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Valutazione di una nursery in mare per individui giovanili di *Ostrea edulis*: effetti della densità di allevamento sulla crescita e sopravvivenza

Evaluating a deep-water nursery for *Ostrea edulis* spat: stocking density effects on growth and survival

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Abstract in Italian

L'ostrica piatta europea (*Ostrea edulis*) è una specie di bivalve di grande rilevanza ecologica e commerciale, storicamente abbondante nei mari europei. Questi organismi svolgono funzioni di vitale importanza, tra cui il miglioramento della qualità dell'acqua, la creazione di habitat per numerose altre specie e la stabilizzazione dei sedimenti marini. Attualmente, la presenza dell'*O. edulis* risulta in forte declino a causa di fattori quali la pesca eccessiva, il degrado dell'habitat, l'introduzione di specie invasive e l'impatto delle malattie.

Negli ultimi anni, numerosi progetti di ripristino vengono avviati per reintrodurre le ostriche nei loro habitat naturali, ma la riuscita di tali iniziative dipende da molteplici fattori, tra cui la corretta selezione del sito, la densità di allevamento e le condizioni ambientali associate ai siti selezionati. In questo contesto, il presente studio valuta l'efficacia di una nursery in mare per individui giovanili di ostriche, analizzando in particolare gli effetti della densità di allevamento sulla crescita e sulla sopravvivenza.

L'area di studio si trova nell'isola di Helgoland, nel Mare del Nord, un sito di particolare interesse per il ripristino delle popolazioni di *O. edulis*, data la presenza storica di questa specie nell'area. Lo studio, della durata di cinque mesi, coinvolge tre siti di allevamento: un sito a terra e due siti in mare a profondità differenti. L'obiettivo principale della ricerca è comprendere come la densità di

allevamento influisca sulla crescita e sulla sopravvivenza degli individui, al fine di ottimizzare le pratiche di acquacoltura e migliorare le strategie di ripopolamento.

Nel primo sito, situato presso le strutture a terra dell'Alfred Wegener Institute, gli individui vengono allevati in tre differenti vasche con parametri ambientali controllati e con la somministrazione di tre diete differenti (3 µg Chl L^{-1}, 6 µg Chl L^{-1} e 9 µg Chl L^{-1}). Il secondo sito, in mare aperto, si trova a una profondità di 25-28 m, mentre il terzo sito, sempre in mare aperto, è situato a una profondità di 8-11 m. In questi due siti, l'alimentazione è naturale e i parametri dipendono dall'ambiente stesso. In ogni sito, le ostriche vengono suddivise in tre diverse gabbie con diverse densità di allevamento: bassa, media e alta.

L'analisi dei dati alla conclusione della sperimentazione mostra che la densità di allevamento ha un impatto significativo sulla crescita delle ostriche. A basse densità, gli individui mostrano una crescita relativamente elevata, ma lo spazio non viene utilizzato in modo efficiente. Alla densità media, gli individui registrano i migliori risultati, con una crescita ottimale e una buona sopravvivenza. Infine, gli individui allevati alla densità maggiore presentano una crescita ridotta, probabilmente a causa di interazioni competitive per il cibo.

I risultati evidenziano differenze significative tra i diversi siti di allevamento. Il sito in mare a profondità di 8-11 m fornisce le migliori condizioni per la crescita

delle ostriche, grazie a temperature più elevate e maggiore disponibilità di fitoplancton, mentre il sito a maggiore profondità (25-28 m) registra una crescita più ridotta, probabilmente a causa di temperature più basse e minore disponibilità alimentare. I risultati ottenuti nel sito a terra permettono di stabilire la migliore densità di allevamento (comparata e confermata con i risultati dei due siti in mare), l'impatto e l'importanza dei parametri ambientali e, infine, la concentrazione di clorofilla più adeguata per la crescita degli individui (6 µg Chl L^{-1}).

I risultati di questo studio forniscono indicazioni importanti per il miglioramento delle strategie di allevamento e ripopolamento dell'ostrica piatta europea, confermando l'importanza della densità di allevamento in quanto essa influisce ampiamente sulla crescita degli individui. Viene inoltre sottolineata la rilevanza della corretta selezione del sito di allevamento, che gioca un ruolo fondamentale nel fornire le condizioni migliori per la crescita.

Per studi futuri, risulta necessario analizzare a lungo termine l'adattamento delle ostriche dopo il trasferimento nei siti di ripopolamento, approfondendo ulteriormente il ruolo di fattori ambientali, come la qualità dell'acqua e la disponibilità alimentare, sulla crescita delle ostriche.

FIRST CHAPTER - *Ostrea edulis* characterization

1.1 Biology of the European flat oyster, Ostrea edulis

One indigenous species of oyster in Europe is *Ostrea edulis* Linnaeus, 1758, also called European flat oyster or European oyster (Pogoda, 2019). This bivalve is a filter feeder organism, preferring estuarine or marine water (Sander et al., 2021), associated with firm bottoms (as mud, muddy gravel with shells, rocks or hard silt) (Hayer et al., 2019). It can be found in different countries, mainly in Britain, Ireland, north part of the Norwegian sea, but also in the Mediterranean Sea, Black Sea and the Iberian Peninsula (Pogoda, 2019). This bivalve is indigenous in the North Sea where, in the past, it was possible to find large sized population in the sublittoral environment (Sander et al., 2021).

O. edulis is characterized by an oval shaped shell with a thin periostracum which has a rough surface with a color pattern characterized by nuances of brown and cream with also the possibility to have some blue bands that can be located on the right valve. The internal surfaces of the shell present a color that ranges between white pearl and grey with the occurrence of some darker areas that are bluish. They present an inequivalve shell, where the left one, which is the one fixed to the surface, is concave while the right one is flat (Perry and Jackson, 2017).

One of the characteristic organs of bivalves is the mantle, a tissue responsible for the shell formation, which is made of two thin skin layers. The mantle is thicker

at the edges and it contains and protects the internal organs. The mantel is in direct contact and attached to both the internal faces of the shell, but the edges of it are not attached to the shell itself. The mantle possesses a sensory function, it is in fact able to start the closing or opening process of the valves depending on the stimulation coming from the external environment. Between the previously described functions, the mantle could be involved in the respiratory process, and it can also directly influence the water inflow in the body chamber. Under the mantel, as previously mentioned, there are other internal structures. One of them is the adductor muscle that in oysters, a monomyarian species, is a single muscle located in the center part of the shell. The muscle presents different areas: the "quick muscle" (anterior part of the muscle) which can close the valves and the "catch muscle" (smaller and smooth) that is able to keep valves in position when they are just partially closed. The ligament and resilium determine the valve opening when the adductor muscle is relaxed, while during the contraction the valves are closed. Important for this class of filter feeders are the gills, a structure involved in both respiratory and feeding processes (Gosling, 2002; Helm et al., 2004). The feeding process involves the water passage through the gills and thanks to the presence of a filter in the gills chamber, all the present organic particles in the water are removed. Also resuspended particulate matter coming from the bottom can represent an important food source (Grant et al., 1990). Gills are in pairs and located on both sides of the body and, at their anterior end, they

present the labial palps that are involved in directing food to the mouth (Gosling, 2002). At this point, food particles removed from water are bound with mucus and directed to the mouth by the labial palps. After entering the mouth, the bolus reaches the stomach, which is a sac-structure with different openings, through a short esophagus which is connected to the mouth opening and the stomach. The stomach is enclosed by the digestive diverticulum, which is a dark colored gland, and from here, through an opening, the food mass reaches the much-curled intestine that ends up in the rectum. A second opening in the stomach leads to a tube-shaped sac that contains the crystalline style. This structure is made of mucoproteins and it is shaped like a transparent bar with a gelatinous texture, where one of the ends is round and the other one is pointy. The round part of the crystalline style is located in the stomach and the main function of this structure is mixing the stomach contents and releasing useful enzymes for digestion, especially the ones involved in the conversion of starch into usable sugars (Gosling, 2002; Helm et al., 2004). Out of water, after a couple of hours, the crystalline style can be reduced in size or even disappear, but once the individual is in water again, the structure is quickly built again (Langton and Gabbott, 1974).

Another important structure is the foot that is used by larvae to move and then settle on a surface, while in adults this structure is vestigial. The circulatory system is pretty basic, and the heart is located in a transparent pericardium. In oysters, it is located in the proximity of the adductor muscle, and it is composed

of two auricles with an irregular shape and just one ventricle. The hemolymph is carried to all the body parts thanks to the anterior and posterior aorta that comes from the ventricle. In general, the venous system is represented by sinuses with thin walls that bring blood back to the heart (Gosling, 2002; Helm et al., 2004).

Oysters are able to form big populations where, in the case of high densities (250 individuals per square meter) a reduced growth and weight is generally observed due to the general poor condition of the individuals. Since oysters grow by cementing themselves to the next present oyster, competitive phenomena in terms of space and resources are frequently witnessed and the main result of this situation is the development of a distorted shell shape. Concerning growth, it is especially fast in the first year and a half, and it is evidenced that it can be especially faster in protected sites compared to exposed ones. After the first fast period of growth, the growing process stays more stable where adult individuals tend to gain, more or less, 20 g every year. Then, after a period of around 5 years, the growing process slows down in a more gradual way (Perry and Jackson, 2017).

