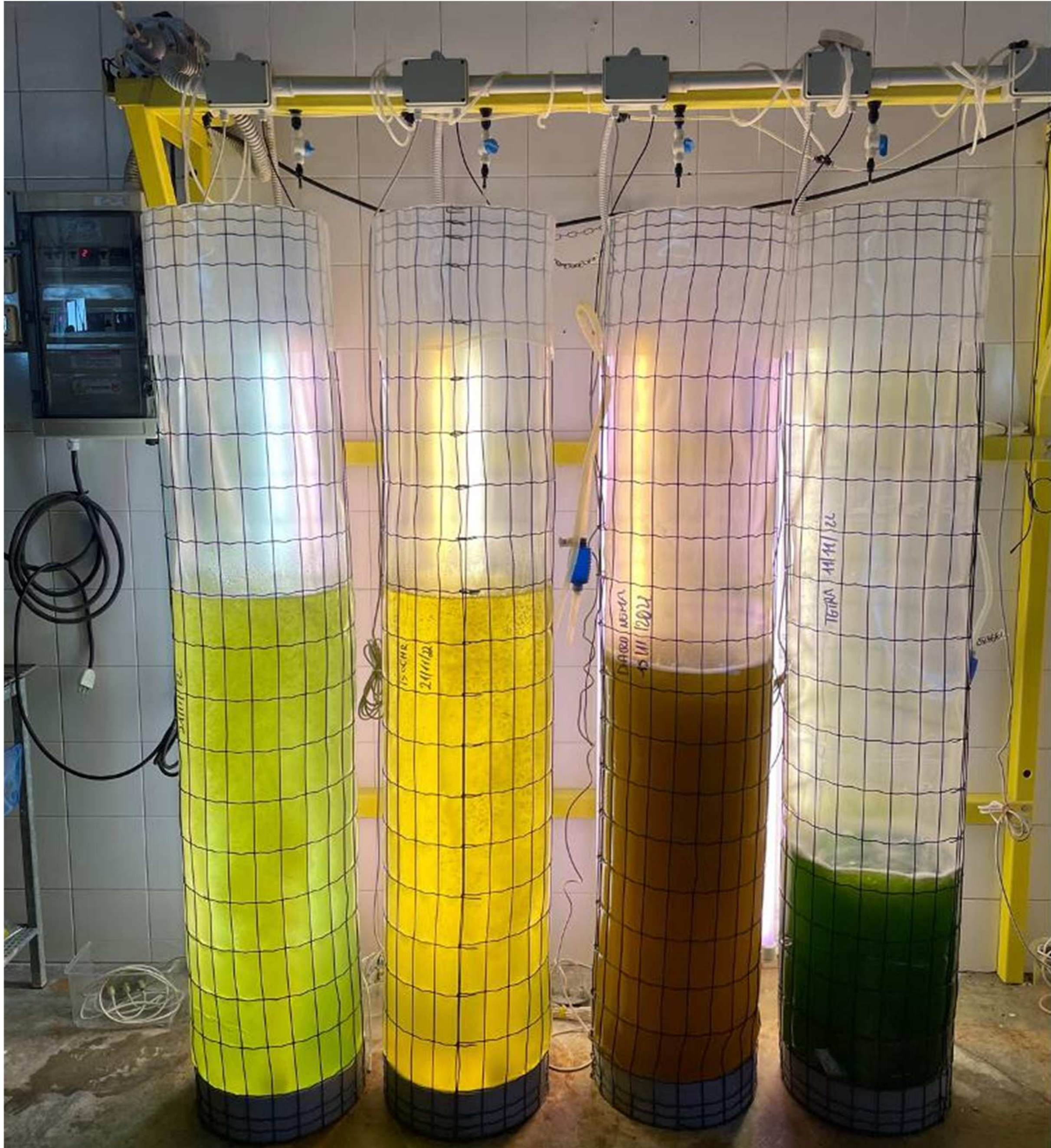


Figure 11: Massive cultures in 100l bags.

Mother culture

Containers: sterile 50 ml Erlenmeyer flasks
Medium: F2 Guillard (0.22 μ enriched filtered seawater)
Aeration: no
Inoculation: 2-5 ml from mother culture
Duration: approx. 70 days
Final density: 2.8x10^6 cells/ml
Light: 3000 lux

Multiplication culture (Phase I)

Containers: sterile 250 ml Erlenmeyer flasks
Medium: 200 ml F2 Guillard (sea water filtered at 0.22 μ enriched)
Aeration: no
Inoculum: 1 Erlenmeyer flask of mother culture
Duration: approx. 8 days
Final density: 2.8x10^6 cells/ml
Light: 5000 lux

Multiplication culture (Phase II)

Containers: 2L sterile flasks
Medium: 1800mL F2 Guillard (0.22 μ enriched filtered seawater)
Aeration: 0.3 bar
Inoculum: 250mL Erlenmeyer flask of phase I culture
Duration: approximately 3 days
Final density: 1.9x10^6 cells/ml
Light: 5000 lux

Multiplication culture (Phase III)

Containers: sterile 5L flasks
Medium: 4800 ml F2 Guillard (0.22 μ enriched filtered seawater)
Aeration: 0.3 bar
Inoculum: 2L culture flask in phase II
Duration: approx. 3 days
Final density: 3x10^6 cells/ml
Light: 5000 lux

Multiplication culture (Phase IV)

Containers: 10 L flasks
Medium: F2 Guillard (0.22 μ enriched filtered seawater)
Aeration: 0.3 bar
Inoculum: 2.5 L from phase III culture
Duration: approx. 4-6 days
Final density: 3.5x10^6 cells/ml
Light: 5000 lux

Production culture

Containers: 150 L bags
Medium: F2 Guillard (enriched seawater)
Aeration: 0.3 bar
Inoculation: 10 L flask of phase IV culture
Duration: approx. 7 days
Final density: 4-5x10^6 cells/ml
Light: 5000 lux

Table 1: Diagram summarising the succession of algal cultures, from the maintenance of primary cultures to the progressive multiplication phases.

Evaluation of gonadal maturation of the breeders of Ostrea edulis and Paracentrotus lividus

In order to assess the state of maturation of the gonads for specimens of *O. edulis* and *P. lividus*, randomly selected organisms were collected and subsequently analysed every two months. Different methods have been identified to determine the stage of gonadal maturation and thus to assess possible gamete release. The most accurate is the preparation of histological departments of the gonad, but it is very expensive, long and involves the sacrifice of the animal. Instead, the most commonly used techniques are to smear the gonad or extract small samples from some individuals and examine them under a microscope. In hermaphrodite species, male and female follicles usually do not mature synchronously, in order to minimize the possibility of self-fertilization. The assessment of the stage of maturation of *O. edulis* individuals was then carried out by biopsy sampling of portions of the gonad and subsequent microscopic observation. After opening the valves, using a Pasteur pipette, a sample was taken directly inside the gonad (Fig.12). The material thus taken was placed on a slide and observed under a microscope with 10x and 40x objectives. Depending on the appearance of the gonads and the presence or not of mature gametes (sperm and eggs) and/or embryos (Fig. 13) it was possible to classify the state of maturation in 5 different stages (*Marteil et al*.1976) (Tab. 2). The presence of spermatozeugmata has been observed, that is spherical structures in which the heads of the spermatozoa are grouped in a central nucleus with the tails extended outside; after the dilution in seawater, the spermatozeugmata dissociate and the free-swimming spermatozoa are released (Fig. 13).

