Compilation of Culture Experiences

cockles

COCKLES PROJECT

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EXECUTIVE SUMMARY

The common cockle, Cerastoderma edule, is a bivalve mollusc species that lives in temperate waters, in intertidal and shallow subtidal areas, and its geographic distribution extends along the north-eastern Atlantic coastline, from the western region of the Barents Sea and the Baltic Sea to the Iberian Peninsula, and south along the coast of West Africa to Senegal (Hayward and Ryland 1995).

The common cockle is widely exploited and supports important commercial bivalve fisheries throughout its geographical distribution (Freire et al., 2010; Dare et al., 2004; Malham et al., 2012; Martinez-Castro and Vázquez, 2012), particularly in coastal areas of the north-eastern Atlantic. However, according to FAO (2017) a sharp decrease in cockles' catch was observed in the last years and studies point at several causes.

The production of bivalve molluscs is a strategic activity since it contributes significantly to the preservation of coastal economies, generating distributed income and employment opportunities on the littoral areas. The bivalve aquaculture industry depends significantly on the availability of high-quality juveniles, with capacity to grow rapidly to commercial size, and which are less dependent on environmental conditions or on the fluctuations of the natural populations recruitments (Ojea et al., 2004).

The artificial production of bivalve juveniles has the potential to satisfy the needs of the aquaculture industry since it will allow obtaining a product under controlled conditions. For hatcheries to consistently produce seed it is essential to develop robust protocols for broodstock conditioning, larval, and post-larval techniques (Pernet et al., 2004; Pronker et al., 2013). The on-growing phase of bivalve aquaculture industry depends also on the availability of ground plots with adequate environmental conditions for the adequate growth of these organisms. This report compiles information on three different cockle culture initiatives carried out recently by three different research institutions in different locations, the first of them in Ria de Formosa in the South of Portugal, and the other in two southern rias of Galicia (Vigo and Arousa, in this order). The first one was carried out as part of the activities of the Interreg Atlantic Area COCKLES project while the other two are initiatives promoted under different projects and under the auspices of other funding mechanisms.

The three experiences demonstrate that rearing of C. edule juveniles is possible in hatcheries. The technology applied is that normally used for other bivalve culture. In addition, the short larval period of the species and the great contribution of yolk reserves during the larval period are undoubtedly great advantages in aquaculture.

Both, a system with open sea water circulation (FTS) and a system with recirculation (RAS) are feasible culture systems. Among other conclusions it is advisable to start the conditioning phase with breeding individuals who have already started their gametogenesis in the natural environment, in order to avoid a prolonged conditioning period that causes excessive stress on the conditioned individuals. For a quick response, we recommend conditioning these individuals in an open circuit system (FTS) while for longer maintenance and later spawning we recommend the use of the RAS system.

More studies are needed to optimize the production methodologies and consequently, maximize the success of cockle culture. The results achieved within the scope of COCKLES project and with the two other initiatives presented can effectively contribute to generate an interest to implement cockle's aquaculture and make significant progress. The production of this species can have a very favourable impact in terms of population preservation in areas where this species supports important fisheries, such as the cockle areas in Ireland, UK, France, Spain and Portugal.

INTRODUCTION

The common cockle, Cerastoderma edule, is a bivalve mollusc species that lives in temperate waters, in intertidal and shallow subtidal areas, and its geographic distribution extends along the north-eastern Atlantic coastline, from the western region of the Barents Sea and the Baltic Sea to the Iberian Peninsula, and south along the coast of West Africa to Senegal (Hayward and Ryland 1995). Cockles are particularly abundant in sandy substrates such as estuaries and bays (Dabouineau & Ponsero 2009), where population densities can reach several thousand individuals per square meter (Coosen et al. 1994, Tyler-Walters 2007).

The common cockle is widely exploited and supports important commercial bivalve fisheries throughout its geographical distribution (Freire et al., 2010; Dare et al., 2004; Malham et al., 2012; Martinez-Castro and Vázquez, 2012), particularly in coastal areas of the north-eastern Atlantic. However, according to FAO (2017) a sharp decrease in cockles' catch was observed in the last years. The synergistic action of fishing pressure coupled with the rapid growth rate, short lifespan and huge mortality of C. edule caused by different factors (mostly infectious diseases and extreme environmental conditions) leads to large inter-annual fluctuations in stock abundance and periodic recruitment failure (Ramón, 2003). In some years, the abundance of this species decreases dramatically, threatening the biological and eventually, the economic sustainability of this fishery. The development of restocking programs supported by advances in C. edule culture could represent an efficient strategy to rebuild stocks (Joaquim et al., 2007; Zhang et al., 2021). Particular attention deserves marteiliosis, a highly pathogenic disease that caused cockle fishery collapse in the southern rias of Galicia (NW Spain, Villalba et al., 2014; Iglesias et al., 2015). Producing marteiliosis-resistant cockle strains appears to be a promising approach to overcome this disease in endemic areas, considering the difficulties to fight against marteiliosis in an open sea context, and because selective breeding programs have proved successful to increase molluscs' resistance against various diseases (Dégremont et al., 2015; Smits et al., 2020; Potts et al., 2021).

The production of bivalve molluscs is a strategic activity since it contributes significantly to the preservation of coastal economies, generating distributed income and employment opportunities on the littoral areas. The bivalve aquaculture industry depends significantly on the availability of high-quality juveniles, with capacity to grow rapidly to commercial size, and which are less dependent on environmental conditions or on the fluctuations of the natural populations recruitments (Ojea et al., 2004). Aquaculture also enables the selection of the size and weight of most suitable seeds to initiate the growing phase during all year long. The control of the zootechnical conditions allows the production of juveniles with certain features of interest such as optimal survival and growth rates, disease resistance, etc. In this sense, the artificial production of bivalve juveniles has the potential to satisfy the needs of the aquaculture industry since it will allow obtaining a product under controlled conditions. For hatcheries to consistently produce seed it is essential to develop robust protocols for broodstock conditioning, larval, and post-larval techniques (Pernet et al., 2004; Pronker et al., 2013). The on-growing phase of bivalve aquaculture industry depends also on the availability of ground plots with adequate environmental conditions for the adequate growth of these organisms. The seasonal environmental changes and the localization of the ground plots have a great influence on the bivalve population development, namely in survival, growth, and reproduction. Indeed, the quality and food availably are factors of great importance to the bivalve ground plot productivity. Due to the high social and economic importance of C. edule it seems essential to optimize the production conditions of this species to ensure the economic viability of its culture and develop restocking and selective breeding programs.

The culture of cockles both in hatchery or shellfish plots is not a common practice as for other bivalves, such as clams and oysters. Usually, in addition to fishing this species, bivalve producers collect cockle specimens that grow naturally in their licensed areas in the intertidal zone, but there is no practice of deploying hatchery-produced seed. However, the interest of the industry to develop reliable hatchery production and land-based grow-out techniques for cockles has increased considerably.

Currently, there is no commercial hatchery that can regularly provide cockle seed for growing out. Moreover, research in cockle culture is very scarce and only 3 reports have been found on this subject. Pronker et al. (2013) described for the first time the cultivation of Cerastoderma edule on a commercial scale. They concluded that the best diet for broodstock conditioning is 100% of the microalgae Tetraselmis suecica, that induced 12% of female to spawn about three thousand eggs. A mixed diet of Phaeodactilum tricornutum and Skeletonema costatum, fulfilled the dietary requirements of seed to obtain an average growth rate of 168 µm day-1 and an average final size of 19.0±1.9 mm. Also, these authors demonstrated that hatchery produced cockles (F1) can act as broodstock, thereby, closing the production cycle. It was possible to obtain several millions of eggs and larvae at each spawning event, although this part of the production cycle still needs further improvement. This information suggests that large scale cockle cultivation in hatchery seems technically feasible, but still many questions must be answered before this culture becomes economically viable. Ferreira et al. (2015) also found a progress in the gametogenic cycle when broodstock was fed with diet incorporated with Rhodomonas lens. According to the authors, this diet increased gonadal condition index and extended the stages of gonad maturation and spawning. Finally, Fuentes et al. (2015) also reported good results in inducing sexual maturation of cockles and successful spawning, however, without specifying which microalgae species were supplied in conditioning.

Restocking shellfish plots with the release of juveniles produced in hatchery to rebuild the biomass of the wild populations to a level where the fishery can provide regular harvests can be a solution to reach the carrying capacity of the habitat and, consequently, to increase the productivity of these areas. Also, the transplantation of juveniles from natural cockle beds to shellfish plots (within the same area) to rebuild relatively high-density patches can be a solution for cockles' economy.
This report is aimed to compile information on three different cockle culture initiatives carried out recently by three different research institutions in different locations, the first of them in Ria de Formosa in the South of Portugal, and the other in two southern rias of Galicia (Vigo and Arousa, in this order). The main objective of this report is not to recommend one or another approach among those described, but to provide evidence of recent progress and to inform on support infrastructure and research teams with demonstrated specific background and capacity to help future attempts by the industry; or for any other stakeholders willing to further invest and foster the recovery of cockle culture and production, building upon the lessons learnt from these past experiences on what it concerns to cultivation strategies.

Finally, it is important to state that the only research action, of those the three further on described, that was funded and performed within the COCKLES project (funded by EU INTERREG, Atlantic Area programme) was the one developed by IPMA. The descriptions of the experiments by CIMA-Xunta de Galicia (Fuentes, 2016, 2020) and CIM-ECIMAT University of Vigo (Costas Costas D. et al., pers. comm., 2021) were compiled and adapted to be integrated in this COCKLES' project deliverable, to provide the stakeholders with more comprehensive information on this subject.

PRODUCTION EXPERIENCES

I . Instituto Portugês do Mar e da Atmosfera (IPMA) COCKLES Project
C. edule culture experience at Estação Experimental de Moluscicultura de Tavira.

The studies developed in COCKLES project intended to contribute to restoring and increasing cockle's production. One of the project objectives was to develop efficient culture procedures, including hatchery protocols.

IPMA led a Research and Development action (for the project's Work Package 7.1) that comprised several sets of experiments which specifically investigated reproduction, nutrition, growth and zootechnical aspects of cockles, aiming for a successful control of the different culture stages and to establish an optimized culture protocol. The results obtained allowed to contribute to this report with the most important baselines for the aquaculture of cockles.

This section of the report is expanded with more details on the experimental developments publicly available at the COCKLES project website through the following LINK.

I.1 Broodstock conditioning

I.1.1 Seasonal considerations

Broodstock conditioning is a key step in the process of rearing bivalves in hatchery and is directly connected with the nutritional needs of the species when aiming to have sexually mature individuals out of his natural reproductive season. This process consists in maintaining breeders under conditions where different parameters such as water temperature and food addition are controlled to achieve the complete ripening of the gonads or the extension of the spawning season.

Normally, the length of the conditioning period can vary from two to ten weeks, depending on the initial gonad condition of the individuals. In some bivalve species, best results are obtained when broodstock conditioning starts at a resting gonad stage, however, it is also possible to achieve ripe gonads starting the conditioning when gametogenesis is relatively advanced, with an adequate diet. In Galicia and Southern Portugal, the onset of cockle gametogenesis takes place in early autumn, progresses throughout the winter, and the mature stage is finally reached in spring, then the spawning period runs from mid spring to mid-summer (Martínez Castro and Vázquez, 2012; Maia et al., 2021). In this context, cockles conditioning should be initiated in late summer or early autumn, at resting gonad stage or in the onset of gametogenesis.

I.1.2 Broodstock pre-treatment

Adult cockles were collected in natural banks and transported dry to hatchery. Once in the hatchery, cockles were rinsed to eliminate some detritus and placed in quarantine tanks with seawater at 2 to 4°C below the environmental temperature for at least one week before conditioning. During this period, the minimum amount of food was administered for their survival.