Concerning life history, European flat oysters have usually a lifespan of 5-10 years (Roberts et al., 2010). This estimation comes from the fact that most of the individuals forming populations are usually between 2 and 6 years old. Regarding reproduction, oysters are protandrous alternating hermaphrodites (Perry and Jackson, 2017) where individuals reach sexual maturity between the age of 3 or 5

years old (Cole, 1951). Starting as males, they reach sexual maturity at around 3 years old and after the production of sperm, they switch sex to become functional females and produce eggs. Gametes start to maturate around March or April but the whole process is influenced by temperatures (Perry and Jackson, 2017). During the reproduction season, gonads can represent till the 50% of the total body mass of the individual (Helm et al., 2004). In some warm geographical areas, the production of gametes can be continuous due to the high temperatures. Considering the fecundity, big individuals can reach the production of 2,000,000 larvae. *O. edulis* eggs are characterized by a round shape with a diameter of ~150 microns. Female individuals, once they are mature, produce eggs that are kept in the mantle cavity and gills. The fertilization occurs once the males release the sperm in water and, when the water is drawn inside the female organism through the water flow used for feeding and respiration, the eggs are fertilized. After the fertilization they are retained for 7-10 days until they reach the veliger stage. At this point they are released to the external environment and due to the internal keeping of the larvae, oysters can be defined as larviparous (Perry and Jackson, 2017).

Oyster settlement is characteristically sporadic and with a high mortality rate that is deeply influenced by different parameters, like food availability, temperatures and presence of a suitable substrata for the settlement. Also, the presence of predators or other adverse situations can determine high mortality in larvae (Cole,

1951). Like many other types of larvae, *O. edulis* larvae can respond to the different stimulus coming from the surrounding environment and thanks to them, they are able to find a valid location to settle. Chemical cues coming from conspecifics are extremely important for the settlement process since larvae tend to settle in substrata where other larvae are already located (Perry and Jackson, 2017). Larvae newly attached to a substrate are defined as "spat" until a size of around 4 mm, after that size, they are defined as "juveniles" (Tetrault, 2012). Once they are settled, they will stay for the rest of their life in the chosen position (Helm et al., 2004),

The European flat oyster can be found at depths of 20 m, but in some cases, it can be found at depths of 50 (Hayer et al., 2019) or even 80 m (Perry and Jackson, 2017). The optimal salinity is over 30‰, but in estuarine habitat they can tolerate lower salinity between the 16‰ and 19‰ but only for a short period of time (Hayer et al., 2019).

The presence of flat oysters in certain areas is influenced by different factors, where the most important are feeding conditions and temperature. Considering that they are active filter feeders, the diet is mainly represented by phytoplankton, bacteria and suspended organic matter (SOM), but also the resuspended materials that come from the sea bottom. The growth of the *O. edulis* occurs in the warmer months with a minimum temperature of 15°C, while the optimum can be higher.

In the Helgoland Bank, sea water can reach temperature between 16°C and 20°C in warm summer and in these conditions, characterized by a general low temperature, the development of the larvae and the settlement is negatively influenced, with effects on the survival (Bennema et al., 2020).

Helgoland is located 60 km from the mainland, specifically in the area named German Bight. This area represents a transitional point between coastal waters (well mixed and with low salinity) and deep waters coming from the Southeast of the North Sea. The characteristic of the seawater around Helgoland variates along with the meteorological conditions (Gebühr et al., 2009) that are getting closer to oceanic conditions, determining changes in the compositions of the present communities. Because of this, the species present on the different rocky shores of the island can be defined as isolated compared to other ones present in the North Sea and, this situation can be seen as an indicator of the progressive warming conditions (Reichert et al., 2008).

The area around Helgoland is unique and very important, from an ecological and geological point of view, characterized by the presence of rocks, both in the intertidal and subtidal area. The island is isolated, by several kilometer of soft bottoms, from other similar hard bottom areas located in Britain and Norway. There is anyway a scarce knowledge concerning the isolation level of the animal community at genetic level from the other close community in England and

Norway. The Helgoland hard-bottom macrozoobenthos presents variability in terms of composition and abundance of species (Franke and Gutow, 2004).

Originally, *O. edulis* was abundant in the offshore grounds of the North Sea but nowadays the decline of the species is clearly evident. Information comes from fisheries data but there is no extensive knowledge concerning the ecological baseline or ecological role and density that the species covered in the offshore area (Merk et al., 2020).

1.2 History of O. edulis exploitation in the North Sea (Helgoland)

In these days, all the knowledge regarding not only the distribution, but also the ecological state of *O. edulis*, remain incomplete despite the continuous increasing interest towards flat oyster habitats (Sander et al., 2021). Despite this, there is still enough availability of historical record that allows us to reconstruct part of the history of oysters' exploitation. In the past, during roman times, flat oysters in Europe were heavily used and collected as a food source. Without the current knowledge in terms of species conservation, humans in the past continued to remove adult flat oysters and this, along with the development of a more efficient way to collect these bivalves, led not only to the progressive weakening of the reproduction success, but also to the destruction of very important biogenic structures that provide a valid substrate for the settlement of their larvae. Ultimately, the loss of these structures determined an alteration in the ecosystem

composition. Currently, we know that in the North Sea during the 13th century, in the subtidal coastal waters, deep water locations and offshore waters till 50 m depth, there were abundant natural beds of European oyster (Pogoda, 2019). Only between the 12th and 13th century oyster fishing practices were on the first place as commercial activity in the field of fisheries practices in the German North Sea (Hayer et al, 2019).

Along German coast, oysters were located along North and East Frisian Wadden Sea, in the Helgoland Oyster Bed and in the North Sea Oyster Ground, covering area of 20,000 - 25,000 km^2, located at a depth of 40 m. Also in these areas, oyster fishing practices (performed with iron dredges and sailing cutters) were an important money income and, from the end of 17th century, the over exploitation of oyster beds was a huge problem (Pogoda, 2019).

From historical records, it is known that flat oysters, in the North Sea, were found in places where the bottom was made of sand rich in silt and broken shells, where it was possible to observe around 3 or 5 oysters organized in a clump. The biometric parameters were different between the different specimens, where it was possible to observe single oyster with a width of 1.35 cm, a length of about 1.20 cm and a thickness of about 3.2 cm, while the shell shape was spherical (Bennema at al., 2020). Considering the low temperature of the sea water, which

determines a low energy investment in reproduction, oysters were bigger and with a high tissue weight (Orton, 1928).

Following the progression of history, at the end of the 18th century, it was possible to find huge amounts of flat oysters close to the island of Helgoland. Anyway, due to the absence of proper fishing equipment, these oysters were not collected. Flat oyster beds were discovered in the open North Sea around the middle of the 19th century and from that moment, there was a constant increase in the use of this resource (Sander et al., 2021).

With the development of steam trawlers, that happened in the industrialization period, oysters were heavily collected because they were a cheap source of food and this, along with the development of more efficient infrastructure, has enabled the transportation of huge amounts of flat oysters in different places. As previously stated, without the current knowledge regarding European oyster conservation, overexploitation was not only a common practice, but it was also widely accepted. All these factors led to the decline of flat oysters in Europe (Pogoda, 2019).

For the Helgoland area, the highest yields were during the 1875-1877, with approximately 350,000 to 500,000 oysters harvested in a single year. From this, the situation progressively got worse in a short time: the result was the closure of the fishing season between 1879 and 1882 (Pogoda, 2019). During the 19th

century, oyster beds located in the Helgoland area, were defined as old and characterized by a lack of juveniles (Sander et al., 2021).

From what the Alfred Wegener Institute located on Helgoland has estimated, during the 19th century the oyster beds in the area comprised about 1.5 million individuals (Boos et al., 2004). The reason behind the fast decrease in oyster bed, as previously stated, is behind the fact that there was the possibility to use paddle steamers with the presence of multiple dredges to fish them. Along with the loss of oyster beds, all the associated fauna and flora were lost too. Considering all these multiple factors that led to the decline of the flat oyster along the German coast, the practice of fishing oyster ended in 1920 because not only it wasn't profitable anymore (Pogoda, 2019) but also because during the World War I it wasn't possible to continue this activity. Even though oyster harvesting stopped, any process of recovery of the natural beds was observed (Sander et al., 2021). From the 1950s, it is commonly believed that oysters are functionally extinct in German waters (Pogoda, 2019).

During the 1960s the arrival of the *Magallana gigas* or Pacific oyster was witnessed and then, in the 1970s, the introduction of the oyster parasite *Bonamia ostreae* was observed: both boosted the progressive decline of the flat oyster population (Bennema et al., 2020).

During the 20th century, it was common to find flat oysters in an area comprised between the Mediterranean Sea, Black Sea, northeast Africa till Norway and the Atlantic coasts. It was recently observed the presence of flat oysters in Denmark, in particularly in offshore wind farm. This discovery can point out the possibility of a re-colonization process of the North Sea (Hayer et al., 2019).

1.3 Oyster reefs functions and importance of restoration practices

Oysters are considered ecosystem engineers due to their ability to form a biogenic reef habitat, providing vital ecosystem services and functions (Colsoul et al., 2020). *O. edulis* beds are defined by OSPAR as "beds of oyster specimens belonging to *O. edulis* occurring at densities of 5 or more per m^2" (OSPAR, 2009; Sander et al., 2021). These beds present a very complex structure of the external surface, making them able to provide shelter and settling substrate for different organisms. Due to these characteristics, oyster beds are fundamental in the process of increasing biodiversity at local level. It can be stated that oyster beds provide ecosystem services by improving environmental quality, as well as several cultural and commercial values. Nowadays we are assisting to the progressive decline in flat oyster populations due to several factors such as overfishing (Sander et al., 2021), harsh winters, invasive species, diseases (Hayer et al., 2019), pollution and habitat destruction, that frequently lead to extinction at functional level (Helmer at al., 2019).