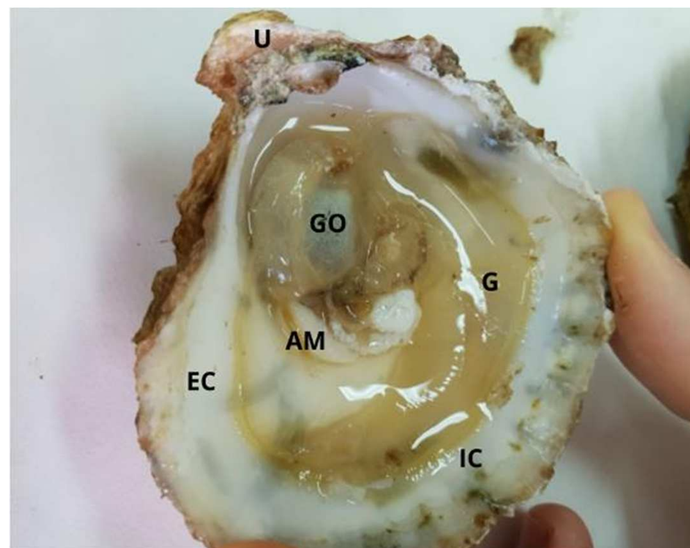


Figure 12: Soft tissue anatomy of Ostrea edulis. AM: adductor muscle, G: gills: GO: gonads (M and F), U: umbo, IC: inhaling chamber, EC: exhaling chamber.

STAGE	DESIGNATION	GONAD	APPEARANCE SMEAR
0-1-5	Undefined	Sexual resting stage: gonad empty	No gametes observed
2	Onset of gametogenesis	Gonad visible under epithelium	Sperm morulae immature
3	Advanced gametogenesis	Gonad hypertrophied; thick white layer surroding visceral mass	Gametes highly abundant in various stages of maturiry
4 a, b	Spawning	Pallial cavity filled with a white milky mass turning slate grey	Female incubating embryos

Table 2: Classification gonadic maturation according to *Marteil et al.*,1976

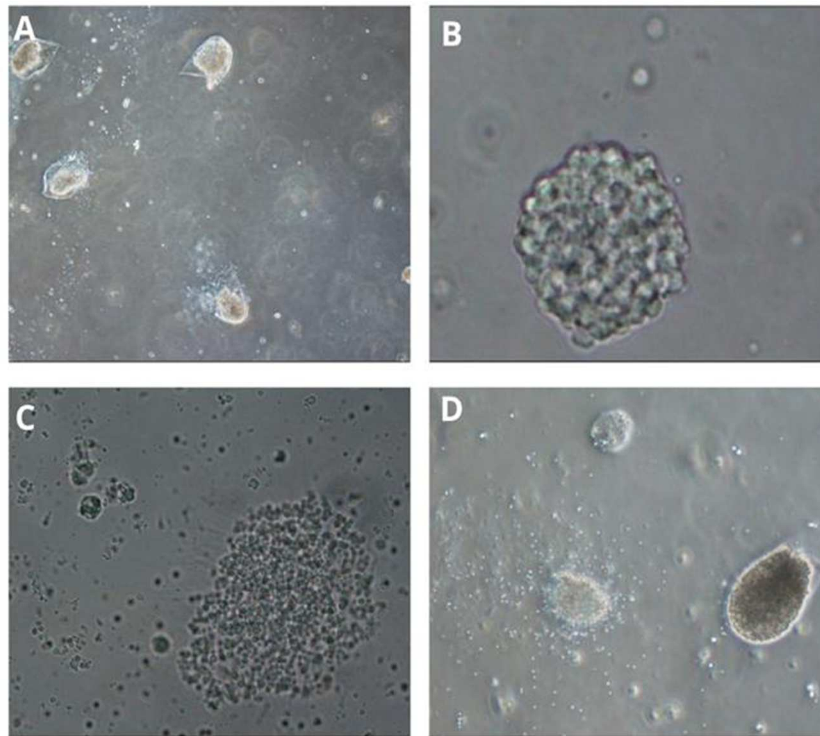


Figure 13: A: Image of eggs displayed with objective 10x; B: Image of an embryo displayed with objective 40x; C: Image of a spermatozeugmata displayed with objective 40x; D: Image of a dissolving spermatozeugmata after dilution in seawater displayed with target 10x.

With regard to the evaluation of the state of maturation of the gonads of the sea urchin, the analysis of the gonadal index was carried out. An organism was gently removed from the relaying tank and placed on an absorbent sheet of tissue paper in order to remove all the water present; any fragments of shells and food residues between the spines have been removed and the whole organism has been weighed. Once the measurements were taken in terms of diameter (excluding spines) and weight, the specimen was sacrificed, with a sterile scissor the peristomial membrane has been engraved near the lantern of Aristotle and a circular cut has been made, dividing the organism in two along the horizontal axis of the body. As a result of the opening of the organism, any excess water and faecal material has been eliminated, taking care not to damage the gonads (this would cause the release of the gametes if present). Thus the 5 gonads were separated from the individual and then weighed (Fig. 14). The collected data were used in the following formula to obtain the result of gonadic index per individual.

GI = Gonad wet weight (g)/sea urchin wet weight (g)*100

Values greater than 8 correspond to a good gonadic index. Once the gonadic index had been evaluated, a portion of the tissue was taken for microscopic observation of the gametes to assess their quality and stage of maturation.

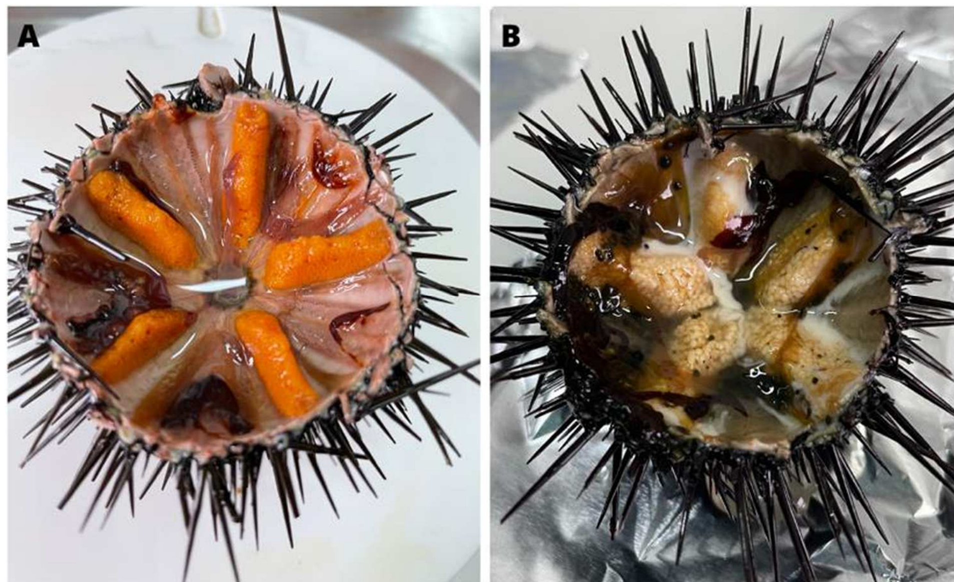


Figure 14: - Soft tissue anatomy of *Paracentrotus lividus*. A: gonads female organism; B: gonads male organism, well visible sperm cluster.