I.1.3 Tank systems and water treatment

The conditioning was performed in 25 to 60-L plastic tanks in a flow-through system (Figure 1) at a flow rate of 0.8 L min-1 with natural sand-filtered seawater and aerated (0.5 L min-1). Adult cockles were placed in the tanks inside perforated trays to allow the passage of faeces and debris at a density of 340 ind. m-2 per tank. Sieves of 20 and 100 µm were kept at the end of the tanks for the collection

of eggs. Survival of progenitors and spontaneous spawning were evaluated daily.
The maintenance of the adequate conditions for cultivation, hygiene and animal health are critical during the conditioning. Tanks were sniffed to remove faeces every two days and a complete cleaning for tanks disinfection was performed once a week. The remaining system components, such as filters, water and/or food pipes, etc., had a cleaning schedule that could ensure water and food quality.

Figure 1
Conditioning tanks

I.1.4 Broodstock feeding

Likewise for other bivalves' species, in the exponential growth phase, microalgae were added daily with the help of a pump, at a ratio of 4% of cockles' dry weight (g) in microalgae dry weight (mg). Microalgae concentration and the proportion of the different species (diet formulation) were calculated daily according to the number of individuals in each tank.

Aiming to identify the most adequate diet for cockles conditioning phase, three different food regimes previously tested for conditioning other bivalve species, were tested:

- Diet 1 – Isochrysis aff galbana (T-iso) 75%+ Chaetoceros calcitrans (C.cal) 25%
- Diet 2 – Isochrysis aff galbana (T-iso) 25%+ Chaetoceros calcitrans (C.cal) 75%
- Diet 3 – Tetraselmis suecica 100% (Pronker et al., 2013)

The Diet 1 and Diet 2 with different proportions of each microalgae had been normally used for clams (Matias et al., 2016) and, Diet 3 constituted only by the microalgae Tetraselmis suecica, was chosen with the objective to compare with published data which suggest this diet as the most adequate for conditioning cockle species (Pronker et al., 2013).

Food flows were regulated to 0.05 L min^{-1} to ensure a continuously and adequate concentration of microalgae in each tank during all day, allowing a proper feeding of individuals.

Results obtained in COCKLES project showed that the best sexual maturation process was obtained with a bi-specific diet, which contained a superior component of diatoms (Diet 2 - Isochrysis aff galbana 25% + Chaetoceros calcitrans 75%). With the referenced diet for cockles conditioning, the condition index decreased significantly and there was no gonad development.

I.1.5 Temperature regime

Temperature was registered and controlled daily and kept constant at 21±1ºC.

I.1.6 Photoperiod

The effect of photoperiod is important for the success of gonadal development of the bivalves. An alternated combination of temperature and photoperiod according to natural conditions revealed a clear development of sexual products and enabled spawns out of natural periods. The photoperiod proposed by IPMA was 9L:15D hours (Light/Dark).

I.1.7 Gamete quality evaluation

The gonadal development was determined by the evaluation of condition index and gonads' smear of some individuals. During the conditioning, condition index should rise reflecting the increased weight of the gonad, resulting from the gonadal development process. At the end of conditioning, most part of the broodstock individuals should present the gonad

on the ripe stage, corresponding to the maturity of most of the gametes. In the mature oocytes the rupture of the peduncle occurs, and the oocytes consequently occupy the follicular interior. In males, the gonadal acini mainly contain spermatozoids.

I.2 Spawning and fertilization

Spawning induction allows obtaining gametes when individuals are sexually mature. Breeders are stimulated to release their gametes in response to the stimulation that is applied.

During COCKLES project experiments, the spawning by thermal shocks (Figure 2) after the broodstock conditioning was tried without success. So, spawning occurred spontaneously in conditioning tanks.

Figure 2
Spawning by thermal shocks

Fertilization took place immediately after gametes released in tanks. Fertilized eggs and embryos were retained on a 20 µm sieve after passing through a 100 µm sieve to remove debris (Figure 3).

Figure 3:
Sieves for eggs collect

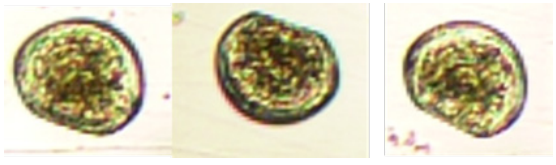
I.3 Larvae culture

I.3.1 Embryonic phase

C. edule fertilized eggs and embryos were collected on a sieve, washed with filtered (0.35 µm) and UV-treated seawater, and redistributed in a known volume of filtered seawater. Subsamples were taken and counted aiming to determine the total number of fertilized eggs and embryos.

Embryos were put to incubate in fiberglass cylinder-conical tanks at a density of 100 embryos ml^{-1} with seawater filtered at 0.35 µm cartridge filter and ultraviolet treatment, at 21º C with very slight aeration. In general, we do not recommend aeration during this early stage because the mechanical effects of the disturbance can lead to abnormal development. However, for embryos incubation in the cylinder-conical tanks, a very slight aeration should be used to avoid the aggregation of embryos at the bottom of the tank.

Fully shelly D-larvae were recovered 24 hours later, and tanks containing newly developed D-larvae were drained to a 20 µm mesh screen and subsequently drained to a graduate beaker (Figure 4). The D larvae retained in a beaker were homogenized and small sub-samples (approximately 1 mL) were often taken do determine the total number and to measure the shell length of veliger. No food was added at this phase (incubation) because bivalve embryonic phase and trocophora larvae do not have the ability to feed. During this period, energy comes from reserves laid down during egg development (oogenesis) by the maturing females.



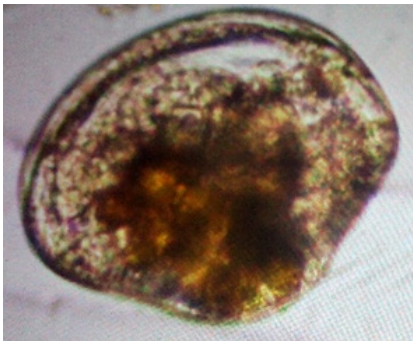


Figure 4:
Cerastoderma edule larvae

I.3.2 Larval phase

At the post-incubation phase, C. edule larvae need to be fed with unicellular cultured microalgae.

Two systems were tested:

- Batch rearing system

Batch rearing is the traditional system for larval rearing. Larval culture tanks and all equipment used was thoroughly cleaned and then rinsed, it can be done with either freshwater or filter seawater.

After incubation, larvae were transferred to the same cylinder-conical tanks (Figure 5) with seawater, filtered with a 0.35 µm cartridge filter and ultraviolet treatment, at 21º C and with aeration (0.5 L min^{-1}), at an initial density of 7 larvae ml-1.

Figure 5
Batch system. Cylinder-conical tanks for larval rearing

During larval culture, seawater was changed three times a week. In general, the procedure for water-change days was like that described for embryos incubation. The tank was emptied by a bottom drain, delivering the discharge flow into a sieve battery with different mesh size gradient. In the top of the battery sieve, it is essential to use a mesh with aperture to retain large debris, and the bottom the mesh should be of smaller aperture size than the size of the larvae. In this way, larvae were retained on the mesh of the lower sieve.

- Recirculating Aquaculture System

Another methodology utilized for rearing larvae is RAS (Recirculating Aquaculture System). This system can be expected to improve productivity since it enables larvae rearing at higher densities (densities can be doubled or trebled) than in traditional Batch System and to a better and more efficient use of water, labour-time, and space.

Time can be saved in raising the density of larvae without the need to drain statically operated tanks 3 or 4 times each week. The RAS system used in the experimental hatchery of IPMA is composed by culture and sump unities (Figure 6). The sump consists in a reservoir which contains the biofilter. Seawater from the culture unit was collected in the inlet section of the sump and flowed upwards into the decanting cabinet. Thereafter, seawater flows from the top to the biofilter section. The filtered water passes to the resting cabinet where it is pumped to the culture unit at 1000 mL per min. after passing through a U.V. system. The culture units contain a filter whose mesh size varies according to size of the larvae. In this system only 10% of the water is changed daily, essentially to avoid salinity rises.

Figure 6
Recirculating aquaculture system (RAS) for larval rearing.

- Nutrition regime

At this stage, larvae need feeding with unicellular cultured microalgae. Usually, progressive diet is used to meet growing biomass. Normally in their first days, bivalve larvae are mixotrophic, that means that yolk reserves contribute to the maintenance of the larvae, so the input of phytoplankton should be low

Consistently, commercially species used in hatcheries worldwide typically include the flagellate Isocrhysis aff galbana (Clone T-iso) and the diatom Chaetoceros calcitrans; both have good nutritional proprieties as feed for many aquaculture organisms (e.g. Gouda et al., 2006). The quality of the food provided to culture bivalve larvae is indeed a critical factor in the quality and health of the larvae and is a major contributor to the success of aquaculture operations (Brown, 2002; Gouda et al., 2006). A key to this success is the identification of dietary regimes that result in maximum growth, survival, and settlement, which in turn will reduce hatchery operating costs.

Aiming to provide crucial information on its nutritional requirements and to identify the most adequate diet for this stage of cockle lifecycle, four diets were tested under the COCKLES project: two progressive monospecific diet (flagellate Isocrhysis aff galbana (Clone T-iso) and the diatom Chaetoceros calcitrans), one progressive bispecific diet (flagellate I. aff galbana (Clone T-iso) plus the diatom C. calcitrans) and a control without food (unfed give the knowledge of the reach of the yolk reserves) (Figure 7):

- **Diet 1 – *Isochrysis aff galbana (T-iso)***

50 cells.µl^{-1}	75 cells.µl^{-1}	100 cells.µl^{-1}
2º – 5º day	5º – 10º day	10º – 11º day

- **Diet 2 – *Chaetoceros calcitrans (C.cal)***

68 cells.µl^{-1}	100 cells.µl^{-1}	135 cells.µl^{-1}
2º – 5º day	5º – 10º day	10º – 11º day

- **Diet 3 – *Isochrysis aff galbana + Chaetoceros calcitrans (Tiso+Ccal)***

100 cells.µl^{-1} T-iso	60 T-iso + 40 C.cal cells.µl^{-1}	40 T-iso+ 60 C.cal cells.µl^{-1}
2º – 5º day	5º – 10º day	*10º – 11º* day

- **Unfed**

* Larvae were fed daily to provide equal biomass proportions of T-iso and C. cal in a ration cell number

Figure 7 :
Recirculating aquaculture system (RAS) for larval rearing

These diets were based on other nutritional regimes normally used for bivalves rearing. Larvae were reared at a Batch system (at the condition described above) and fed daily at a concentration according to the described nutritional regimes. At each water change day, sub-samples were taken to evaluate survival and growth, and the percentage of settlement (presence of foot).

The mono-specific diet T-iso proved to be the most suitable diet for larval rearing in COCKLES' experiments. The settlement was initiated after the 11th day of culture. The bi-specific diet is normally the best one for larval stage in other bivalve species such as clams and oysters. However, the survival results of this diet did not work so well when the metamorphosis phase began. The introduction of C. cal. at an early stage or at the metamorphosis phase did not produce better results.

The unfed revels that the yolk reserves have a long reach in larval development and that the introduction of food can be a disturbing factor for survival in detriment of its benefit. However, taking into consideration that the use of a monospecific diet during hatchery production could greatly facilitate routine management, a specific recommendation, based on results, to achieve optimal larval growth, would be to use I. aff galbana monospecific diet during all larval phases.

- Batch System vs. Recirculating Aquaculture System

Temperature (approximately 2 ºC) and salinity (approximately 2ppm) requirements increase in RAS and oxygen (approximately 2 mg/L) decreases, in comparison to the Batch system. The increase in temperature in RAS is generally beneficial in larval rearing, since it normally speeds up larval growth without detriment of survival, since the sump unit of the system maintains water quality, in COCKLES project experiments the results showed that survival and growth of C. edule larvae was higher in RAS than in the Batch. Larvae culture in RAS could be performed at an initial density of 30 larvae per mL without significant losses in survival, compared to the low density, however growth can be affected. At day 11, after fertilization, over 70 % of larvae reared in the RAS presented foot, while in Batch the percentage was much lower (49 %). Furthermore, larvae grown in RAS were ≈ 30 µm larger in length than those rearing in Batch. Therefore, the C. edule larval rearing performed at high stocking densities in RAS presented a reduction in the operating costs to produce this species.