Considering the actual status and presence of the European flat oysters' population, it is possible to reach the conclusion that in general, there is an evident lack in the recovery process. The reasons that can explain the current situation can be found, for example, behind the complex life cycle of flat oysters. In *O. edulis*, there are different stages that comprehend different phases: first the external sperm release, followed by the internal egg fertilization with the obtain of a vulnerable free-swimming larva that in a second moment will settle on an optimal substrate. Also, the introduction of *B. ostrae* severely impacted the different populations of European oysters worldwide. *B. ostrae* is an intrahaemocytic protozoan parasite introduced in Europe from infected seed that was imported originally from California. This parasite has caused mass mortalities all around Europe (Helmer at al., 2019). Another important disease that has also caused mass mortality events across Europe is marteiliosis, caused by the protozoan *Marteilia refringens*. Mortality is mostly detectable in two years old individuals and the mortality rate can reach 90% (Colsoul et al., 2021).

Nowadays, natural *O. edulis* beds are going towards a progressive decline in all the regions where they can be found or, in general, they are heavily threatened (Pogoda, 2019). At the current status it is evidenced that 85% of the oyster reefs around the world are lost (Ermgassen et al., 2020).

The progressive decline of European flat oysters represents also a huge loss in terms of economic income. At the same time, the disappearance of filter feeder organisms determines a general decline in the quality of the surrounding marine environment. In fact, oysters play a major role in dissolved nutrient cycling by removing phytoplankton, suspended solids and organic particles from the water column: due to these activities, oyster reefs play a major role in the control of eutrophication phenomena in marine ecosystems (Sawusdee et al., 2015). Between the different positive impacts of oyster habitats, it is important to mention the protection action towards the shoreline due to their ability to lower the impact of waves and currents. Oyster reefs are also involved in the process of creating and reinforcing adjacent habitats such as, for example, seagrass beds. Due to all of these functions, it is easy to understand that the degradation and loss of these habitats is a serious conservation threat. (Baggett et al, 2015).

Today, the ecological restoration of *O. edulis* habitats is being addressed by several projects in Europe (Pogoda et al., 2019). The key point of the different restoration projects is to focus not only on the single species, but also on the habitat and reefs that *O. edulis* is able to form. Working not only on the target species is an important matter to achieve the objectives reported in the OSPAR Convention for the Protection of the Marine Environment of the North-East Atlantic, the EU Habitats Directive (Directive 92/43/EEC) and the EU Marine Strategy Framework Directive (Directive 2008/56/EC) (Colsoul et al., 2020).

Thanks to the EU Habitats Directive (Council Directive 92/43/EEC), in some countries flat oyster habitats are indirectly protected because they are included in the category of reef or, in other cases, they are defined as key species in estuarine areas. Due to the possibility of widely applying the legislation, there are currently different projects to protect and restore flat oyster habitats. In general, compared to others restoration projects involving other species, oyster restoration is cheaper, but it doesn't mean that it is low cost. In Europe, to achieve the goal of making the best progress in the topic of oyster restoration, in 2017 the Native Oyster Restoration Alliance (NORA) was established. NORA is composed of a network of people with the aim of coordinating projects and exchanging knowledge across 13 European countries regarding the restoration processes of *O. edulis* and its habitat. Thanks to NORA, it had become clear that European countries are facing challenges that are similar. At the beginning, NORA first purposes and efforts were towards oyster production, site selection, disease management and monitoring but nowadays, the main focus is towards the identification of questions that can help to identify and overcome the current barrier in the oyster restoration field in European countries (Ermgassen et al., 2020).

In the USA, oyster restoration has seen its first try 50 years ago. In this case, the main subject was the *Crassostrea virginica* and nowadays there is a high availability of knowledge regarding its restoration practices. Thanks to this knowledge, it is possible to use and apply all the obtained results as a main

guideline for restoration processes, regarding both management but also interactions between different policies. Other knowledge deriving from other countries and different oyster species can be used in the restoration of the European flat oyster too, but it is necessary to collect species-specific and location specific information to properly develop effective flat oyster restoration plans. Among the new challenges that need to be faced to obtain efficient flat oyster restoration, it is necessary to point out the arrival of new diseases but also the different coastal settings and complex cross-border issues present in Europe. Considering the reproduction of the European flat oyster, it is important to guarantee and support the genetic diversity to have a long-term adaptation, which is possible thanks to the avoided inbreeding events (Ermgassen et al., 2020).

The field of oyster restoration made huge progress, but one of the main problems that still persists is the fact that in practice there is often a lack of clear and defined goals. Restoration plans often show no further monitoring after the project realization, which can impact the evaluation of the success rate and the comparison of the data deriving from other projects. An important part of every restoration project is in fact the evaluation of the restored habitat performance: this can be translated into the evaluation of the persistence and effectiveness of the habitat and any other goal. Considering these issues, the restoration process was defined as “the process of establishing or reestablishing a habitat that, over time, can come to closely resemble a natural condition in terms of structure and

function", which is a modification of the original definition from Turner and Streever (2002). Thanks to this definition, all the projects involving the building process of habitats with different materials that can be human or natural made, but also those activities that try to determine the return of a habitat to its prior condition before the disturbance can be part of the same definition. At the same time, all the activities of restoration that have the main purpose of harvest are excluded. Considering the importance of oyster habitats and their functions, it is important to set specific goals for their restoration in order to have a good success rate. Once the goals are clear, there are different parameters to determine the performance and success, for example the density of specific species of different faunal groups (Baggett et al., 2015).

The success and placement of oyster restoration project is heavily influenced by physical factors that are related to the construction of the reef and that can influence the single individual's growth, settlement and survival (Coen and Luckenbach, 2000). An area can be limited in terms of substrate or recruitment rate, or in some cases also both. It is then important to consider that the availability of adapt substrates is the most important factor in the success and development of an oyster population. Along with that, it's also important to gain specific knowledge regarding the larvae preferences towards substrata and the specific mechanism of settlement (Colsoul et al., 2020). This situation of limitation in both substrate and recruitment is common in the European area, where there is a high

interest in having settlement material which results, as much as possible, suitable for larvae. An important point to guarantee the success of a restoration process is to ensure the sustainability of the oyster reef itself. To achieve that, site selection is a very important step: the key is to select an area that is adapt not only for growth, survival and settlement but also for the whole habitat growth itself. To determine if there is a sustainable situation in the reef system, it should be possible to notice a positive shell budget (where the present oysters provide shells to the habitat), but also phenomena of movement and erosion can be considered as a part of the indicators. Among the major obstacles in restoration projects regarding *O. edulis*, it is possible to point out issues regarding biosecurity, related particularly to diseases and invasive species. It is clear that where it is possible, it is mandatory to make all possible efforts to maintain the status of disease-free area, but when or where it is not possible, it is necessary to apply the restoration strategies considering the present diseases. The key to conserve oyster populations that are free from pathogens and invasive species is to have biosecurity measures that comprise scientific knowledge regarding the vector of disease and invasive species. Since it is not possible to eradicate diseases in the open sea, the management of the restoration projects must include the natural presence of pathogens, and the same concept should be applied for invasive species. Regarding the European flat oyster, *B. ostrae* can be considered one of the biggest threats and at the actual status, the disease's impact on natural conditions is not

very well defined: this must be one of the main points to study to develop some form of resistance to it. Genetic diversity and its maintenance are another important highlight in the restoration projects involving, along with the reconstruction and understanding of all the evolutionary processes that took place and determined the genetic structures in the present population. It is extremely important that the potential population source is genetically distinct but also adapted to the receiving environment. In this way, it is possible to introduce individuals with high fitness. Even if it is clear that genetic variability is an important requirement for the success and maintenance of a restoration project, there are still some issues remaining due to the discovery of emerging, or rediscovered, populations that have not been characterized. To facilitate the process of understanding the different genetic and population structure of the *O. edulis* in Europe, it was proposed to formulate a common language (in terms of genetic markers) to speed up the knowledge gaining process. This idea came due to the experience with *C. gigas* and *Pecten maximus* where with the facilitation of the genome studies, it was easier to define the factors that determine the different traits in the individuals (Ermgassen et al., 2020).

In Germany, restoration projects involving *O. edulis* and its reefs mainly focus on furnishing appropriate oyster seed meant to be used in different restoration (PROCEED project) and the creation of an oyster reef in German waters with the

aim of boosting and protect the present biodiversity (RESTORE project) (Pineda-Metz et al., 2023).

SECOND CHAPTER - Oyster aquaculture practices

2.1 Oyster aquaculture: hatchery, rearing process and technologies

The hatchery design is not rigid since it changes with different parameters like location, produced species, personal preferences and available economic resources to invest. The dimensions of the structure itself, for example, can depend on whether the seed production is to supply the hatchery for its own bivalve culture operations or if the seed production is meant to be sold to other hatcheries. Hatcheries could potentially include a nursey part, others can produce larvae for selling purposes, while others can produce and sell juveniles up to a size of 12 mm (Dupuy et al., 1977; Helm et al., 2004). It is then clear that all the hatcheries are different among each other, but there are two parts that are fundamental in all the structures: a well-structured water system and the facility physical structure (Helm et al., 2004).