Gamete collection and fertilization

Once a stage of optimal gonadal maturation was reached, the gametes were inducted. The emission and the subsequent fecundation for the specimens of *O. edulis* have been reached in the tank, following the increase of the daily feeding regimen, at a constant temperature of 20,0 ºC with light/dark range 16L/8B. On the contrary, for the organisms of *P. lividus,* the fecundation was of an artificial type with a direct collection of gametes and subsequent fertilization between sperm and egg cells. The recovery of the specimens from the aquariums was carried out with great caution, avoiding damaging the ambulatory pedicels and the organisms were placed in a large container covered with bibula paper to prevent them from sticking to the container. It was necessary to recover and identify at least 6 organisms (3♂3♀) in order to ensure a good starting pool. The organisms were handled at a temperature of 19 +/- 1 ° C away from direct air sources. For all dilution, egg washing and larval housing steps, autoclaved synthetic seawater at 120 ºC (ASW) were used after salinity, temperature, pH and dissolved oxygen (salinity 34-36 %o, t 18-20 ºC, pH 7.8 - 8.3, O2 8-9.5 ppm). Once autoclaved, the test water was oxygenated for 24h, in order to stabilize the pH and salinity after sterilization. Gamete emission was induced by injection of 0.5 ml KCl (0.5 M) through the peristomial membrane. To try not to puncture the digestive tract, the injection was made with the needle tilted outwards in order to release the saline solution into the celomatic cavity. To facilitate the distribution of KCl in all the gonads the body has been turned over gently for a few seconds. For some specimens, it was necessary to proceed with a second injection of 0.25 of KCl (0,5 M) through the peristomial membrane. As there has been no gamete release 5 minutes after the first. Following the injection, the individuals were positioned, turned with the genital pores downwards, in glass petri (90 mm) or beaker (100 ml),dry (Fig.15).

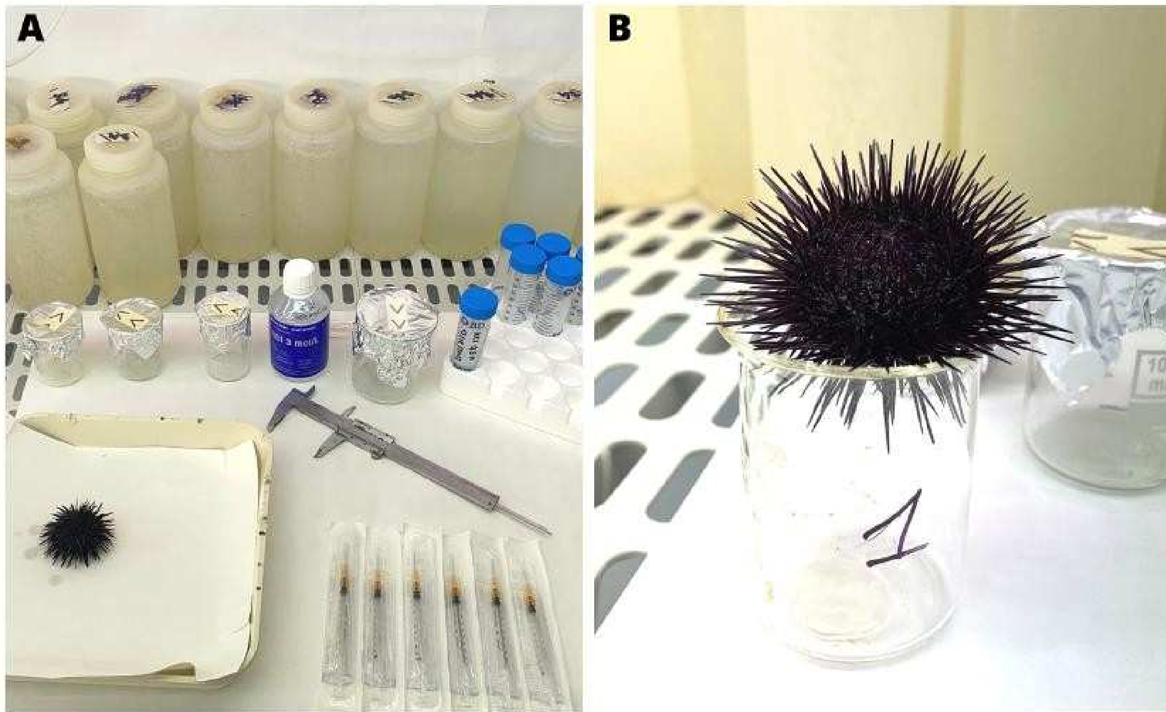


Figure 15: - Induction emission gametes. A: Individual preparation for 0.5 ml KCl 0.5 injection; B: Individual placed on 100ml beaker with genital pores facing down.

The male and female gametes were collected in different ways, taking care to separate the male and female individuals and using different materials (e.g., gloves, pipettes, beakers). At the time of the release of the eggs, female specimens were placed on a collection plate containing synthetic seawater needed to prevent the eggs from drying. The emission lasted for a period not exceeding 30 minutes to ensure a better quality of the gametes and to ensure the survival of the reproducers. The latter, once the collection of the gametes was finished, was again inserted into the aquarium, taking care to use a different tank than the one where they were previously housed in order to exclude fertilization with the remaining organisms. The eggs recovered from the Petri dishes were then transferred to 50 ml falcon tubes; the petri dish was rinsed with a few drops of ASW in order to recover the eggs remaining attached to the walls; the eggs were then stored at a temperature between 18 -20° C.
They were subsequently allowed to decant for a period of at least 15 minutes in order to separate viable cells from damaged ones (which tend to go to the surface) after 15 minutes, the egg washing and filtration steps were carried out (Fig. 17). The spermatozoa were recovered dry (to avoid their activation) directly from the gonopore of the organism by aspiration with a glass Pasteur pipette (Fig. 16) and immediately stored at 4°C in a 1.5ml tube (Eppendorf-type).

In each falcon containing the eggs, the supernatant containing damaged or poor-quality eggs were removed, and the remaining part was filtered on 100µm mesh and then collected in a 100ml beaker. The contents of the beaker were transferred again in 50ml falcon and allowed to decant so that the eggs concentrated again on the bottom (Fig. 17). This step was timed 3 times. After decanting, washing and filtration the selected eggs were transferred to the general (fertilisation) pool.