I.4 Settlement and metamorphosis

Metamorphosis is a critical time in bivalves' lifecycle, during which the animal changes from a swimming planktonic to a sedentary benthic existence. At this phase considerable mortalities can occur because feeding activity slows down because larvae spend an increasing period close to the bottom and in the bottom of the tank (e.g. Hiddink et al., 2002). This marks the beginning of the settlement. Successful transformation and survival depend on several factors, among those the availability of energy reserves accumulated during the larval phase.

Cockle larvae begin substrate search behaviour with approximately 200 to 220 µm shell length around the 11th and 12th days of larval culture (Figure 8). This phenomenon is evident when it is observed that they attach to surfaces and to each other, with byssus threads and when it can be observed the presence of foot

Figure 8
Cerastoderma edule post-larvae

When larval culture presented more than 30 % of larvae with foot, they were transferred to a sieve with 100 µm mesh at a density around 100 per cm2 of the base area. However, they were kept at the same cylinder-conical tanks for culture larvae with seawater, filter at 0.35 µm cartridge filter and ultra-violet treatment, at 21º C (Figure 9). Each sieve was provided with an air-lift with a down-welling flow.

Figure 9
Post-larvae rearing system

Settlement tanks were treated the same as larval culture tanks. Water was changed 3 times a week draining water through a sieve to retain the remaining swimming larvae. Larvae retained on the sieve were counted, their survival and the numbers of metamorphosed seed estimated before returning them to the tank. Tanks were gently aerated during this period. Food was daily added to the tanks at a concentration of 150 cells.µl^{-1}. Normally, diet was composed of flagellate and diatom at a proportion 1:1. Cockles stood at these conditions for two weeks.

I.5 Indoor seed culture (nursery)

Seed were transferred in cylinder sieves with different meshes, depending on the seed' size, at a density of around 10 per cm2 of the base area, to rectangular tanks with a down-welling flow of water. In this phase water passes only by a sand filter and room temperature is used (Figure 10).

Figure 10
Juveniles rearing system

As in cylindric-conic tanks, water quality was maintained by complete water changes twice or three times each week. For cleaning, cylinders were removed and sprayed with a seawater jet to dislodge and remove detritus adhering to the seed and to the mesh of the containers. The reservoir and holding tanks were cleaned and refilled before returning the seed.

Water with algal food was pumped from the food reservoir into the holding tank. Food suitable for growing cockles seed in closely controlled conditions within hatcheries were the same as that used for larval culture. Diets for seed were constituted by a mixture of different microalgae such as Skeletonema costatum, Chaetoceros calcitrans and Isochrysis galbana. Daily food ration was given in a proportion

of 2 % of the dry meat weight in dry weight of algae and it was adjusted every week.

Figure 11
Seeding in a production area

The on-growing experiments carried out under the COCKLES project, showed that the net protection against predators increased survival between 15 and 20% in seed with an initial size of 19 mm (2.2g. by weight), in the first month after restocking (Figure 12). After one month of culture, juveniles were robust enough and well adapted to the new habitat to remove the net protection. These juveniles increased about 60 µm per day in a mean period of 3.8 autumn/winter months for both densities tested (mean wild density - 1200g.m^{-2}; high density - 1800g.m^{-2}). During the on-growing period the culture area still needed cleaning of macroalgae, detritus and predators. In these conditions, biomass yield was around 1400g.m^{-2} and individuals reached 26 mm of mean length and 5g of mean fresh weight.

Figure 12
Protection net

I.6 Suspended culture

Another way to grow this species it is in a suspended structures in earthen ponds in tanks used for fish production in polyculture. Integrated multitrophic aquaculture is expected efficient, economically attractive, robust, and environmentally friendly. This type of aquaculture takes advantages on the nutrient and energy efficiency gains generated by associating different aquatic species of different levels in the food web.

Two different equipment were used for ongrowing:
- rigid mesh bags with 50x100cm and sets of baskets with 40 cm of diameter and 10 cm of height which were divided by 4 little triangular baskets to allow homogeneous distribution of individuals. In these baskets 2 levels of culture depth (20 cm and 40 cm) were used (Figure 13 and Figure 14).

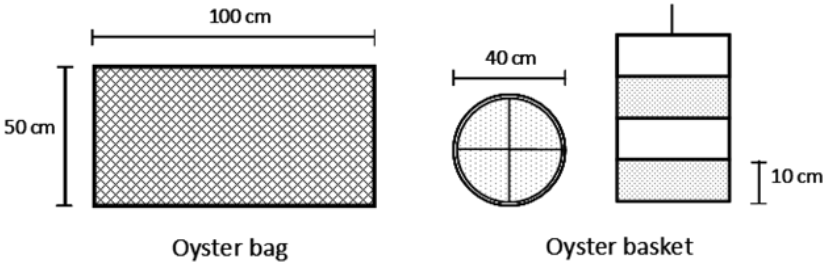


Figure 13
Scheme of oyster bag and basket.

Figure 14
Oyster baskets

Rigid Mesh bags and the sets of baskets were hanged on a cable with floaters (Figure 15 and Figure 16). These structures required cleaning labour once a month or once every fifteen days, depending on the level of encrustation of macroalgae and other fouling organisms. Concerning, rigid mesh bags cleaning, was only necessary to turn them over to maintain the water circulation. However, baskets needed replacement and being washed with fresh water or leaving them to dry in the sun to later remove the attached organisms.

In the experiments carried out under the COCKLES project, polyculture tanks showed better results in terms of growth than intertidal shellfish plots. In the same period (3.8 autumn/winter months), growth rate of cockle juveniles reared in the set of baskets reached 85 µm per day (≈ 29 mm length and 7.3 g of weight). In rigid mesh bags, growth was slower (≈ 26 mm length and 5.3 g of weight), however, this culture structure had the advantage of requiring less cleaning than the basket.

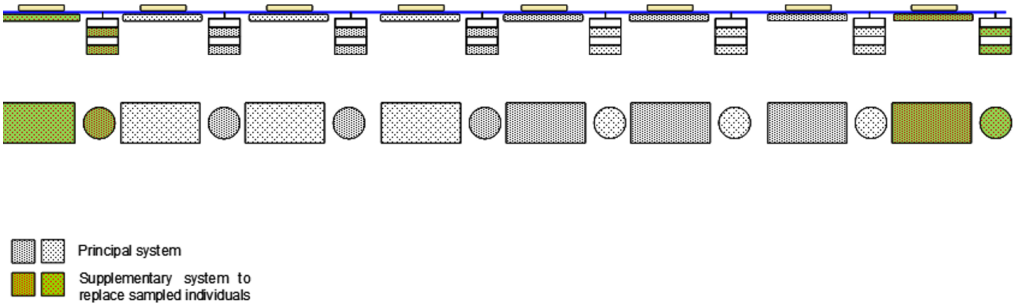


Figure 15
Scheme of the culture procedure in the IMTA

Figure 16
Culture procedure in the IMTA

Despite achieving greater growth increments in the culture of cockles in earth ponds tanks compared to their culture in intertidal shellfish plots, the cockles produced in this system presented shell deformations resulting from the lack of sediment in the culture structures. These shell deformations do not increase mortality but decrease the quality of the product and create obstacles to their sale directly to the consumer. However, this product could be explored as an option for the processing industry.

I.7 Conclusions and/or recommendations

In conclusion, the rearing of C. edule juveniles is possible in hatcheries. The technology applied was that normally used for other bivalve culture. In addition, the short larval period of the species and the great contribution of yolk reserves during the larval period are undoubtedly great advantages in aquaculture. This fact significantly reduces the occurrence of problems during rearing, increasing larval success and consequently making the process cheaper. Indeed, larval rearing performed at high larval stocking densities in RAS system presenting a reduction in the operating costs to produce this species. The larval rearing in RAS system can contribute positively to the promotion of restocking programs based on hatchery produced larvae.

More studies are needed to improve production methodologies and consequently, maximize the success of cockle culture. The results achieved within the scope of COCKLES project can effectively contribute to generate an interest to implement cockle's aquaculture. The production of this species can have a very favourable impact in terms of population preservation in areas where they support important fisheries, such as the cockle areas in Ireland, UK, France, Spain and Portugal.

II Marine Research Centre-University of Vigo (ECIMAT) – CULTURE EXPERIENCES OF THE COMMON COCKLE, Cerastoderma edule (L.) at the ECIMAT (hatchery service)

The common cockle, Cerastoderma edule, has historically been one of the most caught bivalves in Galicia (NW of Spain) (www.pescadegalicia.com). This species is characterized by a marked annual variability in density and biomass. In 2012, an outbreak of Marteilia cochillia caused a massive mortality leading to a drastic decrease in the resource (Villalba et al., 2014). Thus, C. edule has become a species of interest from multiple points of view, for which there are not enough studies on the growth and in general on the zootechnics of the culture. This work presents the results obtained at the ECIMAT-CIM University of Vigo, in culture experiments from 2014 to 2020.

II.1 Broodstock conditioning

II.1.1 Calendar/seasonal considerations

At the ECIMAT-CIM University of Vigo, spawns have been obtained from February to June since 2014 to 2020, using broodstock collected from natural beds in the rías of Noia, Arousa and Vigo. From April to June, spontaneous or induced gamete releases could be obtained with cockles from the beds, with no need for conditioning because many of them would have ripe gonads; in the previous months, after a brief conditioning.

II.1.2 Tank systems and water treatment

Conditioning was carried out in 50-liter polypropylene boxes, at a housing density of 120-140 individuals / box, approximately 15-20g / l, 50% / hour of water renewal and without aeration. Handling was kept to a minimum, reducing it to the daily removal of dead individuals, daily temperature control, weekly flow control, and cleaning the bottom of the boxes.

The temperature gradient started with the seawater filtered through sand at room temperature and progressively introducing seawater at 18ºC, filtered at 0.5 microns, sterilized with ultraviolet radiation, increasing 1 °C each 5 days until reaching 18 °C. The influence of photoperiod and light intensity are not well-studied; anyway, photoperiod was that of the moment of capture at the conditioning start and the daily ratio light hours: darkness hours (L:D) was progressively increased to 18L: 6D. The use of filtered (0.5 µm) and sterilized seawater a few days before spawning induction as well as a controlled feeding enhanced the microbiological quality of the broodstock and limited the transmission of pathogens to the subsequent larval culture.

Figure 17
Broodstock conditioning

II.1.3 Broodstock feeding

The best results were obtained with a diet composed of equal parts in dry weight of Phaeodactylum tricornutum, Chaetoceros neogracile, Tisochrysis lutea and Rhodomonas lens, with a daily ration per individual equivalent to 6% of its dry weight in dry weight of microalgae, by continuous feeding. Average dry weights have ranged from 0.15 to 0.33 g ind-1. Diet comparison studies suggested that including species of high nutritional value such as R. lens can favour gonadal development and larger spawning (Ferreira et al. 2015).

Mortality at 15th day ranged from 40 -75% during conditioning in the period February-May (Figure 18). These values were higher than those described for other bivalves.

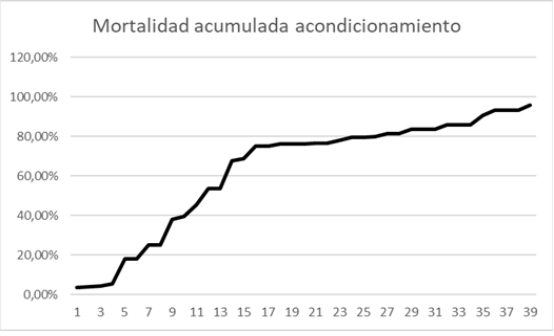


Figure 18
Cummulated mortality during conditioning. (May 2017)

II.1.4 Evaluation of gonadal maturity and spawning induction.

Twenty individuals were taken weekly (at days 7, 14, 21) to determine the gonad condition by examining histological sections under light microscope.

The rapid response to conditioning and restoration of the gonad in just a few weeks can be observed in Figures 19 and 20, starting from dominant ripe (April) or restoring gonad (June), respectively.