Having a high-quality sea water supply is fundamental to ensure the success of all the phases that take place inside a hatchery (Dupuy et al., 1977, Helm et al., 2004). The sea water source, the pump system and the water treating system must be located in an easily accessible and convenient place to maintain the general costs low, along with locating the hatchery at sea-level. To be sure to take high quality sea water, the inlet point for water withdrawal must be located at depth, ensuring in this way water with stable temperature, salinity and free from an excessive

amount of sediment particles. Another advantage is that with a location at depth it is possible to avoid the major plankton blooms (since some of them can be potentially dangerous) and fouling organisms that can damage the reared bivalves and the pipe system by obstructing it. The first step that directly pumped water from the sea must go through is usually a series of sand filters with pores dimensions bigger than 20-40 μm. This first passage allows an efficient removal of fouling organisms and other present particles, if the filter is well maintained. After this first filtration water is collected, with the help of pumps, in storage tanks positioned in locations that are higher than other structures, so the gravity maintains a sufficient water flow in the hatchery. The whole piping system should be located in a thoughtful way to allow proper cleaning without accessibility problems; the same concept must be applied to all the present outlets and valves. At this point there could be the necessity to heat or chill the sea water and for this purpose heat exchange units which are commonly made of titanium are used. Frome here, water goes through other filtration steps where the goal is to obtain a final passage through 1 μm filters. The last step is the disinfection of water thanks to ozone or, most commonly, UV lights. The size of the UV light unit depends on the water volume. At this point, harmless water can be distributed through the hatchery. For wastewater discharge and treatment, it must be consulted and accurately follow any eventual government regulations. Depending on them, it could be necessary the presence of quarantine facilities to avoid the discharge in

nature of non-native species, diseases or parasites. In this case, it is required a specific separate drainage system for the quarantine water/area, where water is treated with hypochlorite solution (then neutralized using thiosulphate) before discharge (Helm et al., 2004; Karydis, 2011).

As previously mentioned, another important point that is shared between the different hatcheries is their physical structure. Hatcheries should present a physical structure that allows efficient operations, considering also the possibility of future changes that should take place without expansive rebuilding operations. For example, it is a clever choice to use fiber glass or plastic material instead of concrete to build tanks, in this way if they need to be moved in a different area of the hatchery, it is easy and less time-consuming. All the areas and rooms in a hatchery are related among each other, but they can be divided in algae culture room, broodstock room, larval rearing area, juvenile culture room and services areas (Bourne et al., 1989; Helm et al.; 2004).

Associated with these rooms and areas, in a hatchery some other spaces are required: for example, spaces dedicate for the quarantine tanks but also laboratories, offices and rooms for the static machinery (e.g. pumps and filters). The general rule to keep in mind is that all the spaces must be divided as much as possible, so in case of disease outbreak it is easier to manage the situation (Helm et al., 2004).

One of the most important rooms in a hatchery is the algae culture room. Inside hatcheries, the most common form of feed used are unicellular marine microalgae, since they adapt to the different nutritional necessities of bivalves at different life stages (Dupuy et al., 1977; Bourne et al., 1989; Helm et al.; 2004). The success of a hatchery is directly related to the continuous presence and availability of high-quality feed sources in big volumes. The dimensions of the room designated for algae production depend on the production level of the hatchery and the culture method, while the room temperature is around 20°C. It is also required a proper carbon dioxide and compressed air supply and, to maintain the temperature stable especially during warm months, an air conditioning system is necessary (Dupuy et al., 1977; Helm et al., 2004). A second algae room, with a cool temperature and proper light sources, is then required to maintain the master algae culture in good condition. It is also necessary the presence of an incubator to store axenic culture in proper conditions (Helm et al., 2004).

The broodstock room and spawning area are the places where broodstock is kept and where the conditioning process takes place (Dupuy et al., 1977; Bourne et al., 1989; Helm et al.; 2004). Due to the conditioning operations, this area requires heaters exchangers since, at a certain point of the year, chilled or heated water is required. To influence gonad maturation and promote spawning, it is necessary to have the possibility to control the photoperiod, so it is necessary to have the possibility to easily create total darkness in tanks (Helm et al., 2004).

The larvae rearing area has a dimension that depends on the production purposes of the hatchery itself but also on the density used to rear larvae, since it influences the dimension but also the number of the tanks. Inside this room, all the tools and equipment necessary to measure, count and collect larvae should be present (Helm et al., 2004).

Once the larvae settle and start the metamorphosis, another specific area for their growth is required, which is the spat culture area. Individuals are kept in this area till they reach a shell size of ~2 mm after which they are moved to the nursery area. Once the spats are obtained, they are moved again into other tanks where they can grow until they surpass the shell length of 2 mm (Helm et al., 2004).

At this point a nursery area is required, which can be defined as the interface between the grow out phase and the hatcheries structures. These systems are very cost effective since their purpose is to quickly grow individuals. Nurseries evolved as an auxiliary part in hatcheries, and they can be considered as the final production stage inside a hatchery or the initial stage of the grow out phase. In this case, oysters seed are kept at different densities and with different strategies (e.g. submerged or floating trays or upwelling containers) (Helm et al., 2004).

For the grow out phase, until oyster individuals do not reach the minimum weight of 10 g, there is the necessity to keep them in trays or bags for example, until they reach a sufficient size to be located on the substrate. This necessity comes from

the fact that bigger sizes are less sensitive to strong waves and tidal action, but also siltation and predation. In systems that involve the presence of baskets or mesh bags, the mesh size must be increased when the oyster size increases to ensure sufficient water flow, which can be translated also into sufficient feed amount for the individuals. While choosing the more suitable techniques for the grow out phase, it must be kept in mind that between the different options, the systems that keep constantly all the individuals underwater are the most efficient ones in terms of growth, considering also the fact that oysters perform generally better when located on the bottom. Another important consideration must be made concerning the stocking density, since it is necessary not to exceed the carrying capacity of the body of water in which the selected site is located. If this happens, there won't be a sufficient algae population to support the oyster individuals, impacting growth and leading to mortality. As mentioned previously, growth is also strongly influenced by immersion time during the tidal cycle where it was evidenced that if oyster individuals, during production time, are left in contact with air for more than the 35% of the total production time, the growth stops. This fact can be anyway used by producers to temporarily slow down the growth for production reasons (Laing and Bopp, 2018).

For the grow out phase it is possible to recognize two main methods: bottom culture and off bottom culture. In the first method, as the name suggests, oysters are grown at the bottom or also sub-tidally or intertidally (in this last option it is

required a firm substrate to avoid the sink of the individuals in it). Considering that specimens are still young, it is necessary to provide protection from strong waves and tide action to avoid the removal from the selected location. To start a bottom culture technique, it is necessary to plant seed which is usually on a collector made for specific type of bottom. If the seed is collected on heavy shell and then is planted, for example, in soft bottoms it can sink causing mortality events. The translocation on a soft bottom can happen only in a second moment, when the size is sufficiently big (Quayle, 1980).

As previously mentioned, the second possible strategy that can be used is the off bottom rearing method (Walton and Swann, 2021). Off bottom culture can be done by using different structures such as, for example, raft or suspended culture (realized with trays or strings), rack (realized with trays, sticks or strings) and then the stake method (Quayle, 1980). This technique implies the culture of the oyster individuals inside containers characterized by a mesh, as bags or basket, that are kept above the sea bottom. With this technique it is possible to obtain a single set of oysters and not clumps of individuals as happens in nature. The used containers, if properly structured and operated, can provide sufficient protection against predators while, at the same time, reducing the burial in sediment, allowing in this way cultivation on soft substrate too. This kind of method can be used both for commercial purposes and restoration efforts to reestablish the different ecosystem services through the creation of oyster reefs. Off-bottom systems rely on the

microalgae present in the water column to obtain a sufficient production and, compared to bottom culture, it is characterized by a higher control level on the farming steps that leads to different advantages as a fast growth due to the control on the stocking density, control on fouling organisms and increased survival thanks to the possibility to manage better anoxia phenomena at the bottom and presence of predators. Even if there are several advantages, off-bottom culture techniques require big investments in terms of money, labor and time. On the other hand, on-bottom techniques tend to be less expensive since they consent to obtain huge quantities of oysters by using the already present oyster reefs or other private oyster grounds (Walton et al., 2013; Walton and Swann, 2021).

As mentioned before, density is a very important parameter. The density at which shellfish are reared can impact both survival and growth because of the competition between the present individuals for space and food (Capelle et al., 2020). In nature, it is identified for *O. edulis* a maximum distance, usually under 1.5 m, between individuals that allows the best larvae production (Smyth and Roberts, 2019). It is important to also consider the reproduction strategy of *O. edulis* where female individual brood the new young individuals: this makes them more subjected to the Allee effect at low densities compared to other oyster species that have a different reproduction strategy (Hughes et al., 2023). It is then clear that for restoration purposes, a minimum stocking density is required to obtain positive results, in terms of increased production, in oyster restoration

(Reeves et al., 2020). In this perspective, also extremely low stocking densities can determine economic loss because of the available space not being fully used, while high densities can cause competition between individuals, with bad outcomes in terms of growth and survival. It is then necessary to establish an intermediate density that can represent a compromise between the use of the available space and the optimal performance of the individual (Macedo at al., 2021). Macedo et al. (2021) conducted a study on both juveniles and adults of *Crassostrea gasar* reared at three different stocking densities. For the juveniles, low density (D) presented 500 individuals per bag, medium density (2D) 1000 individuals while high density (3D) 1500 individuals. For adults, density D presented 216 individuals per bag, density 2D presented 462 individuals, while 3D presented 648 individuals per bag. The used bags, both for juveniles and adults, had an area of 0.5 m^2. The best survival, at the end of the experiment, was for the juveniles at low density, while for adults it was in D and 2D. Considering growth, measured as increase of shell height, was observed only in juveniles at densities 2D and 3D (Macedo et al., 2021).