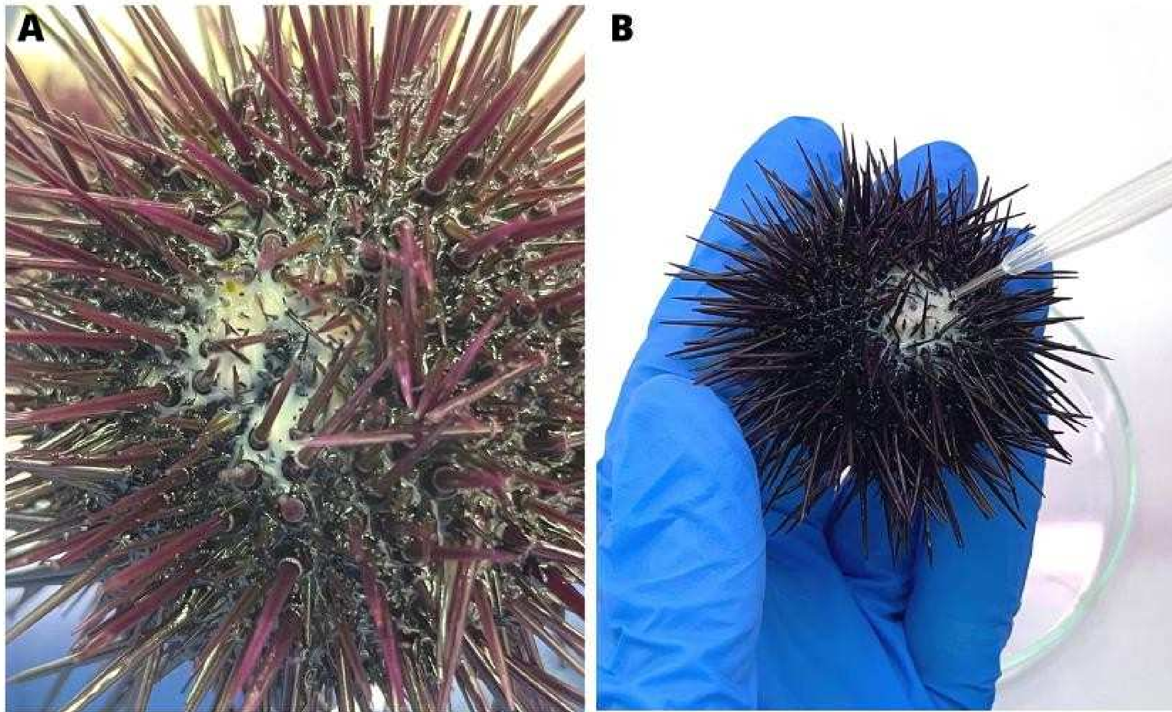


Figure 16: - Sperm collection. **A:** Male individual immediately after release; **B:** Collection of gametes directly from the gonopore.

Figure 17: - Egg cell collection. **A:** Individual female immediately after emission; **B:** Collection gametes in 50ml falcon; concentration of eggs on the bottom allowed to decant for 15 minutes.

Both male and female gametes were preliminary observed under the light microscope to evaluate their quality and select them for the fertilization pool. A small number of eggs, taken with a pipette Pasteur from the bottom of the falcons, was placed on a slide and observed under an optical microscope to evaluate its morphology (objectives 4x-10x-40x). They were discarded eggs, which were found to be irregular, vacuolated or damaged by optical inspection. A small amount of sperm was subsequently observed, and adding ASW was to evaluate sperm motility. Good quality gametes were transferred to falcons/Eppendorf containing fertilisation pools. Gametes from at least three organisms per sex have been selected to ensure good genetic diversity and a higher likelihood of reproductive success. The sperm pool was diluted 1:200 with ASW (100 µl spz in 19.9 ml ASW). At this stage we proceeded with an empirical test of fertilization by adding a few drops of egg and sperm cells on a slide, going to observe the formation of the fertilization membrane. A correct formation of the latter in a few seconds indicates a good quality of the selected gametes (Fig. 18).

Figure 18: - Formation of the fertilization membrane, a few seconds after contact between egg cells and sperm.

Artificial insemination was then carried out. A fertilisation solution was prepared using a 250 ml beaker; 1.4ml (so as to obtain a 1:100 dilution) was taken from the pool of concentrated eggs and 400 µl of sperm from the diluted pool (1:200) brought successively to a volume of 140ml with ASW (138,2 ml). After 5-6 h a first check was carried out to assess the stage of development of the embryos (Fig. 19) which were subsequently diluted with ASW and brought to a volume of 3L to obtain a final concentration of 4000/L. Slight oxygenation and a photoperiod of 12h light and 12h dark were guaranteed. After 48-72h, the first check was carried out to evaluate the achievement of the 4-arm pluteus stage (Fig. 18 I)

The pluteus were fed daily with a dose of 30,000 cells/ml of *Dunaliella tertiolecta* and *Isochrysis galbana*. Reaching the stage of 6 arms pluteus was doubled the dose of algal cells.

Checks were carried out every two days to assess the presence of microalgae in the digestive system.

Figure 19: - Stages of development *P. lividus*: A: Egg cell with fertilisation membrane - B: First segmentation - C: 4 blastomeres - D: 8 blastomeres - E: 16 blastomeres - F: Early blastula - G: Blastula with obvious blastocele - H: Gastrula - I: Pluteus 48h - L: Pluteus at 10 days with microalgae in stomata - M: Pluteus at 8 arms - N: Pluteus metamorphosis.

Collection and maintenance of O. edulis larvae, settlement and spat.

For the collection of the larvae of *O. edulis*, in correspondence with aquarium drain valves (Fig. 20), have been placed containers with at the base a mesh of 120 µm to retain the larvae emitted by the adults (Fig. 20). The containers were observed daily and the presence or absence of larvae at the veliger stage was assessed under a microscope. In case of presence, the larvae were measured and transferred to containers with filtered sea water at 0.22 µm and maintained at a constant temperature of 20°C. Slight continuous aeration of the growth medium has been ensured. The veliger larvae were then fed daily with a diet of 1-2x108 algal cells of *Isochysis galbana* and water replaced every 48h. At the water change, measurements were made under a microscope to evaluate larval growth and metamorphosis. The veliger larvae upon release from the pallial cavity measure about 150-160µm (Fig. 21 B) after about 2 weeks from the collection evolve to the stage of pediveliger (length of about 250µm) during which they tend to stand on the bottom in search of a substrate suitable for fixation (Fig. 21 D). Subsequently, the larvae attach to a solid substratum completing the metamorphosis (settlement) and reaching the stage of spat (Fig.21 D).

Figure 20: Collectors with mesh 120μ for the collection of veliger (160μ).

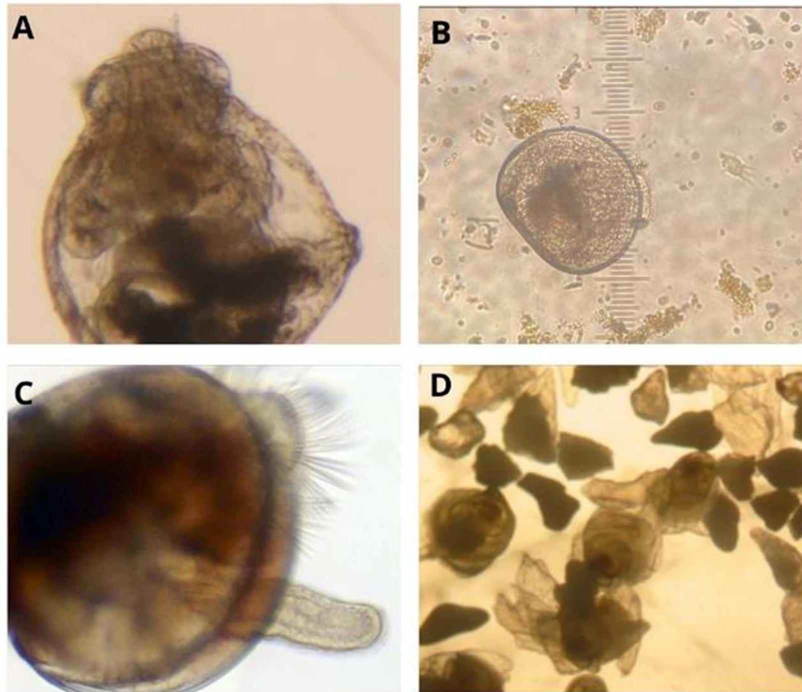


Figure 21: Stages of larval development *O. edulis*. **A**: trochophore - **B**: *veliger* – **C**: *pediveliger* – **D**: *spat*

Figure 22: *Larval development.* ***A:*** *trays and beakers 1L for larval growth O. edulis -* ***B:*** *Substrates identified for settlement spat. (Whole oyster valve, strings, shattered valve).*

Larval development and metamorphosis of the Plutei

Between the 19th and 25th day, the first tests of metamorphosis in larger volumes (10 - 50 L) or experimental tests in multiwell plates were carried out, ensuring a substrate /metamorphosis inductor composed of the green macroalgae *Ulva lactuca* (fresh or frozen). Once a proper metamorphosis has been achieved, the pluei will be transferred to small tanks in series with light aeration. The following parameters will be checked: synthetic seawater with temperatures between 18 and 25°C, salinity between 32-36%o, pH between 7.5-8.5, dissolved oxygen values between 8-9.5 ppm and lighting conditions between 0L/24B and 16L/8B. For the first month the organisms will be fed ad libitum with a quantity of protein feed equal to 2% of the biomass. For the first month the organisms will be fed ad libitum with an amount of protein feed equal to 2% of the biomass, after which the diet will be supplemented with portions of *Ulva latcuta*, *Zea mays* and *Daucus carota*.