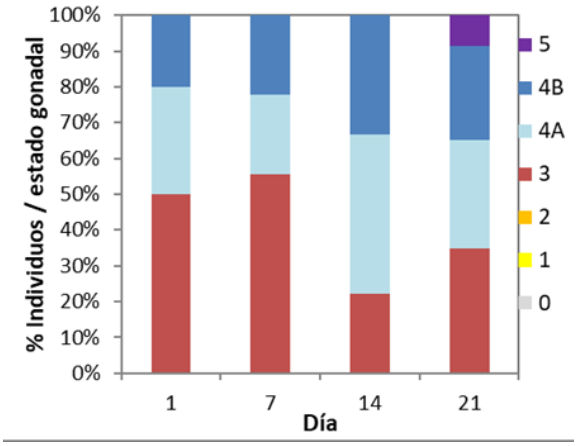


Figure 19
Distribution (%) of individuals in gonad condition stages at days 1st, 7th, 14th and 21st of conditioning in April..

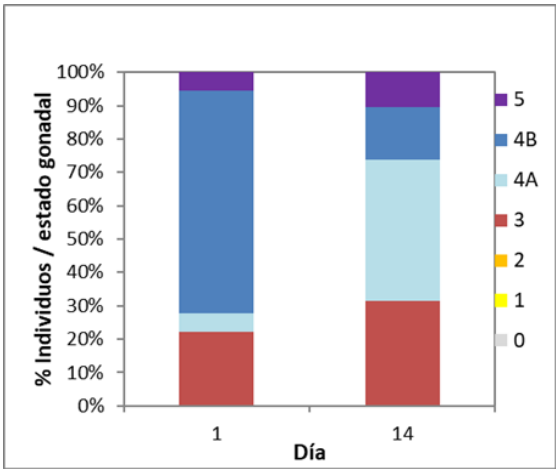


Figure 20
Distribution (%) of individuals in gonad condition stages at days 1st and 14th of conditioning in June 2020.

Satages of gonad condition (Martínez-Castro and Vázquez, 2012)

0	Resting gonad
1	Initiation
2	Advanced gametogenesis
3	Ripe
4A	Post-spawning
4B	Gonad restoration
5	Gonad resorption

II.1.5 Gamete quality evaluation

Mobility and density have been considered as indicators of good quality sperm. In the case of oocytes, they must be perfectly spherical, turgid and without damage to the chorionic envelope. Generally, after fertilization, the main quality indicator of the larvae is the percentage of transformation to D larvae stage 24 hours after fertilization.

We attempted to assess rapidly the laying quality through stress bioassays. The quantity and quality of eggs or larvae corresponding to each diet, female, origin, time of the year could not be properly verified. The composition of the gonad, the fatty acids, etc. are relevant, as well as the experience and knowledge about the animal husbandry and the species' behaviour by the technicians in charge of the hatchery. However, we have unsuccessfully searched for a simple, fast, and cheap indicator that allowed us to evaluate quality of D larvae 24 hours after fertilization (1ddf), as a response of the larvae as a whole, and not based on a single parameter, in presence of a stressor. Thus, (1) a salinity stress test was carried out involving the evaluation of the response of 24-hour D larvae exposed for a period of 96 hours to decreasing salinity values (S = 33, 17, 12, 10 and 8.5 ‰); (2) a starvation test in which the same methodology as in the salinity test was used, except that all individuals were exposed to a salinity of 34 ‰ and only the larvae of the control vials were fed at time 0 and 48 h, with the rest starving; (3) a copper test, in which salinity remained that of seawater (34 ‰), and organisms were exposed to increasing concentrations of dissolved copper in filtered seawater. Likewise, the larvae were fed with Tisochrysis lutea at time 0 and 48h.

The results showed that starvation for 96 hours implied survivals above 94% in all cases, salinity above 17 ppt implied survivals above 92%, while at 12ppt it stood at 13-23%, causing a total mortality when it went below 10ppt; and finally, in the copper test, survival rates were higher than 95% after 96h, even at values of 2560 µg / l. Thus, we were not able to reach the objective, being the salinity test for the range between 10-20 ppt a candidate to find, through further study, some significant difference in larval quality.

II.2 Spawining and fertilization

II.2.1 Fertilisation procedures

The spawns were obtained in two ways:

- Spontaneously, in the conditioning tanks:

The water in the conditioning tanks was poured into a 40 µm sieve with a 150-200 µm pre-sieve to remove faeces and other debris. Gamete releases were obtained at night and generally occurred on the day of the arrival of the cockles because many of them had ripe gonad or were in advanced gametogenesis (late spring). Foam, which normally appears on the sieve, is a good indicator of spawning. The pre-sieve was then removed, and a minimum water change was very gently made on the 40 µm sieve, with UV treated, 1 µm filtered seawater. Manipulation, even minimal, can cause damages in the chorionic envelope of the oocyte, which makes the development of the trochophore larvae unviable; manipulation drastically reduces the transformation to D larvae below 10%. In the case of spontaneous spawning in tanks, the eggs stick to the inner side of the tank as well as to the cockles, so part of the spawning is directly collected in the form of D larvae, after 24 hours.

- Controlled spawning induction.

The controlled induction was carried out in a traditional way alternating dry periods with sudden variations in water temperature between 10 and 22ºC. This can be performed in groups, in special induction tables, or individualized in glasses.

The day before induction, broodstock were left without food, they were removed from the water at

6:00 am, vigorously washed in freshwater and kept dry at 4ºC for about 2 hours to subsequently start the temperature shocks.

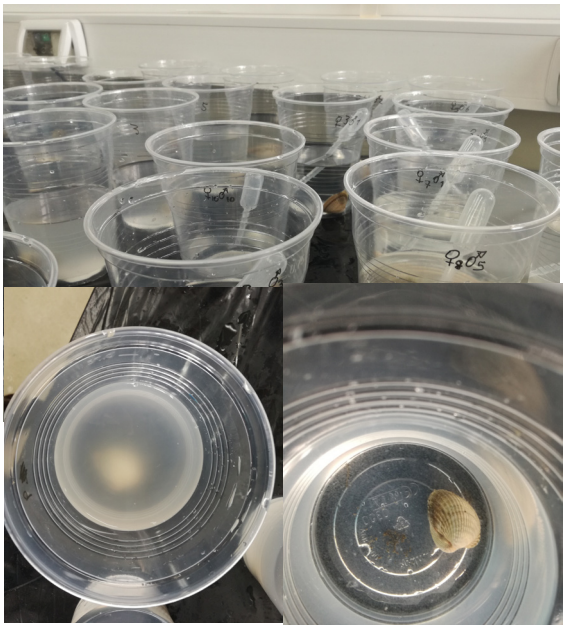


Figure 21
Individualized spawning induction. Male and female

Once the spawning had started, each female was identified and allowed to spawn for a maximum of 1 hour. Then the female was removed, and controlled fertilization was carried out with little amount of sperm filtered through a 20 µm sieve. We added 100-500 µl per spawning in several steps, the mix was gently stirred and the appearance of corpuscles on the oocytes or the presence of sperm passing through the chorionic envelope was checked every 5 minutes. Without practically observing sperm surrounding the oocytes (1-3 sperm / oocyte), fertilized oocytes were seen. Fertilization occurs extremely rapidly and adding too much sperm leads to polyspermy and aberrant development. The corpuscle appeared at 15-20 min and the first division 1 hour after fertilization, at 18 °C.

The number of eggs per female ranged from 20K to 1400K, with a mean value of 550K eggs per female (N=25)

II.3 Larvae culture

Approximately 1 hour after fertilization, with the first division, the fertilized oocytes were added to culture tanks with 0.5 µm filtered, UV-sterilized water, at 18-20 °C; the embryos and larvae were kept 24-hour in closed circuit with gentle aeration. At 22 hours after fertilization, most larvae were at D stage, thus and easily handled. The larval culture was carried out in both closed and open circuits.

The closed circuit larval culture was carried out in 150 l fiberglass, cylinder-conical tanks. The water was changed 3 times a week; initial density was 1-10 larvae / ml (mean of 6 larvae / l), temperature was 19ºC ± 0.5 and food consisted of between 40-80 equivalents of Isochrysis galbana (Eqlg) in a diet composed of equal parts of Tisochrysis lutea, Chaetoceros neogracile and Rhodomonas lens. No differences were found with diets initially composed of Tisochrysis lutea the first week, 50% Tisochrysis lutea + 50% Chaetoceros neogracile the second week and introduction of Rhodomonas lens before metamorphosis.
The open circuit larval culture it was performed under the same conditions, but with a daily renewal, upwelling water intake, 6 daily feedings through dispensers, a weekly screening of the larvae, and a greater quantity of feed.

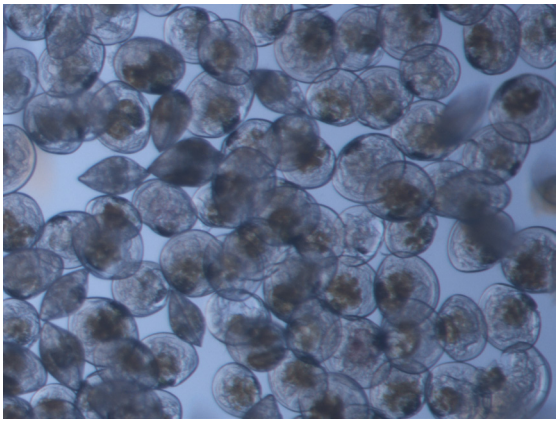


Figure 22
Larvae at 14 days after fertilization

Larval culture lasted 12-15 days and survival ranged between 0 and 60.8%, with a mean value of 25.21%. The following table shows the mean values of the distribution (percentage) of larvae into sieves at 14-15 days postfertilization (dpf):

Sieve (µm)		
120	150	180
12,88%*	27,91%*	68,75%*

* Mean percentages of larvae

II.4 Settlement and metamorphosis

Culture through settlement and metamorphosis was carried out in PVC cylinders with 150 micron nylon bottom mesh, without the need for stimulants or substrate, with downward flow. The same culture tanks used for larvae were used to hold those cylinders for the settlement-metamorphosis stage, either in a closed circuit with water change and seed cleaning three times per week or in open circuit with daily water renewal and weekly screening of the larvae/postlarvae. The maximal initial density was 200 larvae / cm2; other densities were not tested.

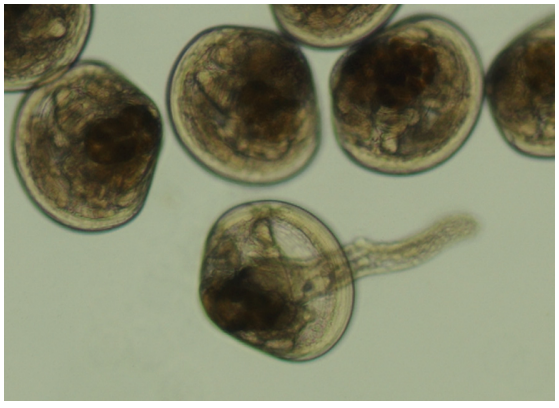


Figure 23
Semilla at 17 days after fertilization

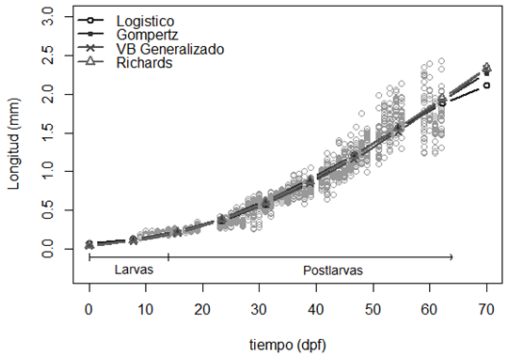
II.5 Indoor seed culture (nursery)

Once the settlement had finished, the postlarvae were kept in cylinders with bottom mesh of variable size depending on growth. Generally, the cylinders were hold in rectangular tanks with flat bottom, with upward or downward flow depending on availability. Initially, the same diet was maintained, progressively increasing the amount of food and including new species of microalgae such as Phaeodactylum tricornutum and Tetraselmis suecica. The seed remained very active, climbing the walls of the cylinders. The seed required daily cleaning on the cylinder itself and was sieved weekly. The seed was maintained in open flow with continuous feeding, which increased as seed grew. In case of appearance of biofilm on the valves, cleaning had to be intensified. The final survival for the period 14-63 days after fertilization (dpf) reached a maximum of 88.05%.