To obtain the best results, it is necessary to select the best site to practice shellfish culture. Shellfish aquaculture is limited by the requirement of a suitable site, high sea water quality, carrying capacity of the body of water and the ecological impact of the activity itself. In the specific case of intertidal shellfish farming, the activity turned out to be not only intensive from a work force point of view but also limited

concerning the possibility of automation and other improvements in term of industrial equipment. Because of these specific requirements, it came up the necessity to move aquaculture practices to offshore sites. Before establishing bivalves farming in offshore location, there is the necessity to determine all the traits that characterize the new environment to assess the possibility to establish an activity there. Along European waters, it is already possible to find bivalve production in open waters and also oysters seem to be suitable for this purpose, especially associated with wind farms. Few examples of offshore farming can be found in France, both with off bottom and on bottom techniques. Offshore mussels farming has good results and this, in association with the overexploitation of intertidal areas (resulting in reduction of growth) led oyster farmers to look for new and unexploited sites offshore, where the first experiments were done using cage at the bottom. What is important to keep in mind is that producers are looking for a site that allows both survival and growth of the reared individuals. Considering that oysters are suspension feeders, it is necessary to correctly evaluate the temporal and spatial variation on which phytoplankton and other suspended materials are subjected. Along with the evaluation of the spatial temporal variability related to feed availability, it is necessary to also evaluate water temperature to determine the possible response, in term of growth, coming from the individuals. Finally, in selecting new offshore possible sites suitable for shellfish farming, it is necessary to identify the socio economic and environmental

restrictions related to industrial development, shipping activities and fisheries, as well as marine biodiversity protection and sea water quality maintain (Barillé, et al., 2020).

An extremely valuable tool that can be used to identify the best site for oyster farming in offshore sites is the Coastal Observing System for Northern and Arctic Seas (COSYNA). This system allows a better understanding of all the complex processes that happen in the North Sea and German Bight due to the constant change in the environment because of the exploitation of the coastal area. COSYNA is a modelling and an automated observing system made to execute real-time monitoring of the environmental conditions as well as short term forecast data and data products to understand human activity induced change. These observations are possible thanks to data collected through satellite, in situ platforms and remote radar. To better understand the interactions that happen between ecology, physics and biogeochemistry of coastal areas, there is the use of instruments, sensors and specific algorithms. It is evident that currently, in the North Sea, there are a lot of changes taking place that influence, for example, the food webs determining the disappearance of native species. The data obtained are available and free for the public and they are used by different national authorities to fulfill the requirements of the European Water Framework Directive and the MSFD (Baschek et al., 2017).

2.2 *Oyster nursery: feeding strategies*

According to the development stage of oysters, both quality and quantity of feed variates, since the administered diet should meet the needs of the animals (Robert and Gerard, 1999). For example, larval stages require small amounts of feed of high quality, while at post-larval stage lower quality feed is suitable but at higher quantities compared to larvae stage. During broodstock conditioning stage, both feeding quantity and quality should be high since they are extremely important (Muller-Feuga, 2000).

Generally, the administered diets are made by mixing two or three different species of microalgae since they apport more benefits compared to diets made by a single type of microalgae. In this way, by choosing appropriately sized flagellate and diatoms, both growth and survival are enhanced. It is anyway fundamental to consider both digestibility and the possibility of ingestion of the chosen microalgae (Helm et al., 2004).

In calculating the required diet, it is important to also consider if the rearing system is extensive or intensive. This is because it is extremely hard to apply the same calculation used for determining the feeding ratio in indoor systems to extensive ones. In natural environment locations, density and nutritional values of microalgae greatly varies during the years with different seasons (Helm et al., 2004). In the determination of the feed ratio, the spat biomass in the tank must

also be considered, as well as the water exchange system adopted. Considering the algae dry weight, the ratio can be determined in the following way:

$$F = \frac{(S \times R)}{7}$$

In the formula, F corresponds to the required algae dry weight in one day, R represents a seven-day ratio of algae dry weight per live weight of oyster individual, while S corresponds to the weight, at the beginning of the week (7), of live spat. All the factors are in the unit measure of mg. Considering the used formula, it is then evident that the water volume used for their growth is not considered at all. If the diet is not well determined and the food supplied is too much, spat produces an excessive amount of pseudofaeces and in this case it is necessary to correct the amount of feed. For spat, a good general ratio that can be used corresponds to 0.4 mg of dry weight of algae per every mg of live weight spat weekly. This ratio, in hatcheries, is useful because even if it is not particularly high, it provides enough energy and nutrients with good results in terms of growth (Helm et al., 2004).

Instruments as a Coulter counter and/or spectrophotometer can be useful to evaluate cell number or chlorophyll content to administer the correct ratio. Spectrophotometers or fluorometers are necessary to evaluate the chlorophyll-a content present in an algae culture, but the content of it is strongly influenced by

the nutritional state of the culture. Because of that it is not advised to use these instruments to determine the culture density (Bourne et al., 1989), instead a Coulter counter, or multisizer, is more suitable for the purpose (Helm et al., 2004). The Coulter counter is an optical method that estimate cell particle diameter and, like other optical methods, it is sensitive, fast and it can be used for non-destructive sampling (Sheldon and Parsons, 1967; Kranck and Milligan, 1979; Günerken et al., 2017). The Coulter counter, anyway, does not consent to make any discrimination between the phytoplankton to be counted and other abiotic particles or eventual contaminants (Günerken et al., 2017). To effectuate the counting, all the particles present in a small volume of water pass through a small aperture that separates two electrodes (Sheldon and Parsons, 1967; Marie et al., 2005). The small aperture size is extremely important, and it should have a diameter between 50 and 100 μm to count cells with a dimension of 2 to 10 μm (Helm et al., 2004). Between these two electrodes there is a flow of electric current and, when the passage of the particle happens, it moves a certain amount of conducting liquid that increases, temporarily, the impedance of the aperture. This process determines a signal that is converted into a voltage pulse. By summing the amounts of pulses generated by a specified amount of sample it is possible to know the concentration of the particles in the processed sample. Since the passage of a particle determines a pulse, through the evaluation of the amplitude of the

pulse itself, it is possible to determine the spherical volume of every cell (Sheldon and Parsons, 1967; Marie et al., 2005).

To evaluate the quality of the administered microalgae diets, a hemocytometer can be useful. A hemocytometer is made of thick glass slides that present on the upper layer two chambers, both with a dimension of 1.0 x 1.0 mm. Every chamber, which is marked at the bottom with a grid to allow the count, has a total volume of 0.1 mm^3. To count motile species in an accurate way, it is necessary to add to the sample to process a drop of 10% formalin (Helm et al., 2004) or Lugol solution. At this point, a specific coverslip can be positioned and a couple of drops of the sample can be added with a Pasteur pipette to fill the chambers. At this point, with the support of a microscope and the grids in the chamber, it is possible to count and check the quality of the sample (Bourne et al., 1989; Helm et al., 2004; Tetrault, 2012).

2.2.1 Algae production

The most used microalgae in oyster production are diatoms and flagellates that represent, in marine natural environment, the base of the food chain. In hatcheries, they can be cultured by using treated sea water, a carbon dioxide source and nutrients such as vitamins, nitrates, phosphates and other essential elements (Bourne et al., 1989; Helm et al., 2004). It is not possible to use the natural microalgae population present in the sea water took from the natural environment,

mostly because it requires a high density and a well-controlled microalgae population to fully satisfy the requirement of the bivalves reared species in the hatchery. Also, using artificial algae cultivation allows a major control on the apported nutrients (Helm et al., 2004).

Algae production starts from a stock culture (Helm et al.; 2004) of 100 ml. Those cultures are maintained in condition of isolation where the parameters (temperature and light) are controlled. In these cultures, there is no form of aeration (Bourne et al., 1989; Helm et al.; 2004). When there is the necessity, stock culture can be inoculated to have a starter culture where the culture is grown for maximum 14 days (Bourne et al., 1989; Helm et al.; 2004). The volume used by the HOH for the starter culture is 1 L. In this case, growth is fast compared to stock cultures because they are cultured with carbon dioxide addition, higher temperatures (over 18°C, while stock cultures are usually maintained at temperatures between 4 and 12°C) and higher light conditions. After maximum 14 days, part of this culture is used to have a new starter culture, then another part of it is used to have an intermediate-scale culture and, for this purpose, 20 L carboys are used. These cultures can be directly used for larvae feeding or, in alternative, they can be used for large-scale culture (Helm et al., 2004) with a volume of 450 L.

During algae culturing process, independently from the volume or the used species, the cell population goes through different phases that allows to distinguish a different life stage of the population itself. Those phases are defined as lag phase, exponential growth phase and stationary phase, and they cover all the progression, from inoculation until the collapse of a culture (Bourne et al., 1989; Helm et al.; 2004; Tetrault, 2012). The lag phase covers a time span that starts from the culture inoculation till 2-3 days after and at the beginning, cell density corresponds to maximum 50 cell μL^{-1} (Tetrault, 2012). The following phase is the exponential grow phase: during this period, that lasts more or less 6 days, the culture is fully acclimatized and adapted to the present conditions, so the growth is faster (Helm et al., 2004). During this period, the cell number of the algae population increases in a logarithmic way (Bourne et al., 1989). The last phase is the stationary phase, where the cells concentration is higher and the available resources as light and nutrients are scarce (Helm et al., 2004). In this phase the cell division rate progressively slows down until it stops. Flagellate microalgae can remain in this phase for a long period of time since they are able to reuse nutrients released from other dead microalgae cells, while diatoms can stay in this phase only for few days since they release specific metabolites that have a self-inhibition function. These metabolites tend to attract bacteria and with their growth, the culture gets progressively damaged till it collapses (Helm et al., 2004; Tetrault, 2012).