Growth of juveniles of Ostrea edulis and Paracentrotus lividus in long-line structures for mollusc farming.

Once the juvenile stage for both species is reached, the latter will be transferred to a natural environment in long-line structures for mollusc farming (Fig. 23).

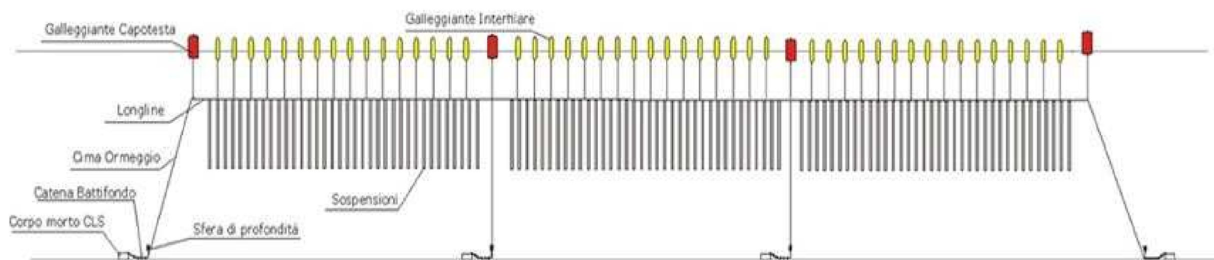


Figure 23: Long-line structures scheme for shellfish farming

Initial experimental transfer tests were carried out to assess the suitability of the area selected for accretion (Fig. 25). The aim of these tests was to investigate the productive potential of the two target species (*O. edulis* and *P. lividus*) in order to promote product diversification in shellfish farming. In particular, in August 2022 some organisms were transferred inside a basket ''Ostriga'', placed at a depth of 10m. The organisms have been subdivided following the distribution scheme represented in figure 24. A total of 120 adult organisms were transferred to the 4 floors of the basket and divided into the 4 different compartments of the same floor.

For each of the 4 floors, 30 organisms (15 oysters and 15 sea urchins) were placed: first compartment in 10 oysters (*O. edulis*) - second compartment in 5 oysters (*O. edulis*) and n in 5 curly (*P. lividus*) - third compartment in n. 10 sea urchins (*P. lividus*) - fourth compartment (control), no individual. The control compartment was left empty in order to assess the differences in fouling/organic material present, compared to the compartments containing the organisms.

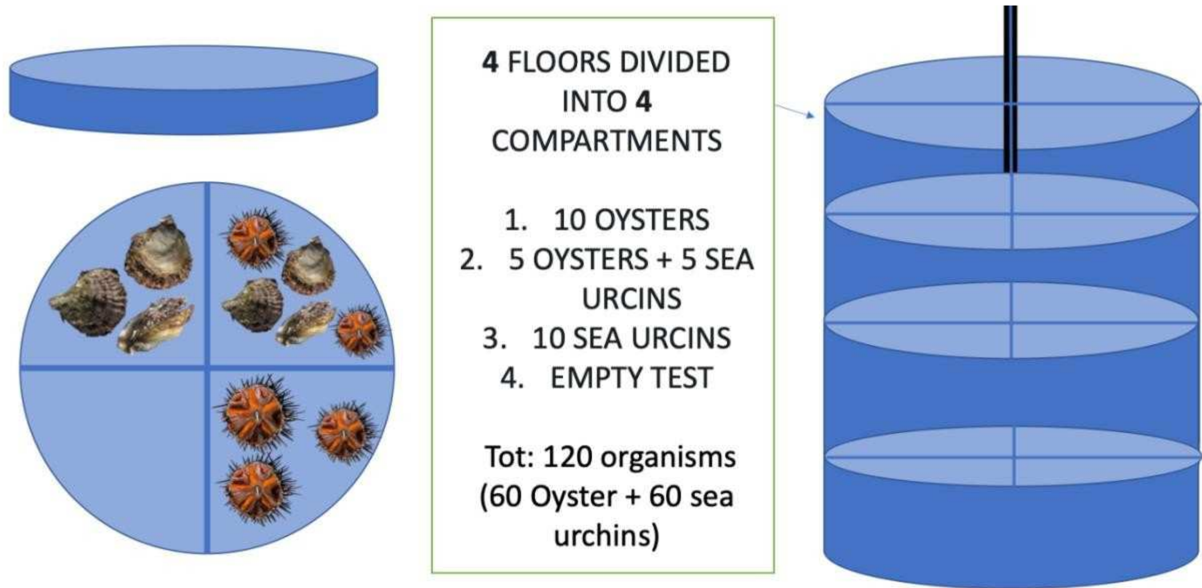


Figure 24: Experimental model for the transfer of organisms at sea; dissemination and distribution of organisms

Figure 25: First sea transfer tests beginning August 2022

The following monthly evaluations were carried out:

• Observation of mortality organisms (n° living/dead organisms);

• State of wear of the structure;

• Presence of epibiont organisms on oyster shells;

• Differences in terms of incrustations between the various compartments and in the different levels of housing of the organisms.

TRANSFER OF TECHNOLOGY TO PRODUCTIVE ENTERPRISES

The fourth phase of the project includes the dissemination of the results obtained, through seminars for practitioners and through dissemination events. A first event, which falls within the activities and objectives of this phase, was organised in October 2022 (Fig.26).

1st Cross-border training workshop and exchange of experiences between Croatian and Italian operators.

- Training workshop for fishery operators in the Molise region ;
- Conference with presentation of the pilot project ;
- Visit to the workshops where some phases of the pilot project were presented and demonstrated ;
- View of the mussel culture/purification plants at the port of Termoli .

These activities were essential, together with the organisation of workshops to raise awareness of the proposed new aquaculture technologies.

Figure 26: Demonstration activities - dissemination and presentation of first results.

RESULTS

Adaptation, growth and maturation of the specimens of Ostrea edulis and Paracentrotus lividus under confined conditions.