Figure 24
Cockle seed

II.5.1 Growth

The growth of C. edule was monitored and modelled for 63 dpf at 18ºC by daily measuring the antero-posterior length of the shell, reaching 1.5-2.7 mm at the end of this period. The data were adjusted to the 4 best known growth models (Gompertz, Richards, Logistic and generalised Von Bertalanffy), being the Logistic model the most appropriate to describe the growth during the first 2 months of life of C. edule in culture. (Cueto-Vega et al., 2015)



II.6 Outdoor culture

The seed was kept in passive suspended systems such as lantern nets, boxes or baskets, maintaining the usual tasks of mesh cleaning, sieving and density adjustment, starting from densities of 0.5-1 g / cm2. A phase of rapid growth occurred from April to October and a phase of slow growth from November to March, reaching up to 2.7 mm at 2nd month, 18.7 mm at 6th month and 23.4 mm at 12th month. The growth pattern obtained agrees with that previously described for this species (Molares et al., 2001; Ramón, 2003) and is related to hydrodynamics and the phytoplankton cycle in the Ría de Vigo (Ramón, 2003).

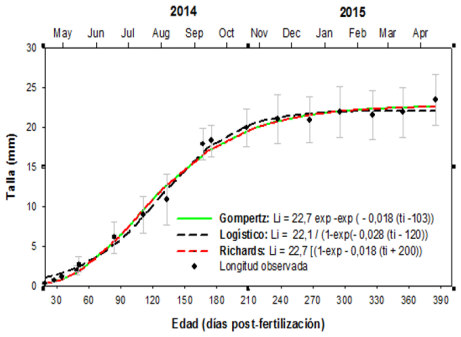


Figure 26
Growth curves and parameters estimated for C. edule. Symbols and error bars represent the mean and standard deviation values of the observed data. The lines represent the estimated values. (Hernández Otero, A, 2015)

The results suggest that obtaining spawns at the end of winter would enable the production of seed of more than 2 mm after 2 months and, by means of suspended growing out (hanging from a raft), to obtain seed suitable to be deployed in the field (18 mm) in 6 months, taking advantage of the entire period of maximum seasonal growth. Despite the presence of deformities during cultivation (7% with 175 dpf and 27% with 175 dpf), the survival obtained was reasonable (42% when cultured in a shellfish bed and 38% with suspended culture). Thus, obtaining market-size individuals in suspended culture without sand is possible. However, the high and growing number of deformed shells would make it difficult to sell these specimens in the fresh market, although it might represent an alternative for the canning industry.

- Deformities

Given the high number of deformities, especially accumulated during the winter period, a study on the deformities and their ability to recover was carried out. The study evaluated the growth, mortality and deformation of normal and deformed cockles (size> 20 mm) grown out in suspended systems without substrate or in natural sand (Martínez et al., 2016) up to close to the market-size.

Because a scale for deformed bivalves had not been previously established, individuals were first described according to different qualitative descriptors (shell opening, presence of a dented or spherical shape, presence of a bulge at the end of the shell, etc.) and finally were classified in five groups (Figure 27).

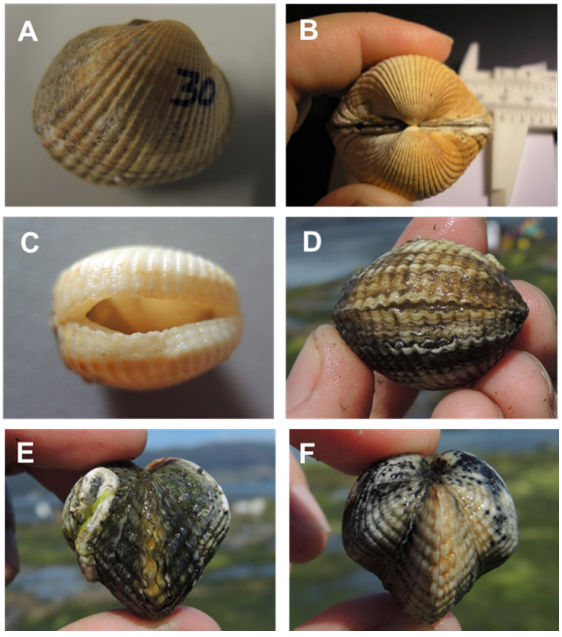


Figure 27
Photographs showing cockle shell deformities. Normal shells (Figs. A, B), open shells (deformity 1, Fig. C), recently closed shells with sharp edges or "sharp shells" (deformity 2, Fig. D), heart shaped shells (deformity 3, Fig. E), and shells with a bulge or "pointy shells" (deformity 4, Fig. F).

The deformed individuals did not recover their normal shape with any of the culture systems during the experiment. Contrary to what has been described for other bivalve species (Royo et al., 2005), we did not find recovery in the deformed cockles during on growing stages.

Cockles kept in suspended culture have a lower prevalence of pathogens than cockles from nearby natural beds. The relation of pathogens present in the cultured cockles is not altered by the presence of malformations in the shell. (Rodríguez et al, 2015).

Regarding the oceanographic conditions for the seed, it is important to point out mortalities of 100% at salinities below 15ppt after 4 days of exposure. Below this salinity, the cockles are inactive and the physiological rates (oxygen consumption, filtration and excretion) fall almost to zero. As salinity rises above this threshold, physiological rates progressively increase until they stabilise at salinity 20-25 ppt. (Peteiro et al., 2016).

II.7 Conclusions and/or recommendations

- A rapid gonad development of broodstock and obtaining spawns controlled by thermal shock is possible during late winter and spring. Conditioning outside this period should be studied. Studying the feasibility of obtaining spawns of conditioned individuals starting from gonadal rest would be interesting.

- The cultivation of cockle seed in a suspended system without substrate allows obtaining individuals suitable for deployment in the natural environment (~ 20 mm) in 7 months, taking advantage of the entire period of maximum seasonal growth. Obtaining market-size cockles (≥25 mm) in suspended culture without substrate is possible. However, the deformities and epibionts would make difficult selling these cockles as fresh product, although their use could be explored for the canning industry. Therefore, it is recommended to address further studies and to explore production strategies.

- According to these results, we advice using young, non-deformed specimens for seeding in shellfish beds or culture plots.

- The improvement of each of the cultivation procedures is possible and together with other results, allows establishing the bases of the zootechnics for the cultivation of cockles with multiple purposes.

III Cetro de investigacións mariñas (CIMA), Consellería do mar da Xunta de Galicia – IMPROVING COMMON COCKE CULTURE METHODS

The experiments next reported were carried out by a research team led by Dr. José Fuentes, within the projects "Posta a punto das técnicas de cultivo en criadeiro das especies do xénero Cerastoderma de Galicia (CROQUE)" ('Fine tuning of the hatchery culture techniques for species of the genus Cerastoderma in Galicia (CROQUE)') and "Engorde en batea de semente de berberecho producida en criadoiro. Mellora das metodoloxía de cultuvo (CROQUEDOUS)" ('On growing from raft of cockle seed produced in hatchery. Improvement of the cultivation methodology (CROQUEDOUS)'), funded by the Consellería do Mar da Xunta de Galicia.

III.1 Broodstock conditioning

III.1.1 1st Assay.

The first objective was to compare the conditioning process of cockle broodstock conditioning between an open circulation system (flow-through system, FTS, hereinafter) and a re-circulation system (RAS, hereinafter). We set a total of 8 x 30 L trays with perforated base and sloping bottom (Figure 28), 4 of them for the FTS and the other 4 for the RAS, as shown in Figure 29. Seawater in FTS came from the CIMA hatchery general circuit. This water was filtered through a battery of 25 µm, 5 µm, and 1 µm cartridge filters and UV sterilized (Figure 30).

Figure 28
Conditioning tray with perforated base and sloping bottom

Figure 29.
Setting of the 8 conditioning trays, with the 4 FTS trays (left) and the 4 RAS trays (right). The upper red taps correspond to the RAS circuit and the lower ones to the FTS.

Figure 30
Cartridge filter battery (25 µm, 5 µm and 1 µm) for FTS continuous circuit seawater filtration. The UV sterilization unit is shown on the right side of the image

The RAS consisted of a head tank, a reserve pond, a bio-filtration column, a protein separator skimmer, a UV sterilizer unit, and a heat exchanger (Figure 31). The system is filled with seawater from the general pipe circuit of the CIMA hatchery. Once the four conditioning trays were filled, re-circulation began through them. Before returning to the reserve raft, the water from the trays was first filtered by a mechanical perlon filter and then driven by a magnetic pump through an external battery of 20 µm, 5 µm and 1 µm filters. This external circulation was regulated by a pressure switch acting on two electro-valves. The renewal of recirculating water in the RAS was set at 10% per day (≈50 L / day for the whole system).

Figure 31
Seawater recirculation system (RAS) with reserve pond (BR), head tank (TC), biological filter (FB), heat exchanger (IC), "skimmer" (SK) and ultra-violet unit (UV)

Once the two systems (FTS and RAS) were set up, on Dec. 1st 2016, we started the conditioning of 800 cockles picked from a natural bed in the mouth of Rio Anllóns, in Cabana de Bergantiños (batch CE-RA). The mean length of these specimens was 29,96±0,38 mm (mean±s.e.) and the gonad index (GI) was 0,5 with 50% of the individuals in rest. The 800 individuals were distributed in groups of 100 in each tray (4 FTS trays and 4 RAS). The initial temperature of the seawater was 15,2ºC e 15,6ºC for RAS and FTS, respectively. Temperature was increased progressively to a maximum of 17ºC in both systems. The initial photoperiod of 9/15 hours (Light/Dark) was progressively altered until 12/12 hours L/D alternance. Cockles in the 8 trays were fed with a mix of microalgae of the genera Isochrysis, Diacronema, Rhodomonas, Tetraselmis, Chaetoceros e Skeletonema at the proportion 20:20:25:10:10:15. This mixture was distributed to the 8 trays from a 560 L capacity flat bottom cylindrical tank using an 8-channel peristaltic pump (Figure 32), with a flow rate of approximately 11 mL / minute / tray. The daily amount of food supplied (dry weight of microalgae) was equivalent to 2% of the dry weight of the cockles in the 8 trays.

The release of gametes occurred spontaneously, so a 40 µm sieve was placed in the drain of each tray to collect fertilized oocytes, embryos and even larvae that could have been produced in the trays after external fertilization of oocytes (emitted by females) by sperm (released by males). Each sieve was examined at the beginning of each working day and, occasionally, throughout it, to detect the presence of "eggs" (set of fertilized oocytes, embryos and larvae) in them, assess their quality and estimate the number of embryos and larvae. With this procedure, the same "spawn" could be derived from gametes provided by more than one male and more than one female.

Figure 32
Cylindrical tank of 560 L for microalgae storage. Three pumps for feed distribution are set above the tank; an 8 channel peristaltic pump is set in the middle

III.1.2 2nd Assay

On February 3rd 2017, we started a new comparative conditioning between the two systems. In this case, cockles collected from a shellfish bed in the inner side of the Ría de Muros- Noia (batch CE-N) were used. The mean length of these individuals was 32.65 ± 0.29 mm (± s.e.) and the state of gonadal development was much more advanced (GI = 1.67) with 67% of the individuals in advanced gametogenesis. In total we used 400 cockles at a rate of 100 individuals per tray (2 FTS trays and 2 RAS trays). The initial seawater temperature was 16.2ºC and 16.1ºC for the RAS and FTS systems, respectively. These temperatures were gradually increased to a maximum value of 17ºC in both systems. The initial photoperiod was 12/12 hours (L/D) and it was kept throughout the conditioning. The rest of the conditions were the same as those described for the previous assay.

The results obtained in these two comparative experiments varied according to both the initial state of the batch and the conditioning system used. The cumulative mortality of individuals in both batches (CE-RA and CE-N) conditioned in FTS was clearly higher than those conditioned in RAS. In the case of the CE-RA batch, 100% of the individuals kept in the FTS died before two months of conditioning, while only 27.8% of those in the RAS did so. The survivors were kept in the trays until March 23rd 2017 with a final cumulative mortality of 59.1%. Regarding the offspring obtained (Table 1), from the CE-RA lot we only obtained 3 spawns, and all of them from the individuals conditioned in the RAS. The first of these 3 spawns took place on March 6th 2017, more than three months after the start of conditioning. In the case of the CE-N batch, which was conditioned later, a similar trend was recorded, albeit in a less notorious way. In this case, FTS-conditioned individuals endured only one month in conditioning with a final mortality of 95.8% (Figure 33). In contrast, individuals conditioned on RAS survived for one more month with final mortality of 75.8% (Figure 33). Unlike the CE-RA batch, in this CE-N batch we obtained abundant offspring, more in the FTS system than in the RAS (Table 1). The first spawning occurred immediately (in less than a week) in the FTS system and somewhat later (four weeks) in the RAS. With respect to the number of larvae per spawn (Table 1), we did not detect significant differences either between batches of cockles or between circulation systems.