2.2.2 Characteristics of the microalgae used by the Helgoland Oyster Hatchery and Nursery

In bivalve hatcheries, microalgae can be fed directly as a living feed, but it is important to consider that only a few of them, around 20 species, are considered as a suitable form of feeding (Brown, 2002; Sirakov et al., 2015). Microalgae used in aquaculture should have some specific characteristics such as great nutritional value associated with an appropriate cell dimension, no form of toxicity and easy cultivation. At the same time, they should be easily digestible, and all the nutrients should be easily available and assimilable (Hemaiswarya et al., 2011). Administrating alive feed like microalgae in aquaculture activities has several benefits for the reared organisms since they increase their resistance to disease and positively impact growth and physiological activities. The most used microalgae in aquaculture for feeding purposes belongs to the genera *Tetraselmis, Chaetoceros, Isochrysis, Thalassiosira* and *Nannochloropsis*. Considering the cultivated species and the growing conditions, algae can have different nutritional value, size, shape and cell structure. Their composition and digestibility change with these factors, along with the biochemical composition too. In general, the quality in terms of nutrients depends on the amino acids and vitamins composition, but also with the polyunsaturated fatty acids composition as docosahexaenoic acid, eicosapentaenoic acid and arachidonic acid (respectively DHA, EPA, AA). The additional presence of antioxidants, pigments and sterols

increase their nutritional quality too (Jisha et al., 2021). The production of microalgae is very important in hatcheries, but at the same time it represents a bottleneck for the operations in the aquaculture field. The major limitations are mainly represented by CO_2 and O_2 availability, light and temperature (Iwasaki et al., 2021).

Two of the microalgae species used by the HOH are *Chaetoceros muelleri* and *Isochrysis galbana*. *C. muelleri* is a diatom distributed worldwide in the marine environment. This microalga is characterized by an easy maintenance and a fast growth, characteristics that made the *C. muelleri* a perfect candidate for feeding purposes in aquaculture. This diatom has also the characteristic to easily adapt to salinity variations (Rahmadi et al.; 2020, Minggat et al.; 2021).

In diatoms culture, one of the biggest problematics is the light attenuation phenomenon. What happens is that light availability decreases with cell density and path length in an exponential way, where physical (wavelength) but also biological properties (e.g. cell size, pigments and morphology) influence the phenomenon. In several hatcheries, to provide light in a proper way with the most advantaging wavelength, LEDs lights are used because of their reduced heat production, long life expectancy, high light intensity and high efficiency of energy conversion (Iwasaki et al., 2021).

Carbon is another important factor for algae culture, and it is usually supplied as gas bubbled into the culture itself. Once it is added in water, it is dissolved as inorganic carbon (DIC) where the relative proportions of its components vary with temperature, salinity and pH values. Microalgae can use CO_2, and they can concentrate it with the carbon concentrating mechanisms (CCMs) to convert bicarbonate back into CO_2 for photosynthesis purposes. The addition of CO_2 via gas bubbling in the culture decreases the pH, which makes the CO_2 and bicarbonates present in high concentration. This is a positive signal since those are the two compounds who can be used for the photosynthesis processes. CO_2 bubbling in cultures allows control on the pH in an easy and economical way (Iwasaki et al., 2021).

Considering the morphological characteristics, *C. muelleri* has a brown-orange color with a cylindric oval shape. The cells present four hairlike projections, one on every present seta of the cell (Brown, 2002). Concerning nutritional composition, protein represents 36.5%, lipids 27.9% and carbohydrates 3.2%. The protein content then varies with the growing conditions of the algae culture and the phase at which the culture is used. Concerning lipids, they can be present at different percentages, usually between 20 and 50%. Carbohydrates are mainly represented by cellulose, starch and other polysaccharides, and they are involved in general digestibility. Concerning pigments, the dominant one is chlorophyll-a, but it is also possible to find carotenoids that can act as provitamin A (converted

in a second moment in vitamin A). There is the presence of phycobiliproteins, which are pigments soluble in water and specifically, in *C. muelleri*, it is possible to find C-phycobilins, callophycocyanin and C-phycoerythrin. The most important poly unsaturated fatty acids (PUFA) present in *C. muelleri* are eicosadienoic acid (EPA), decosahexaenoic acid (DHA), linoleic acid and hexadecatrienoate. Regarding essential amino acids (EAA) it is possible to evidence the presence of histidine, methionine, threonine, phenylalanine, valine, lysine, isoleucine and leucine, making *C. muelleri* a good source of EAAs (Jisha et al., 2021).

I. galbana is a golden-brown marine flagellate (Camacho-Rodríguez et al., 2020) belonging to the phylum Haptophyta (Gnouma et al., 2017). *I. galbana* is a valuable feed source used for years in aquaculture (Camacho-Rodríguez et al., 2020). This microalga, under optimal conditions, can show a high growth rate and as mentioned, it is widely used in aquaculture. One of the reasons is that it is particularly rich in lipids, usually 20–30% of the dry weight (Zarrinmehr et al., 2020). *I. galbana* is particularly rich in chlorophyll (mainly chlorophyll a and c), crisolaminarin and carotenoids, along with a high level of essential amino acids and protein too (Gorgônio et al., 2013).

Concerning the nutritional composition, *I. galbana*, is a valuable source of DHA and EPA, while the main two present classes of lipids are free fatty acids and

triacylglycerols. The growing condition and the culture growth phase at which they are used, strongly influence the presence of fats. The fatty acids profile underlined as a main present group the PUFAs (where omega 6 are less abundant than omega 3), followed by saturated and monounsaturated FAs (Bonfanti et al., 2018).

It is possible to find in high concentration aspartate, glutamate and leucine while histidine, methionine, tryptophan and cystine are present in lower concentrations. Oyster individuals, juveniles in particular, grow well in carbohydrates rich diets (Brown et al., 1993) and *I. galbana* is suitable for the purpose since it contains several monosaccharides, such as xylose, arabinose, mannose, galactose, glucose and ribose (Gnouma et al., 2017).

2.3 Influence of environmental factors in oyster aquaculture site selection

Parameters like organic and inorganic matter, chlorophyll and total suspended matter, strongly influence oysters' growth during the time, while physical factors (salinity, temperature and silt deposition) and biological factors (pathogens, parasites, predators and presence of competitors) influence oysters' survivorship. In this context, reproduction, growth, survival, environmental conditions and biochemical content have a strong relationship between each other, impacting at the same time the presence of oyster specimens (Çelik et al., 2015).

The correct evaluation and understanding of those biotic and abiotic parameters, along with logistical factors, have a key role in the selection of the correct site in which it is possible to realize a successful restoration plan (Pogoda et al., 2023; Pogoda et al., 2020).

2.3.1 Water quality

Seawater should have specific characteristics depending both on the bivalves reared in the hatchery and the geographic location. For the success of hatcheries operations, water parameters must be examinate and evaluated carefully because all the different life stages of bivalves have specific physiological requirements (Helm et al., 2004). The concept of water quality can be described as "a measure of the suitability of water for a particular use based on selected physical, chemical, and biological characteristics". So, it is necessary to determine the different water characteristics to evaluate water quality. Once the characteristics of interest are obtained and measured, they are compared to numeric values present in guidelines and standards that measure if water is suitable for a certain use (e.g. protection of the aquatic organism). Human activities such as farming, fossil fuels use and animal-feeding operations can strongly influence and impact the water quality of natural water sources. At the current status, the amount of chemicals in use is so high that it is extremely complicated to determine the risks on aquatic organisms and environment (Cordy and Gail, 2001).

The problem of pollution was detected for the first time in populations of bivalves in the 20th century and it is recognized as one of the main causes of the decline of natural oyster beds. In fact, pollutants explicate their toxicity by impacting both morphological and physiological characteristics of both adults and juveniles. In the case of hatcheries, two of the main elements that can be found, both in water inlet and outlet, are chlorine and zinc. Chlorine concentration between 50 and 200 mg/L negatively impact both larval growth and survival, while zinc increase the risk of unusual development and death in larvae. Another problem is represented by microplastics, as they tend to adsorb other pollutants from the environment and, through respiration, these microplastics enter the bodies of bivalves and remain there (Colsoul et al., 2021).

Transition elements and organo-metal can be easily found in polluted estuaries, and they cause a greenish color of oyster flesh due to the presence and accumulation of copper. Even if oysters are highly tolerant to high levels of zinc and copper, concentration between 1 to 16.5 mg/g dry weight leads oyster to not be suitable for human consumption. While adults are less sensitive, juvenile individuals are extremely sensible to zinc, copper, cadmium and mercury (Perry and Jackson, 2017).

Another problem is represented by polycyclic aromatic hydrocarbons (PAH) and hydrocarbons. In general, contamination from hydrocarbons affects the growth

rate of both adults and juveniles of *O. edulis*. PAH are substances that derive from the combustion process of fossil fuel. These compounds are extremely water soluble and because of this characteristic, they accumulate in high concentration in the tissues of all the bivalves. PAHs are able to negatively impact oysters' immune system (Perry and Jackson, 2017).

Synthetic compounds like antifoulant (TBT) can also cause contamination. Those compounds are used to maintain equipment or other tools that are in contact with water clean. In the past 20 years, the use of TBTs was not allowed so nowadays it is common to mechanically clean the different equipment. TBTs effects on oysters have been studied: halogenated by-products of chlorinated water cause larvae mortality and reduced growth, bromoform affects gametogenesis and feeding process, cleaning agents used in oil spill cleaning increase the normal larvae development time causing mortality. At local level, TBT contamination negatively impact the natural population size of *O. edulis,* due to the bioaccumulation of the contaminant by adults with the consequent effect of postpone the sex switch from male to female in reproduction season (Perry and Jackson, 2017).

Another threat is the increase of organic and non-organic nutrients (e.g. silicon, phosphorus and nitrogen compounds) in the surrounding environment. In fact, this condition can lead to a situation where specific organisms are no longer limited

by the presence of certain nutrients and the consequence is the variation of the different ecosystem services due to the change in species composition. The variation of the supplied ecosystem services leads in the end to the progression of the negative effects of eutrophication (Perry and Jackson, 2017). Also, the increase of nutrients used by photosynthetic organisms can lead to abnormal microalgal bloom with the consequent possibility of having reduced oxygen levels (Bricker et al., 1999) and mass mortality events (Lenihan, 1999). It is anyway important to underline the fact that a slight increase in term of nutrients can bring some advantages for suspension feeder as they determine an increase of food availability (Perry and Jackson, 2017).