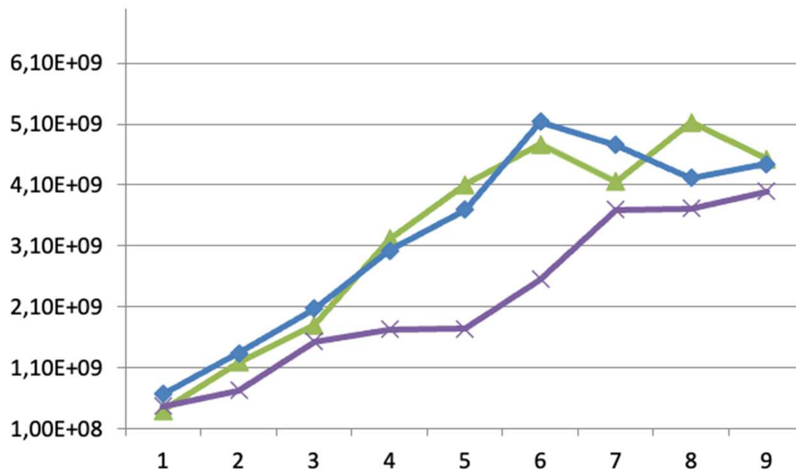
In the first phase of the project, it was possible to implement and verify effective protocols for the collection and transport of specimens, the relaying and the management of tanks. The development of good food diets for the organisms from the first larval stages to the adult ones ensured the transition to the second phase of the project.

The cycle of the culture of microalgae in succession (*Isochrysis galbana*, *Diacronema lutheri*) has been standardized, ensuring a constant supply of food to meet the needs, for both adult and / juvenile larvae of the two species. Oysters responded positively to the relaying after the initial fasting period, and it was possible to assess the excellent filtration rates following high dietary intake (Tab. 3). The massive feeding and the constant control of the chemical-physical parameters (t° C - dry oxygen - pH - salinity) in the tank led the organisms to mature their gonads outside the biological maturation period (June-July). Analyses were carried out during the first month of oyster housing to assess the filtration rate of oysters. Table 3 and Graph 3 show some of the counts made during the first month of housing.

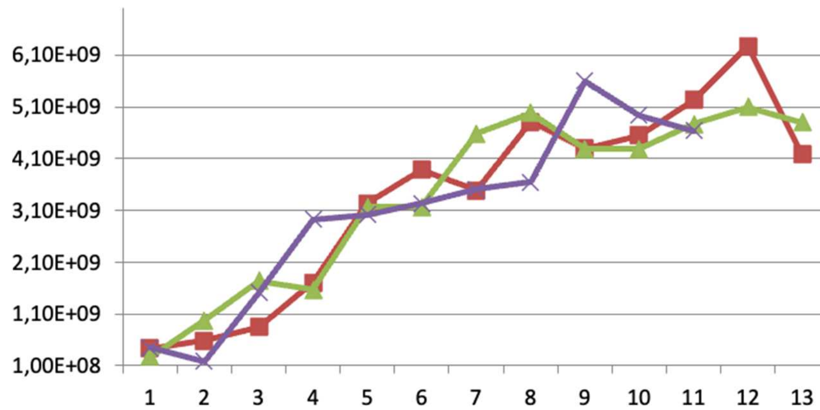
Table 3: Summary diagram of the successive steps of algal cultures, from the maintenance of primary cultures

Staging day	First algal concentration cell/L	Algal concentration 7 hours after dosing cell/L
5	5,7 x10^8	2,5 x10^8
6	6,7 x10^8	2,3 x10^8
7	7,6 x10^8	2,1 x10^8
8	8,9 x10^8	3,2 x10^8
9	9,2 x10^8	2,4 x10^8
10	8,4 x10^8	1,4 x10^8
11	7,1 x10^8	2,9 x10^8
12	7,3 x10^8	3,5 x10^8
13	8,3 x10^8	5,1 x10^8
14	6,1 x10^8	2,8 x10^8
15	5,6 x10^8	1,1 x10^8
16	3,9 x10^8	1,4 x10^8
17	6,52 x10^8	1,88 x 10^8
18	7,42 x 10^8	2,21 x 10^8
19	6,81 x 10^8	1,83 x 10^8
20	7,12 x 108	2,12 x 108
21	8,55 x 10^8	1,53 x10^8
22	9,13 x 10^8	2,11 x 10^8
23	8,86 x 10^8	1,81 x10^8
24	9,15 x 10^8	2,44 x 10^8
25	6,95 x 10^8	2,81 x10^8
26	7,48 x 10^8	2,24 x 10^8
27	8,13 x 10^8	2,08 x 10^8
28	8,72 x 10_8	1,93 x 10^8
29	8,14 x 10^8	2,48 x10^8
30	9,77 x 10^8	1,88 x 10^8

to the stages of progressive multiplication.



Graph 1: growth of Isochrysis galbana crops in 100l.



Graph 2: growth of Diacronema lutheri cultures in 100l.

Graph 3: Evaluation of the filtration rate of individuals after about 7 hours of feeding during the first 30 days of housing

At the same time, also the sea urchins have responded positively to the stabling in the tank having survived all the specimens from the day of collection at sea up to the moment of induction to the emission of the gametes.

The weekly feeding tests with carrots (*Daucus carota*) and maize (*Zea mays*), have proven to be effective for the maintenance of organisms and have allowed the achievement of excellent indices of gonadic maturation in just three weeks of food administration. This has made it possible to carry out tests of artificial fertilization of organisms outside the natural periods of gonadic maturation (Tab. 4).

Below is the table with the results of the gonadic index carried out.

Sex	*Diameter*	*Dry weight (g)*	*Gonad weight (g)*	*GI*
M	*3,9*	*20,49*	*1,82*	*8,88*
F	*4,2*	*19,97*	*1,88*	*9,41*
M	*3,1*	*18,49*	*0,7*	*3,79*
M/F	*3,5*	*14,68*	*0,82*	*5,59*
F	*3*	*14,81*	*0,88*	*5,94*
M/F	*3,2*	*14,8*	*0,64*	*4,32*
F	*4*	*15,53*	*1,25*	*8,05*
M	*4,2*	*26,04*	*2,01*	*7,72*
F	*3,6*	*21,93*	*2*	*9,12*
M	*3,2*	*22,51*	*3,53*	*15,68*

Table 4: results of the gonadic index carried out.

Integrated relaying tests were successful (Fig. 27); already after the first week of relaying, sea urchins began grazing oyster shells. This confirmed the initial hypothesis that urchins housed with oysters ensured cleaner oysters by counteracting the formation of epibiont organisms on the shells (filamentous and coralline algae). This repeated behaviour in sea installations would ensure a large-scale reduction in the costs of maintaining and cleaning the baskets.

Figure 27: Integrated housing tests *P. lividus* and *O. edulis.*

Larval development and metamorphosis from embryos obtained from breeders of Ostrea edulis and Paracentrotus lividus matured under controlled conditions.

The good conditions of relaying and feeding have led the specimens of *O. edulis* to reproduce naturally in the tank. As a result of the emission of the gametes, progress in the growth of the larvae has been recorded, it has been reached on the seventh day of larval development (veliger stage) (Fig. 28). The larval feeding phase is ongoing to ensure the completion of the larval cycle and the settlement of the spat.

Figure 28: *seventh day of larval development O. edulis - veliger stage.*

With regard to sea urchins, the achievement of a good gonadic index and the consequent maturation of the gonads allowed two experimental tests of artificial fertilization.

The first test was carried out in order to identify any critical issues and effectively define the individual steps of the fertilization protocol. The second artificial insemination test yielded very good results with regard to gamete emission.

After 48 hours from the fertilization of the egg cells it was possible to observe the pluteus with 4 arms, well-formed and free of malformations (Fig. 28 A-B-C). The plutei were fed with *Dunaliella tertiolecta* and *Isochrysis galbana*, it was possible to observe the presence of microalgae in the stomach of plutei through microscopic observation (objective 40x) - (Fig. 29 D) / (Fig.30).