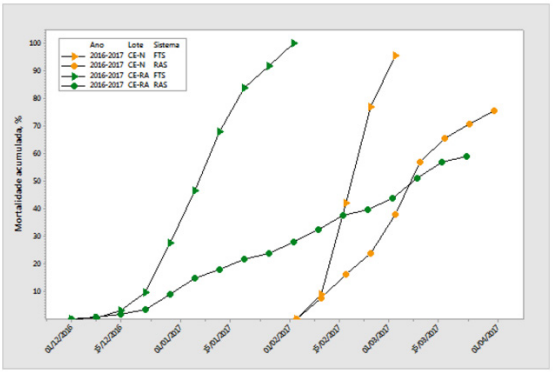


Figure 33
Cumulative mortality of cockles from CE-RA batch (green) and CE-N batch (orange) conditioned in FTS and RAS during 2016-2017

The disparity of results obtained between the two batches is probably not attributable to their geographical origin but to the different state of gonadal development at the start of conditioning. Conditioning involved severe stress, which was more intense (higher mortality and lower ripeness rate) in individuals maintained in the FTS than in those maintained in the RAS. In contrast, most individuals in the CE-N batch showed advanced gametogenesis, near gonadal ripeness, at the onset of conditioning. According to the results obtained, to avoid excessive stress in the conditioning process, it would be advisable to start this process with reproductive individuals which have already started their gametogenesis in the natural environment. For a quick response we recommend conditioning these individuals in an FTS system, while for longer maintenance and for obtaining later spawns we recommend the RAS system.

Batch	System	Spawning runs	Nº of larvae / spawning (mean±s.e.)
CE-RA	FTS	0	*
CE-RA	RAS	3	178500±153500
CE-N	FTS	11	159778±54455
CE-N	RAS	6	204167±83282

Table 1
Number of spawns and of larvae per spawn obtained from the CE-RA and CE-N batches conditioned in FTS and RAS

The disparity of results obtained between the two batches is probably not attributable to their geographical origin but to the different state of gonadal development at the start of conditioning. Conditioning involved severe stress, which was more intense (higher mortality and lower ripeness rate) in individuals maintained in the FTS than in those maintained in the RAS. In contrast, most individuals in the CE-N batch showed advanced gametogenesis, near gonadal ripeness, at the onset of conditioning. According to the results obtained, to avoid excessive stress in the conditioning process, it would be advisable to start this process with reproductive individuals which have already started their gametogenesis in the natural environment. For a quick response we recommend conditioning these individuals in an FTS system, while for longer maintenance and for obtaining later spawns we recommend the RAS system.

III.1.3 3rd Assay

A new process of comparative conditioning between the two systems (FTS versus RAS) was carried out using cockles from the same two origins (CE-N and CE-RA) to validate the previous conclusions. The conditioning began on January 11th 2018, with a total of 528 cockles from each batch (1.056 in total), divided into 8 trays (4 for FTS and 4 for RAS), at a rate of 132 individuals per tray. The gonadal index (GI) of these individuals was 1.0 and 1.7 for the CE-RA and CE-N batches, respectively, with more than 60% of the individuals in both groups with active gonad (initial, advanced gametogenesis, or ripeness). Conditioning was maintained until February 23rd 2018, following the same general conditions as in the previous experiments. In both FTS and RAS, spawning occurred faster in the cockles of the CE-N batch (2 weeks from the start of conditioning) than in the CE-RA batch (3 weeks). This difference was probably due to the higher degree of gonadal development of CE-N cockles. The total number of spawns was very similar for the two batches but very different between the two conditioning systems (Table 2). The values of the average number of larvae per spawn (Table 2) were clearly lower than those recorded in the previous year for the same batch and system combinations (Table 1). Again, no significant differences were detected in the number of larvae obtained per spawn between batches of cockles or between circulation systems.

Batch	System	Spawning runs	Nº of larvae / spawning (mean±s.e.)
CE-RA	FTS	5	134550±12650
CE-RA	RAS	2	29600±7400
CE-N	FTS	7	81971±23376
CE-N	RAS	1	96500

Table 2
Number of spawns and of larvae per spawn obtained from the two cockle batches CE-RA and CE-N conditioned in FTS and RAS

Again, the cumulative mortality of the specimens conditioned in FTS was considerably higher than that for the individuals conditioned in RAS (Figure 34). In the first weeks of conditioning, prior to the first spawn, mortality evolved similarly in both systems. Cumulative mortalities started to diverge once the conditioned individuals started to spawn. For batch CE-N, conditioned in RAS, the experiment had to be cancelled on 5th February 2018 because of a sudden contamination with bacteria, which rapidly increased cumulative mortality (Figure.34)

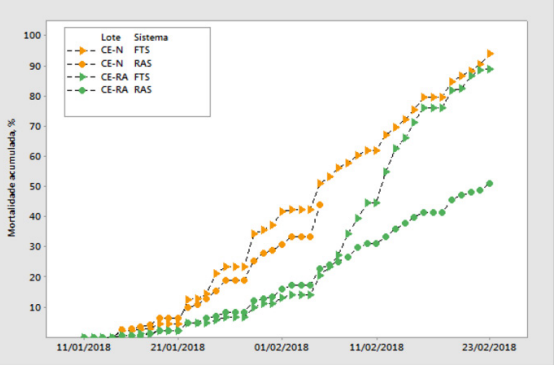


Figure 34
Cumulate mortality of cockles in batches CE-RA and CE-N conditioned in FTS and RAS along the year 2018

III.2 Spawning induction trials

III.2.1 1st Assay

Several attempts were made to induce the spawning of individuals of various batches of conditioned cockles in the CIMA hatchery in 2017. These attempts were made following, with just light variations, the same method of heat shocks (successive changes in seawater temperature) already used with other bivalve molluscs and, also, with cockles in previous experiments at CIMA. The results were unsatisfactory, without obtaining individualised and synchronised spawning of a sufficient number of males and females allowing crossbreeding. Surprisingly, many of these individuals who did not respond to thermal induction in the individual glasses spawned spontaneously some time (hours or days) after they had been returned to the conditioning trays.

III.2.2 2nd Assay

In 2018, we changed our strategy and we used cockles collected in the natural environment in June 2018, in the natural period of reproduction, instead of inducing the spawning of individuals from batches conditioned in the laboratory. We made three attempts to induce spawning with cockles from the mouth of the river Anllóns in Cabana de Bergantiños, and from Vilanova de Arousa (batches CE-RA and CE-VN, respectively). Once in the laboratory, the cockles were cleaned and kept in a dry refrigerator at ≈ 4ºC until the next day (≈15-20 hours). Then we transferred the cockles (≈50 individuals / batch) to individual plastic cups filled with sea water at 20ºC, filtered by 1µm and UV-sterilized. After ≈ 1 hour under these conditions, the cups were emptied and filled with seawater at 10°C. We failed to obtain spawns either from males or females in the first attempt (batch CE-RA). In the second attempt (batch CE-VN), we got spawns of 3 males and 4 females, although not in all cases with good-quality gametes. After selecting the highest-quality gametes (oocytes for their shape and sperm for their mobility), we performed a 3 male x 3 female crossbreeding in a 2L plastic recipient. After 48 hours, we found unfertilized, degraded oocytes but we did not detect embryos or D-larvae. In a third attempt (batch CE-VN), we obtained spawns of 3 males and 15 females; however, we did not obtain embryos or larvae in the crosses.

III.3 Larval rearing, settlement and metamorphosis

III.3.1 1st Assay

The larval rearing protocol, once the eggs were collected in the 40 µm sieves, continued with setting the fertilized oocytes and embryos into 50L truncated cone tanks (Figure 35), filled with 1 µm-filtered seawater, heated to 20°C and UV sterilized, and left for 48 hours without food and with light aeration. After these 48 hours, the content of each tank was passed through a battery of three sieves of 300, 60 and 40 µm, where large debris, D-larvae and unfertilized oocytes, respectively, were retained.

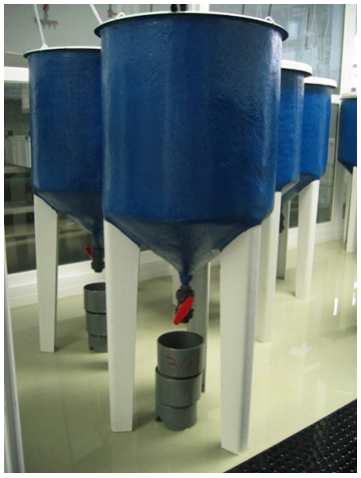


Figure 35
50 L truncated cone tanks for larval rearing

Once the D-larvae were separated, they were counted, and larval culture began in 50 L truncated cone tanks filled with seawater under the same conditions as in the first 48 hours. The larval culture density remained in most cases equal to or less than 10 larvae / mL. At this stage, we added food to the tanks, which were kept with a light aeration. The food provided consisted of a mixture of six microalgae of the genera Isochrysis, Diacronema, Rhodomonas, Tetraselmis, Chaetoceros and Skeletonema in the proportion 20: 20: 25: 10: 10: 15, respectively, and a concentration equivalent to 50,000 cells / mL of Isochrysis. In addition, antibiotics were used routinely to prevent deaths caused by pathogenic bacteria, specifically 8mg / L chloramphenicol. The larval rearing was carried out in open circuit, with a daily renewal of 10% of the seawater; the food was supplied with a peristaltic pump. Two days a week (usually Monday and Friday), the tanks were emptied, and the seawater with larvae passed through a battery of three sieves (60 + 85 + 100 µm, 85 + 100 + 150 µm, or 100 + 150 + 180 µm), depending on the size achieved. Approximately 15 days after the start of larval culture, when most larvae were retained in the 150 and 180 µm sieves, these larvae began to show both a subtle "eye" or eye spot and increased foot activity, unequivocal signs of the proximity of the metamorphosis. Then, the larvae with "eye" were transferred to PVC cylinders (15 cm x 30 cm Ø) with nylon mesh of 100-150 µm at the bottom, equipped with an "air-lift" aeration system to induce water moving through it. These cylinders were introduced into the same 50 L tanks, with seawater under similar characteristics to those used for the larval culture. During this phase of metamorphosis, the same percentage of seawater renewal and the same quantity and type of food was added. Once the metamorphosis of the larvae was completed, the postlarvae obtained were initially cultured inside the same PVC cylinders, in the 50L truncated cone tanks. this protocol was improved using a new polypropylene truncated cone tank with higher capacity (150 L) for larval culture.

During 2017, we launched several larval cultures from broodstock cockles collected from the inner side of the Ría de Muros-Noia (CE-N); from the mouth of the river Anllóns, in Cabana de Bergantiños (CE-RA); from the shellfish bed of O Sarrido, in Cambados (CE-S); from a shellfish bed of Vilanova de Arousa (CE-VN), and from the shellfish bed of Vilaxoán (CE-VX) (Table 3). The total percentage of larval cultures that were started (derived from spawns with good larval quality) with regard to the number of spawns obtained was quite high (Table 3), indicating a good overall quality of the collected larvae. The average number of larvae per spawn was highly variable, both between batches and between spawns of the same batch (Table 3). The percentage of larval cultures that reached the metamorphosis stage with regard to the number of larval cultures that were started also varied among the different batches (Table 3). The mean cumulative mortality from the beginning to the end of the larval culture, considering only those cultures that reached a metamorphosis stage, was 32.08 ± 4.23%.