2.3.2 Temperature

In *O. edulis*, physiological processes as metabolic and growth rate tend to increase with the increase of the temperature. Optimal growth is detected at temperatures of 17°C but it is possible to witness the same results with exposure, for a short period of time, at 25 °C. It has been proved that oysters are anyway able to survive temperatures of 30°C for short periods of time. An environmental parameter that is strongly linked to the temperature is salinity. Situations characterized by a reduced salinity but a high temperature determine mortality events, but it is necessary to specify that in nature those conditions rarely occur. High temperatures occurring in warm summers positively influence the recruitment rate

(that usually start around 15°C) and this influence of the temperature on the recruitment is particularly important for the regions in the north where *O. edulis* occur. Larvae growth is optimal between 12 and 27°C, while at 30°C survival is extremely reduced. From this knowledge, we know that the long-term presence of a natural oyster bed and the recruitment rate, depends on temperature. When in winter temperatures reach 10°C or less and salinity is lower, individuals of *O. edulis* present a reduced metabolic rate that allows them to resist and survive the winter period. This is valid only for adult individuals because at the same temperature larvae survivorship is extremely low. If in summer low temperatures occur, the outcome is a general low recruitment rate: this outcome is deeply linked to the reduced food availability due to the surrounding conditions and a larval development that require a longer time. It is then clear that low temperature can determine huge variation on oyster beds, affecting also the recruitment (Perry and Jackson, 2017).

2.3.3 Fouling impact

Biofouling, also defined as "biofouling communities" or commonly called "fouling" is characterized by the accumulation of different organisms belonging to different taxa (e.g. polychaetes, anthozoans, bryozoans, ascidians, hydroids, sponges and barnacles) on surfaces that are partially or totally submerged in water. Those surfaces can be represented, for example, by bivalve shells but also

aquaculture gears. The composition of organisms can be different depending on the type of colonized surfaces, location, time of the year and surrounding conditions (IOC Technical Series, 2022). Also in oysters, biofouling represented by the epibiota growing on them is considered to be a spoiling factor (Smyth and Roberts, 2010). Once the biofouling is attached and well formed on a surface it can be quite difficult to remove and the present organisms can be competitors for resources meant to be for the reared species in cages (IOC Technical Series, 2022).

In aquaculture activities concerning bivalves rearing, biofouling represents a threat since it can impact growth and the collection of spats. For companies, it can represent economic damage since it affects the aesthetic of the product meant to be sold in the market. The economic threat concerns also the cost of the operations that are then necessary to control biofouling, it is in fact estimated that around 15% of the total annual operating cost are used to control biofouling (Sieversa et al., 2019).

Biofouling is problematic also because it grows on the cages used to grow bivalves, causing in this case a reduced water exchange with the consequent reduction of oxygen. In this way, the accumulation of waste products on the interested area is favored, causing problematics to the other present marine organisms (Arduini et al., 2022).

2.3.4 Wave action

Natural populations of *O. edulis* are usually located in areas where the tidal flow is usually under 1 knot (0.5 m/sec) because in the case of environments characterized by very strong waves, they can be removed from the substrate causing a big loss in the population. In this case, the detachment is due to the erosion of the substrate where the oysters are attached (Perry and Jackson, 2017).

Even if an increased water flow can improve the amount of available particles in the water column, it can also affect the feeding in a negative way by reducing the time window where the individuals are able to feed. In fact, it has been shown that higher water flow leads to an increase of the filtration rate until the point where oysters cannot remove any more particles from the surrounding water. From this point on, every other increase in the flow does not lead to any benefit. It is then clear that a water flow that is too harsh have a strong impact on *O. edulis* specimens. Water currents affect not only the feeding process, but also the success of the reproduction and recruitment rate. It is then hypothesized that oysters tend to grow better in less exposed areas (Valero, 2006).

2.3.5 Depth

Depth is an important parameter to consider because with the variation of it the values of oxygen, temperature and chlorophyll concentration also vary. In experiments conducted on the *Crassostrea gigas*, it was evidenced that the

different species had benefits from being reared at depth between 7 and 13 m, where at this depth the environment conditions were more favorable for their growth and survival (Zrnčič et al., 2007).

In bivalves, the different environmental conditions such as salinity, temperature and in particular food availability, strongly influence the energy storage cycle. In general, with depth increase the amount of available food tends to decrease. In fact, it is common to find oysters in coastal areas where food available for them is higher. At greater depth then, the growth of the individuals tends to be affected negatively. The food lack in deep water impacts fecundity too (by slowing gonad maturation) and, for restoration purposes, it is then better to use a reduced depth for rearing individuals, because a faster gonad development is linked to a faster recovery. In fact, a fast gonad development indicates the presence of high energy reserves. This data suggests that the culture depth of bivalve strongly influence the development of the bivalve themselves due to the parameters change (Ngo et al., 2006).

Since oysters are tolerant to air exposure, in mariculture practices they are reared in trays localized in areas characterized by a low intertidal level. This strategy can cause different problematics since water immersion is reduced. Anaerobic respiration determines oxygen lack and the lack of time in water influence negatively the feeding process. The energy cost of this process determines a

reduced growth but also problematics regarding the reproduction abilities. *O. edulis* specimens can be found at depths of 80 m, and deep-water locations can be considered as sheltered areas for their growth. In shallow water in offshore sites, water currents can determine the previously listed negative effects. In general, areas characterized from a bottom abundant in empty and broken shells, are usually less impacted and more resistant to wave action (Perry and Jackson, 2017).

2.4 Impact of environmental factors on survival and growth

Growth can be evaluated in different ways, for example it can be described as an increase in term of shell length or height, soft tissues weight or a mix between the previous parameters. Oyster juveniles' growth rate is directly related and influenced by water parameters, feed ratio and the nutritional value of the administered diet, while a minor role is covered then by genetic and water salinity level. The feed ratio is strongly related to temperatures: at higher temperature the metabolism is faster, and it is necessary to administer enough feed to support the fast metabolism with some extra energies to devolve to their growth. In fact, growth in the different geographic areas is different and in temperate regions growth is clearly detectable in spring and summer due to the higher water temperature and feed abundance. Growth stops in winter, and this determines a check on the shell that can be used to determine the age. Growth is strongly

influenced by stocking density too and it is required to find a compromise between maximum growth and density. At exaggerated growing densities per volume unit of water, mortalities are frequent and the first detectable symptom is a progressive decrease in color intensity of the shell. Another sign is a low pH value in the tank, usually under 7. The acidity conditions are present due to the accumulation of CO_2 produced by bacteria and oyster individuals in the tank. The acidity determines the discoloration of the shell and if the situation persists it dissolves the crystal of calcium carbonate of the shell. In general, oyster juveniles belonging to *Crassostrea* tend to grow faster than other bivalve species or compared to *O. edulis*, and this could be related to larvae size at settlement phase and the absence of lag phase during the metamorphosis stage of the larvae (Helm et al., 2004)

For restoration purposes, since the major goal is to reestablish an ecosystem and its functions, the biggest challenge is to have high growth and survival rates, while enhancing genetic diversity (Colsoul et al., 2021).

O. edulis individuals are usually located in coastal areas, but it was shown that both growth and survival do not seem to be negatively impacted by offshore sites conditions. Growth increase was confirmed both by the increase of the shell length but also by the increase of the dry mass. The high survival confirms then the suitability of offshore sites (Pogoda et al., 2011).

A mortality cause could be the lack of oxygen. Even if oysters are in general able to well tolerate condition of hypoxia, the lack of oxygen can impact them causing their death (Perry and Jackson, 2017). In fact, it was underlined that in *Crassostrea virginica* a long-time span spent in hypoxia condition, paired with low food availability and general poor condition, leads to mass mortality events (Lenihan, 1999).

Another parameter that can influence oysters' presence is the amount of suspended solids (Perry and Jackson, 2017). It was evidenced that the growth of *O. edulis* was improved at reduced levels of suspended solids (Ray et al., 2005). In situations characterized by high levels of suspended solids, oysters respond with an increased production of pseudofaeces paired with a rapid valves movement to expel the accumulated sediments, process that require a high energy loss. In general, in these conditions, the filtration rate is reduced to avoid this energy loss. High amounts of suspended sediment impact negatively the growth rate, while it causes the thickening of the shell, which leads to the incapacity to properly feed. It is then possible to say that European flat oyster individuals are intolerant to highly turbid waters and that the variations of sediments presence in the environment deeply impact also the juvenile individuals causing long term effects on the present oyster population (Perry and Jackson, 2017).

Third chapter - Aims of the study

Considering the actual status of *O. edulis* in the North Sea and worldwide, the present study aims to determine where to establish a field nursery in the proximity of Helgoland, Germany. The experiment also aims to determine at which depth and density it is better to locate an offshore oyster nursery, basing the decision on the best performance of the reared juveniles. Using information coming from decades of studies on *O. edulis* aquaculture and restoration practices, the main parameters to evaluate to understand its success and feasibility are established.

During the five months of experimentation, the performances of *O. edulis* individuals located at two offshore sites are compared with those from an onshore site. In particular, the field experiments are performed to assess the growth and fitness of oyster juveniles at two different depths, while the onshore experiment specifically determines how temperature, density, and food availability affect oysters' performance. Additionally, at the onshore site, it is possible to evaluate which diet best supports oyster growth and performance. Special attention is also given to the different parameters and characteristics of the offshore sites, such as temperature, feed availability, and wave action, since these factors vary throughout the year. The results from the reared individuals at the onshore site are used to evaluate the performance of those at the offshore sites.