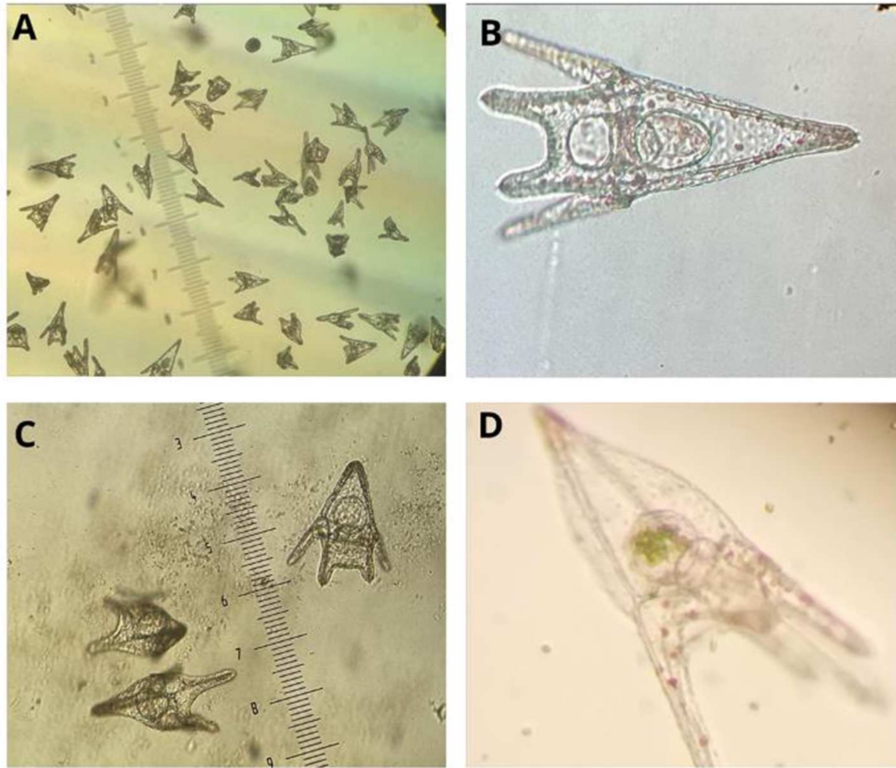


Figure 29: A-B-C Plutei at 72h - D Pluteus day 10, presence *dunaliella* in the stomach.

Samples were taken along the water column every two days and subsequently observed under the light microscope. The observations made allowed to follow the development of the plutei until the 20th day after the fecundation. From the 21st day some critical issues were found, among them:

1. Presence of ciliated organisms on the bottom of 3L Beakers (Fig. 31 A)

2. Concentration plutei on bottom beaker and hint of deformation/ loss arms - Presence of numerous spines radiate without body of the individual (Fig. 31)

3. Between the 11th and 20th day of testing, the formation of other pairs of arms beyond the 4-plutei stage was not observed.

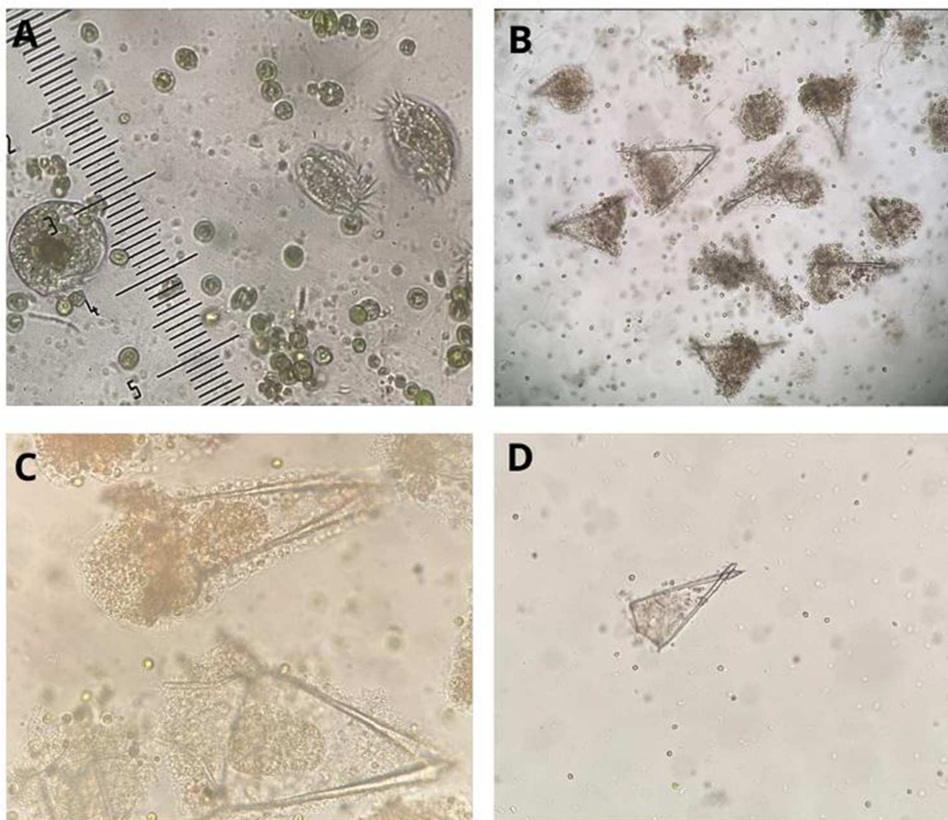


Figure 31: Ciliated contamination; B-C-D: decaying plutei, presence of radiated thorns on the bottom of the beaker

These trials have shown that it is essential to work in controlled environments and that large investments are required to cope with high larval mortality in order to produce huge quantities of larvae and ensure greater reproductive success.

Growth of juveniles of Ostrea edulis and Paracentrotus lividus in long-line structures for mollusc farming.

The basket containing the organisms transferred to the sea during the first week of August was checked once a month for a period of three months (August 2022-October 2022).

At the same time as the activities at sea, measurements of the following chemical-physical sea water parameters were carried out using a multiparameter probe to characterise the breeding site (pH - salinity - temperature - dissolved oxygen) - (Tab. 5).

	pH	***T° C***	***Sal %o***	***O_2 mg/L***
August	*8,12*	*28,4*	*38,9*	*8,07*
September	*8,34*	*25,4*	*39,4*	*8,35*
October	*8,27*	*23*	*38*	*8,62*

Table 5: Chemical-physical parameters of the offshore breeding site.

The following assessments were made during the checks:

- Observation of mortality organisms (n° of dead/living organisms);
- State of wear of the structure;
- Presence of epibiont organisms on oyster shells;
- Differences in terms of incrustations between the various compartments and in the different levels of housing of the organisms.

In general, it was possible to observe an excellent response to the integrated housing between sea urchins and oysters (fig. 32 A), the same behaviours were observed previously in the tank.