Broodstock batch	No. of Spawns	No. of cultures initiated (% per No. of spawns)	No. of larvae / cultured spawn (mean±s.e.)	Nº of cultures reaching metamorphosis (% of initiated larvae culture)
CE-N	26	19 (73.1%)	254974±67861	7(36.8%)
CE-RA	16	9(56.3%)	695856±373213	4(44.4%)
CE-S	5	5 (100%)	392960±204392	4(80.0%)
CE-VN	13	9 (69.2%)	373094±270597	7(77.8%)
CE-VX	1	1 (100%)	2734000	0(0.0%)
Total	61	43(70.4%)	445671±60232	22(51.2%)

Table 3
Number of spawns, of larval cultures that were started, of larvae per spawn and of larval cultures reaching the metamorphosis from different batches of broodstock cockles in 2016-2017

Although the results obtained with the described protocol were satisfactory, the systematic use of chloramphenicol presents serious problems. The European Medicines Agency (EMA) and the Spanish Agency for Medicines and Health Products (AEMPS) include chloramphenicol in the list of banned antimicrobial products for the treatment of diseases in animals intended for food for human consumption. The FAO recommends the use of alternative products. Erythromycin and oxytetracycline are two commonly used antibiotics in aquaculture. For this reason, we conducted an experiment to evaluate the effect of treatment with these two antibiotics on the viability of cockle larvae. For this, we used a spawn of 1,185,000 larvae of the 5 spawns obtained from the CE-S batch (Table 3). We prepared 10 aliquots of ≈50,000 larvae each, which were transferred to each 5L polypropylene cups (≈10 larvae / mL). Eight of the ten cups were filled with 1 µm-filtered, UV-sterilized seawater from the CIMA FTS; two of these eight cups were supplied with 8 mg / L chloramphenicol, other two with 8 mg / L erythromycin, other two with 8 mg / L oxytetracycline, and the other two without antibiotics (FTS control). The remaining two cups were filled with water from the RAS (RAS control). The larvae were fed with a mixture of six microalgae of the genera Isochrysis, Diacronema, Rhodomonas, Tetraselmis, Chaetoceros, and Skeletonema, and each cup was provided with light aeration. Three days a week (Monday, Wednesday and Friday) the water was changed and new food and antibiotics were supplied. At the start of the experiment, and after 14, 21, and 28 days, we estimated the number of normal larvae, the number of deformed larvae, the number of dead larvae, and the number of metamorphosed post-larvae in each treatment. The most relevant results of this experiment are shown in Figures 36 and 37. The cumulative mortality of the untreated antibiotic larvae (FTS and RAS controls) was 100% on the 28th and was already very high on the 14th, even higher in the two replicas of the RAS control (Figure 36). In contrast, cumulative mortality on day 28th of antibiotic-treated larvae was clearly lower (34%, 31%, and 18% for chloramphenicol, oxytetracycline, and erythromycin, respectively).

In addition to the cumulative mortality in the larval period, another relevant variable to consider is the number of larvae that, after undergoing metamorphosis, reached the post-larval state. This variable could be used to evaluate a potential toxic effect of the antibiotics used. Figure 35 showed the evolution of the percentage of metamorphosed post-larvae during the experiment. The first metamorphosed post-larvae appeared 32 days after onset (Figure 37). There were no post-larvae in the cultures corresponding to the controls (FTS and RAS) because the larval mortality was 100% on day 28th. At the end of the experiment, after 42 days from the beginning, the average percentages of post-larvae in the cultures

treated with antibiotics were very similar (28.9%, 30.1%, and 37.0% for chloramphenicol, oxytetracycline, and erythromycin, respectively), with no significant differences. The results obtained in this experiment support the replacement of chloramphenicol by either of the two alternative antibiotics, and even by a mixture of both, as a preventive antibacterial treatment in the larval cultures of this species.

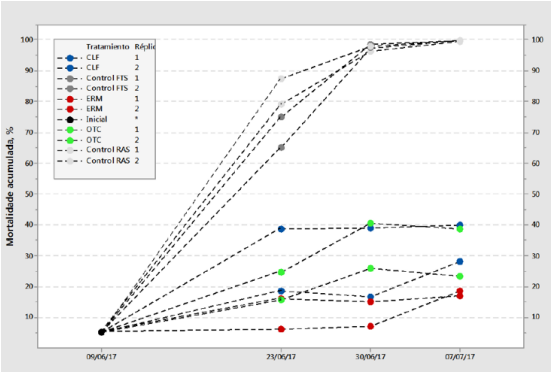


Figure 36
Cumulate mortality of larvae treated with chloramphenicol (CLF), erythromycin (ERM), oxytetracycline (OTC) and without antibiotic (Control FTS and Control RAS).

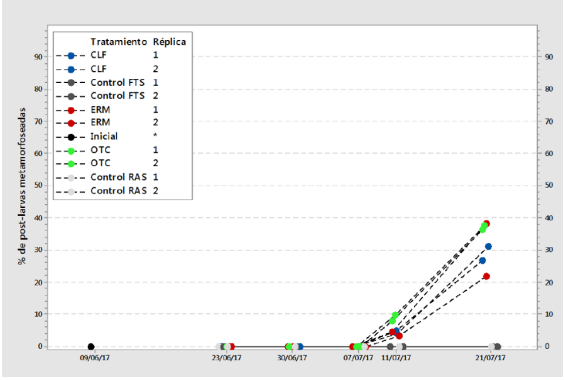


Figure 37
Evolution of the percentage of metamorphosed post-larvae in cultures treated with chloramphenicol (CLF), erythromycin (ERM), oxytetracycline (OTC) and without antibiotic (Control FTS and Control RAS).

III.3.2 2nd Assay

New larval rearing was launched in 2018 using a mixture of 4 mg / L erythromycin and 4 mg / L oxytetracycline instead of the 8 mg / L chloramphenicol supplied in previous years. The larvae were obtained from broodstock cockles collected from the inner side of the Ria de Muros-Noia (CE-N); from the mouth of the river Anllóns, in Cabanas de Bergantiños (CE-RA); and from a shellfish bed in Vilanova de Arousa (CE-VN) (Table 4). For these cultures, the new 150 L grey polypropylene tanks were used primarily, but also the old 50 L fiberglass tanks. In all cases, the cultures were treated with a mixture of 4 mg / L erythromycin and 4 mg / L oxytetracycline instead of the 8 mg / L chloramphenicol supplied in previous years. The average percentage of total larval cultures started with regard to the number of spawns in 2018 was lower than in the period 2016-17 (55.4% compared to 70.4% in the previous period).

The average percentage of total cultures that metamorphosed compared to initiated larval cultures, using the antibiotic mixture (2018), was higher than in the previous period, in which chloramphenicol was used (67.7% versus 51.2%). The average total number of larvae per spawning in 2018 (359708 ± 111,276) was lower than that recorded in the previous period (445671 ± 60232) (Table 4).

Broodstock batch	No. of Spawns	No. of cultures initiated (% per No. of spawns)	No. of larvae / cultured spawn (mean±s.e.)	Nº of cultures reaching metamorphosis (% of initiated larvae culture)
CE-N	21	11 (52.4%)	503982±272243	5 (45.5%)
CE-RA	28	17 (60.7%)	179097±41794	13 (76.5%)
CE-VN	7	3 (42.9%)	854167±477984	3 (100%)
Total	56	31(55.4%)	359708±111276	21 (67.7%)

Table 4
Number of spawns, of larval cultures that were started, of larvae per spawn and of larval cultures reaching the metamorphosis stage for several cockle broodstock batches in 2018

III.3.3 3rd Assay

In 2019, new larval cultures were developed using the same protocol as in 2018, using batches of broodstock cockles with the same origins as in previous periods (CE-RA, CE-VN, CE-VX). We also included another batch of broodstock cockles (CE-VN-456 F2 / 1) consisting of 178 individuals that had been produced in the CIMA hatchery in 2018, from broodstock collected in Vilanova de Arousa, and grown outdoor from a raft (see below, section III.5), having survived a marteiliosis outbreak.

This year, the number of spawns (15) was much lower than the 56 spawns obtained in 2018 (Table 4) due to the lower number of broodstock individuals employed in each batch. The success rates in larval culture in 2019 (Table 5) were significantly higher than in 2018 (Table 4). This improvement was due to two factors: (1) the continued use of the mixture of antibiotics erythromycin and oxytetracycline throughout the cultivation process and (2) a more rigorous management of culture densities

Broodstock batch	No. of Spawns	No. of cultures initiated (% per No. of spawns)	No. of larvae / cultured spawn (mean±s.e.)	Nº of cultures reaching metamorphosis (% of initiated larvae culture)
CE-RA	8	5 (62.5%)	238100±104504	4 (80.0%)
CE-VN	5	5 (100%)	431900±198645	5 (100%)
CE-VX	1	1 (100%)	222000	1 (100%)
CE-VN-456 F2/1	1	1 (100%)	619500	1 (100%)
TOTAL	15	12 (80.0%)	349292±95059	11 (91.7%)

Table 5
Number of spawns, larvae culture initiated, larvae per spawn that reach the metamorphosis stage for several cockle broodstock batches in 2019.

III.4 Indoor seed culture (nursery)

Once the metamorphosis of all the larvae of each cultivated batch was completed, the surviving post-larvae (seed) were pre-grown in the CIMA hatchery until they reached the appropriate size for their transfer to a raft to continue outdoor the rearing process..

When the post-larvae reached a size equal to or greater than 500 µm in the tanks in which they had undergone the metamorphosis, they were transferred to new PVC cylinders (30 cm x 20 cm Ø) with a mesh of 500 µm at the bottom, also equipped with air-lift. All these cylinders were set into a common 560L tank, where the final stage of indoor rearing was carried out. Seawater was filtered, sterilized and heated to 18°C, using RAS. The daily renewal of the RAS was set at 10%. Regarding food, the composition of the microalgal mixture was the same as in the previous culture stages and the concentration of this microalgal mixture was gradually increasing depending on demand and size, from the initial 50,000 cells / mL to the 200,000 cells / mL provided in some cases.

In 2017, 26 seed batches were cultured indoor; only three of them (11.5%) completed the process with reasonable survival and were transferred to the raft for final growing, 19 batches (73%) suffered very high mortality indoor despite being treated with 8 mg / L of chloramphenicol, scarce seed was obtained from three other batches (11.5%), and the seed from the last batch was used for another experiment.

In 2018, only 5 of the 21 cultures that had reached the metamorphosis stage (23.8%) were transferred to the raft. The remaining cultures (76.2%) again experienced unexpected mortality despite being treated at this stage with the same antibiotic mixture used during larval culture (4 mg / L erythromycin + 4 mg / L oxytetracycline). In both years 2107 and 2018, the high mortality was further investigated and samples from the heavily affected batches were analysed using histopathological techniques. Neither parasites, lesions or stress signs that could justify high mortality were detected. Bacteriological analyses were also performed by culturing seed and water samples on TCBS agar-agar plates, for the detection of bacteria of the genus Vibrio. Bacterial proliferation was observed in some plates inoculated with seed tissues from heavily affected batches. A hypothetical cause of the recurrent high mortality in the indoor seed rearing would be a potential cumulative toxicity of the antibiotics used to prevent frequent bacterial infections both in the larval and postlarval stages. To confirm or reject this hypothesis we designed a series of ad hoc experiments. The hypothesis of a toxicity or adverse effect of antibiotics as a cause of seed mortality in indoor rearing was not confirmed. In contrast, an effect of lengthening the transition period of larval-to-postlarval stages caused by the antibiotics, appears to be inferred. Another conclusion of these experiments was the convenience of using antibiotics to increase the survival of the seed in indoor seed rearing.

In 2019, 100% of the batches with which pre-growing began were moved to the sea raft for final growing. The highest success rate in pre-growing stage in 2019 compared to previous years may have been due to the continued use of the antibiotic mixture erythromycin plus oxytetracycline during the larval and postlarval culture stages and to a stricter control of culture densities.

III.5 Outdoor culture

The cockle seed were distributed into 40x25cm rectangular bags made of 1,5 mm mesh plastic net (Figure 18A) for outdoor rearing. The bags were set in perforated 60x40 cm plastic boxes (Figure 38A) which were stacked inside a metal structure (Figure 38B). This setting with the boxes was hung from a

raft (Figure 38C) owned by the local shellfishery association "Confraría de San Antonio de Cambados", which is located in the aquaculture sector "Cambados D" in Ría de Arousa (Figure 38D).