In detail, during the first check at the end of August, the Ostriga basket was intact and covered by various fouling organisms, both vegetable and animal belonging to different taxa (filamentous algae, hydrozoans, bryozoans, tunicates, snakes, polychaetes). There were no particular differences in terms

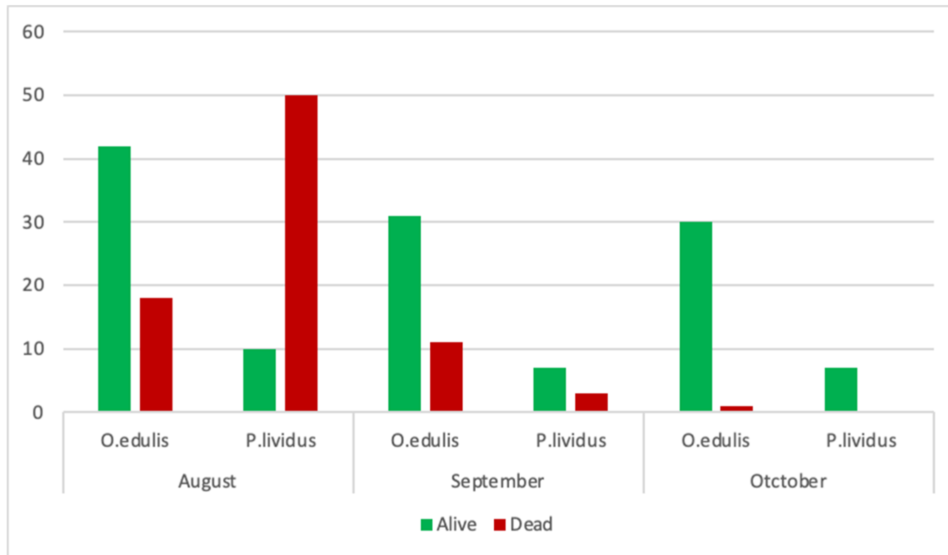
of cleaning the compartments comparing those that had sea urchins inside compared to the compartment with only oysters.

In terms of survival of the individuals, a mortality of 30% for oysters (18 oysters out of 60) and a mortality of 80% for sea urchins (50 sea urchins out of 60) has been recorded (Graph 4). The high temperatures of the month of August 2022 in conjunction with marine weather events of particular intensity, may have affected the survival of *P. lividus*.

Figure 32: Observations during the August (**A-B**) and September (**C-D**) checks

A second check was carried out in September 2022 with a mortality rate of 26% for oysters (11 oysters out of 42) and a mortality rate of 30% for sea urchins (3 sea urchins out of 10) - (Graf. 4). During this check, it was observed that, after about one month of relaying, the compartments presenting the curls were cleaner than those containing only oysters (Fig. 32 C). This observation shows the effectiveness of the grazing activity done by sea urchins against the control of fouling on the breeding structure. Moreover, a good growth rate has been recorded for the specimens of *Ostrea edulis*, which have shown an accretion in terms of length between 0.5 -1 cm depending on the individuals (Fig. 32 D).

A third check was carried out in October 2022 in which a mortality rate of 3% was recorded for oysters (1 in 30 oysters) and no mortality for *P. lividus* (Graf. 4) organisms.



Graphic 4: Assessment of mortality of organisms in the period August-October 2022

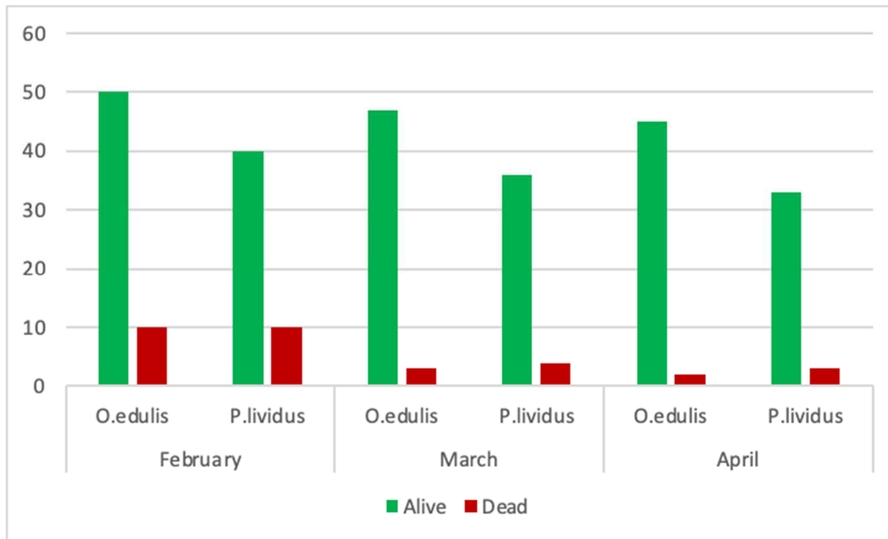
These first tests of transfer to sea have evidenced some criticalities regarding mainly:

- System of anchoring the baskets to long-line structures;
- Positioning along the water column;
- Chemical-physical parameters of water and weather-marine conditions.

In order to ensure greater survival for the sea urchin specimens, a second transfer trial was carried out, following the same distribution scheme in the basket, but this time only the oysters were placed at first and after about a month the sea urchins were added (Graf. 5).

This ensured a considerable increase in survival rates, probably due to the formation of an initial layer of fouling that ensured easier adherence of the urchins to the basket, as well as guaranteeing a food source from the start (Fig. 33).

It is known that sea urchins, being a benthic species, require shaded conditions, which is why fragments and small shells were added to ensure the organisms could cover themselves with them. In addition, the first layer of encrustations helped to keep these organisms in dark conditions.



Graphic 5: Assessment of mortality of organisms in the period February - April 2023

Figure 33: Observations during observations during the second transfer test.

A further transfer trial at different depths is being tested. These trials in the summer months are intended to investigate survival rates in high temperature situations.

FINAL CONSIDERATION

The pilot action was aimed at promoting and developing innovative aquaculture technologies tofacilitate and promote the diversification of aquaculture production towards species that are still under-exploited but of high commercial value. The innovative aspect was represented by the combination of oyster farming with echinoculture, in order to guarantee a reduction in breeding costs for aquaculture operators; maximise yields and reduce environmental impact; and control fouling on structures thanks to the grazing action performed by sea urchins. Today, the promotion of oyster farming combined with echinoculture is a viable alternative, both economically and ecologically, to mussel farming.

During the various phases of the project, a number of critical issues were encountered, including high larval mortality and complex achievement of proper metamorphosis; these are very delicate phases that require large investments in order to work on an industrial scale. Much attention must be paid to the selection of the transfer area to the sea, water quality analyses are necessary (moreover, more sheltered areas such as lagoon areas could play in favour of sea urchin survival and would allow easier product management). The management of the organisms and in particular that of the algal cultures requires adequate facilities and the presence of specialised operators, and this could affect the final production in terms of costs. Further experimental trials will be necessary to define the protocol for the proper transfer and maintenance of organisms in baskets at mussel farms.

Achieving the stated objectives will make it possible to overcome the difficulty of finding seed for these species, increase knowledge in the field of oyster and echinoculture, optimise breeding and marketing with a view to the sustainable management of natural resources, and meet the expectations of business and the market with regard to species of high commercial and biological value.

In conclusion, therefore, we can say that excellent controlled reproduction protocols have been developed, which have made it possible to follow the various developmental stages of the organisms through to metamorphosis into juveniles. This protocol can easily be replicated, and new trials can be developed along both the Croatian and Italian coasts to assess the actual effectiveness of the proposed technique. The flat oyster is a promising candidate for the productive diversification of shellfish farming in Italy. Polyculture with the sea urchin represents an enormous added value both for fouling control and for a possible repopulation project of the species, which is now extremely exploited in the wild. At the moment, controlled reproduction protocols at the laboratory level cannot meet the huge market demand for oyster seed. Therefore, good management of natural resources is essential in order to be able to start collecting natural seed directly and then supplement it with industrially produced seed. All this with the ultimate aim of protecting the resources of the Adriatic Sea.