Figure 38
Cockle seed outdoor rearing from a raft. A: Rectangular plastic net bags with cockle seed within a perforated plastic box. B: Perforated plastic boxes stacked within a metal structure. C: Settling of the metal structure with the perforated boxes in the sea raft. D: Aerial view of the aquaculture sector "Cambados D", in Ría de Arousa.

III.5.1 Year 2017

In June 2017, ca. 7600 cockles (371,27 g) from the batch CE-N-14 and ca. 8600 cockles (472,85 g) from the batch CE-RA-3/6 were transferred from the CIMA hatchery to the raft. The mean size (length) of this seed was 6,40 ± 0,34 mm and 6,70 ± 0,30 mm for batches CE-N-14 and CE-RA-3/6, respectively. Periodically, the bags and boxes were cleaned and cockle density was adjusted the bags as the seed grew, with higher frequency in the warmer months. Growth and survival were assessed monthly and samples were taken for histological diagnosis of pathological conditions. The growth of cockles in these two batches was constant for most of the year but declined sharply after November and only a few individuals reached the minimum market-size of 25 mm (Figure 39).

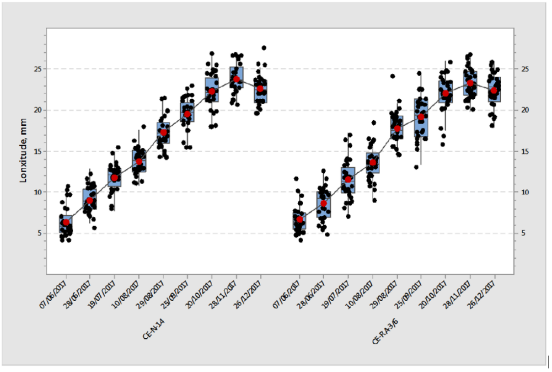


Figure 39
"Box-plot" representing the variance of the length of the cockles of the batches CE-N-14 and CE-RA-3/6 produced in hatchery, during the outdoor rearing from the raft. Red symbols represent mean values and black symbols represent individual values

The halt in growth was due to the fact that the high mortality recorded since September in both batches (Figure 40) particularly affected larger individuals, with a higher energy demand. The sharp drop in the number of individuals recorded between 06/28/2017 and 07/19/2017 (Figure 40) was due to the removal of the "tails" of slower-growing individuals after the screening that was carried out in each monthly sampling.

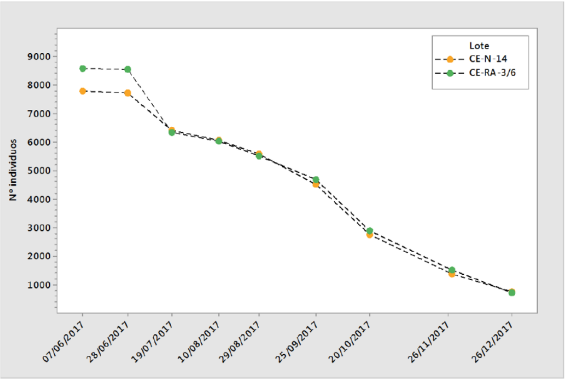


Figure 40
Evolution of the number of alive individuals of the batches CE-N-14 and CE-RA-3/6 during the outdoor rearing from the raft

The mortality recorded during the outdoor rearing was due to a marteiliosis outbreak. This infection had been detected in the histological analysis of samples from August, with intense levels of infection, particularly in the case of batch CE-N-14 (Figure 41). Since that month, the infection progressed rapidly to a prevalence of close to 100% in November and December (Figure 41). It is important to remark, for the purposes of laying the foundations for a later conclusion, that both batch CE-N-14 and batch CE-RA-3/6 were produced from breeders from areas not affected by marteiliosis (Ría de Muros- Noia and Río Anllóns, respectively).

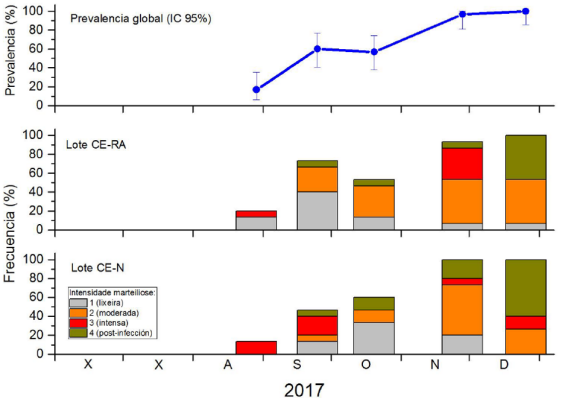


Figure 41
Evolution of prevalence of Marteilia cochillia in cockles of batches CE-N-14 e CE-RA-3/6 during the outdoor growing stage in the raft

III.5.2 Year 2018

In 2018, the cockle seed produced in the CIMA hatchery were transferred to the raft later than in the previous year due to the heavy mortality recorded in the first batches that were being grown. Thus, on August 8th, 2018 we transferred seed of a first batch (CE-RA-27; 181.75 grams;> 1.5 mm) to the raft. Subsequently, on September 20th, 2018, we transferred seed from two new batches (CE-RA-2728 and CE-VN-456, 274 grams and 337 grams, respectively;> 1.5 mm).

The evolution of growth of these three batches was very different (Figure 42). The two batches CE-RA (derived from breeders collected from the river Anllóns, not affected by marteiliosis) did not survive beyond the end of 2018 due to the mass mortality caused by the outbreak of marteiliosis close to the end of that year. In both batches the prevalence of the etiological agent of marteiliosis, Marteilia cochillia in December 2018 was 100%, with many individuals already in advanced stages of infection. On the contrary, the individuals of the batch CE-VN-456 (derived from breeders collected from a shellfish bed in Vilanova de Arousa, an area heavily affected by marteiliosis since 2012) continued to grow, as expected, until October of the year after, when most of them had reached the minimum market-size (≥25 mm; Figure 42). At the time of maximum intensity of the outbreak of Marteiliosis

(December 2018) the prevalence in this batch was only 20%, with most individuals in mild or moderate stages of infection. On October 9th, 2019, 178 individuals from this batch, CE-VN-456, were sent back to CIMA to begin a new conditioning in search of obtaining a new batch of second generation (F2), potentially resistant to this disease.

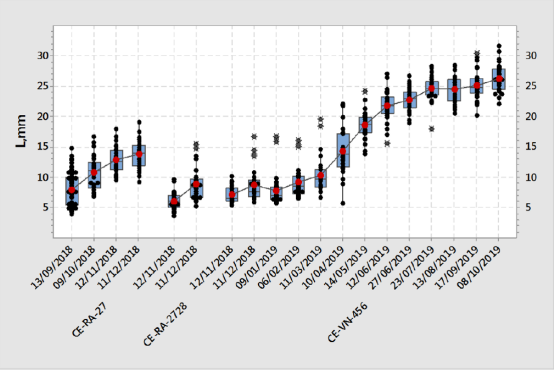


Figure 42
"Box-plot" of the variation of length (maximum distance antero-posterior) of cockles from batches CE-RA-27, CE-RA-2728 e CE-VN-456 produced in hatchery, during the outdoor growing in a raft. Red symbols represent mean values and black symbols, individual values.

III.5.3 Years 2019-2020

The seed produced in the CIMA hatchery during the first quarter of 2019 was transferred to the raft in April, May and June. These were batches CE-RA-5678 (derived from breeders collected in the river Anllóns, area not affected by marteiliosis), CE-VN-12 and CE-VN-345 (derived from breeders collected in a shellfish bed of Vilanova de Arousa, area heavily affected by marteiliosis since 2012) and CE-VX-1 (derived from breeders collected in a shellfish bed in Vilaxoán, an area also heavily affected by marteiliosis since 2012). Biometric checks on these batches were conducted in June 2019 and completed in May 2020 (Figure 43). On November 19th, 2019, approximately seven months after the onset of the on growing in the raft, one-third (≈5 kg) of the individuals in the CE-VN-12 batch reached the minimum market-size (≥ 25 mm) and were sold in the market (auction). These individuals achieved a sale value similar to that of cockles from the natural bank (€ 3.20 / kg), without negative assessments by buyers. Months later (May 4th, 2020) a mixture of individuals who exceeded the minimum market-size of the four batches (≈9.5 kg) were again auctioned in the market reaching a sale value of 2.90 € / kg, slightly lower than the cockles from natural beds. A remarkable result of the behaviour of these four batches in the raft was their differential infestation by the parasite M. cochillia. The three batches (CE-VN-12, CE-VN-345 and CE-VX-1) produced from breeders from areas severely affected by marteiliosis had fairly low prevalences in February 2019 (equal to or less than 7 %). In contrast, the prevalence of batch CE-RA-5678 was significantly higher (47%).

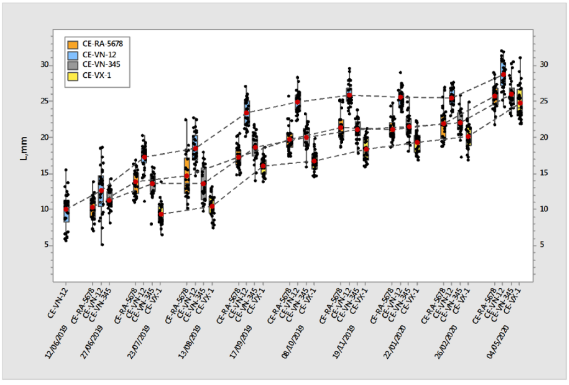


Figure 43
"box-plot" of the variation of the length (máximum distance antero-posterior) of cockles from batches CE-RA-5678 (laranxa), CE-VN-12 (azul), CE-VN-345 (gris), CE-VX-1 (yellow) produced in the hatchery facilities of CIMA, I the outdoor raft on growing stage during years 2019 and 2020. Red symbols represent mean values and black symbols represent individual values (N=30) 47

III.6 Conclusions

1. The conditioning phase of individuals used as breeders can be carried out in both a system with open sea water circulation (FTS) and a system with recirculation (RAS). It is advisable to start this phase with breeding individuals who have already started their gametogenesis in the natural environment, in order to avoid a prolonged conditioning period that causes excessive stress on the conditioned individuals. For a quick response, we recommend conditioning these individuals in an open circuit system (FTS) while for longer maintenance and later spawning we recommend the use of the RAS system.

2. The protocols used for larval culture and indoor seed rearing have been shown highly reliable to produce seed with which successfully addressing the outdoor rearing.

3. The use of prophylactic treatments with antibiotics is advised in the larval culture phase while effective alternative procedures (probiotic agents, immunostimulants, quorum sensing blockers, bacteriophage fighters, etc.) that prevent the proliferation of opportunistic bacteria, especially those belonging to the genus Vibrio, are not available. We propose the use of the antibiotics erythromycin and/or oxytetracycline as an alternative chloramphenicol, which is banned.

4. We propose to extend the prophylactic treatment with these same antibiotics to the indoor seed rearing stage to improve seed survival in this culture stage.

5. Marteiliosis is determinant of success or failure of outdoor cockle rearing in the Ría de Arousa because it causes mass mortality before the cockle seed reaches the minimum market-size in the case of seed from batches susceptible to the disease. However, seed batches with higher resilience to this disease can be successfully grown in cages hung from rafts with high survival rates and being able to reach market-size within eight months after leaving the hatchery.

6. Seed derived from breeders collected in areas heavily affected by Marteilia cochillia, the protozoan causing marteiliosis, since 2012 (year of its first detection in Galicia), have shown high resilience to this disease, while seed derived from breeders collected in areas not affected by marteiliosis showed a very high susceptibility to the disease. This suggests the hypothesis that continued exposure to marteiliosis (specifically in the inner area of the Ría de Arousa) since 2012 is favoring a process of increasing resilience to this disease in this area, by natural selection. It is advisable to use cockle breeders collected in areas severely affected by the disease outbreak if the aim is to produce seed in a hatchery for outdoor rearing in areas affected by the disease outbreaks or for the recovery of shellfish beds affected by this disease. However, it must be avoided moving cockles (including breeders) from affected areas to unaffected areas.

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cockles