In this way, it is possible to determine not only the feasibility of each field nursery site but also the optimal stocking density for growing oyster juveniles for

restoration purposes, as well as the correct depth and site. The collected data and results can be used to address future challenges concerning oyster restoration

Fourth chapter - Materials and Methods

4.1 European oyster juveniles

The oysters used during the experimentation presented a size of 12 mm. However, since oysters are selected by the producer through a grading process, they had a size that could be above or below 12 mm. In fact, grading is a mechanical process of selecting oysters in which they pass through a kind of "mold" that has the size required by the customer. However, since these can pass through the mold following different possible orientations, it is very likely that individuals larger than 12 mm will also be able to pass through. The same concept can be applied to oysters smaller than 12 mm, were in this case, given the small size, effortless passage occurs. The used individuals were ordered from Morecambe Bay Oysters, a company located in the Northwest of England in the United Kingdom, specifically on Walney Island in Morecambe Bay. After their arrival, oysters were stored in basket, hung on poles and located on tanks outside the research facility. At this point, the baseline, i.e. the initial biometric characteristics, were determined. In this way, it was possible to observe that in March, at the beginning of the experiment, the average measurement concerning shell length was 17.12 ± 1.7 mm, while the average weight was 1.09 ± 0.3 g. The average volume was 1.28 ± 0.4 cm^3.

4.2 Oyster baskets and stocking density

The rearing system in the offshore sites and onshore site were different. In the onshore site it was used the shelf system with the Nursery Shelf System Baskets from Casamer with a length of 80 cm, a width of 30 cm and a mesh size of 6 mm, with a total area of 2400 cm^2. The shelf system is realised with a vertical arrangement, allowing a better water circulation and less sedimentation in the tanks, creating the best condition for oyster juveniles' growth while it maximizes the use of space. In the offshore site the used baskets were from SEAPA, with a dimension corresponding to 60 cm length, 21 cm width and a mesh size of 6 mm, with a total area of 1260 cm^2. In this case, baskets were located on landers, durable and stable structures made of metal that can have a different frame. These structures are located on the sea bottom and basket are clamped to them on horizontal poles. This kind of design allow a good water circulation, ensuring to the reared organism enough oxygen, waste removal and feed. Both types of baskets were made of resistant plastic materials with, at both of their extremities, a "door" to allow the positioning of the oysters inside of it. On top of the basket, there were a pair of clamps to position them on poles inside the onshore tanks or on landers in the offshore sites. Inside the baskets, juveniles of oysters were stocked at three different densities, high (Hd), medium (Md) and low (Ld) density. High density corresponded to an initial weight of 2.5 kg with a density of 2 g cm^{-}

^{2} thus corresponding to a 25% increase over medium density. The medium density corresponded to a weight of 2 kg and a density of 1.6 g cm^{-2} and, at the start of the experiment, this density was used as a reference point. The low density corresponded to a weight of 1.5 kg, with a density of 1.2 g cm^{-2}, corresponding to a 25% decrease from the medium density. In the preparation stage, *O. edulis* juveniles (sorted with a grading of 12 mm) were taken from their holding tanks and then cleaned with fresh running water. Before being weighed and placed inside their respective baskets, they were gently spin-dried. The same procedure was applied in the preparation of every basket for every site.

Within each basket, a bag containing approximately 100 g of juveniles was placed inside every basket in order to perform all the required tests prior to the conclusion of the experiment: these, were made with a design such that the bag could be easily retrieved by the divers of the Centre for Scientific Diving (AWI-CSD). These bags were also located in the cages at the onshore site in order to have in each cage at each site the same conditions. Once located in the final baskets, these were identified with a label reporting the density. Based on the site and density, labels had a series of holes and eventual chippings to enable the divers of Centre for Scientific Diving (AWI-CSD) to clearly distinguish them underwater. A temperature logger (UA-001-08, HOBO Pendant, Onset Computer Corporation, US) was then attached to each cage, in each site, to constantly monitor the change in temperature during the months of the experiment. The three baskets for the

offshore shallow site and the three cages for the offshore deep site were then brought in position with the support of the Centre for Scientific Diving (AWI-CSD).

Figure 1. On the left, baskets containing spat (located in the onshore site) and, on the right, bag containing juveniles (located inside the baskets).

4.3 Field nursery sites: description and design

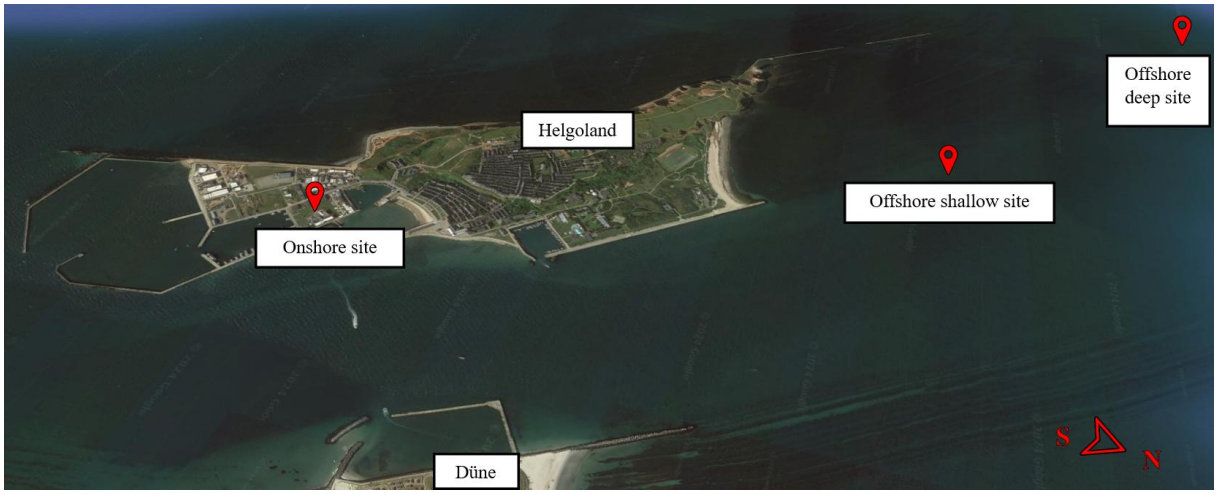

Figure 2. Satellite picture of the island of Helgoland (Germany) with the location of the three experimental sites (picture retrieved from: Google Earth).

The experiment took place in three sites: one site within the Alfred Wegener Institute facilities (onshore site) and two offshore sites (offshore-deep and offshore-shallow) where oyster baskets were located at two different depths. As the onshore site, they again had three baskets each site, also with the same densities (Hd, Md, Ld). The structural design was the same for both offshore sites, that is, a benthic lander made of steel with crossbars for attaching oyster baskets. The offshore deep site was characterized by a depth of about 25-28 m, resulting in low light and a substrate consisting of soft sediment and some cobble stone. As for feeding, this was completely natural. The offshore shallow site, on the other hand, was characterized by a depth of about 8-11 m, with a higher light intensity than the offshore deep site where, light intensity was medium-low. The sediment in this case was represented by mobile sand and pebble bottom.

In both sites, the feeding regime was natural. Information regarding feeding in both offshore sites, comes from the Helgoland Roads long term data series (Wiltshire et al., 2010). The waters surrounding Helgoland are characterized by an incredibly rich phytoplankton community. This is because the area is affected by the high discharge coming from the river Elbe, which determines different water conditions that swings from typical open North Sea conditions to costal conditions with a reduced salinity. In the area, it is confirmed the presence of different phytoplankton species, in total 250, where 147 belongs to diatoms and

97 belongs to dinoflagellate. It is also observed the presence of just few species of silicoflagellates, ciliate and chlorophyte (Kraberg et al., 2019). This primary production is influenced by different parameter, such as temperature, nutrients, water turbidity, water turbulences and light conditions. The species present in the area, during the annual succession, are "slotted" in different phenological time slots who are specie specific. Therefore, the phytoplankton species shows both intra-annual and inter-annual changes in term of growth windows (Scharfe and Wiltshire, 2019).

4.4 Onshore nursery

The oyster baskets located at the onshore site were placed in three tanks with a volume of 420 L and a depth of one meter. Compared to both the offshore sites, the luminosity was medium/high and feeding was not natural. In fact, to each individual tank corresponded a different diet with different concentration (3, 6, 9 µg Chl L^{-1}). Within each tank, different density baskets were then located where, for each tank, one basket corresponded to Hd, one to Md, and one to Ld. Small mesh pouches containing ~100 g of juvenile individuals were also located within each basket, along with a temperature logger (UA-001-08, HOBO Pendant, Onset Computer Corporation, US). The vertical arrangement of the different densities, from top to bottom, was determined randomly, respectively this was the arrangement for each tank during the course of the experiment:

- 3 µg Chl L^{-1} diet tank: Hd, Ld, Md
- 6 µg Chl L^{-1} diet tank: Md, Ld, Hd
- 9 µg Chl L^{-1} diet tank: Ld, Hd, Md

Figure 3. On the left, carboys containing the specific diet, on the right, tanks containing oysters. To each tank, corresponded one of the diets.

4.4.1 Nursery tanks

Each tank had an unheated seawater supply corresponding to 210 L/h. In addition, they were equipped with a circulation pump to ensure the stirring of water inside the tank (5000 L/h). The water discharge flow corresponded to 215 L/h while the feed flow for each individual tank corresponded to 5 L/h. Each tank was related to a specific diet that was administered through silicon lines. With the support of peristalsis pumps, the diet was administered from the 21 L carboy to the tank.

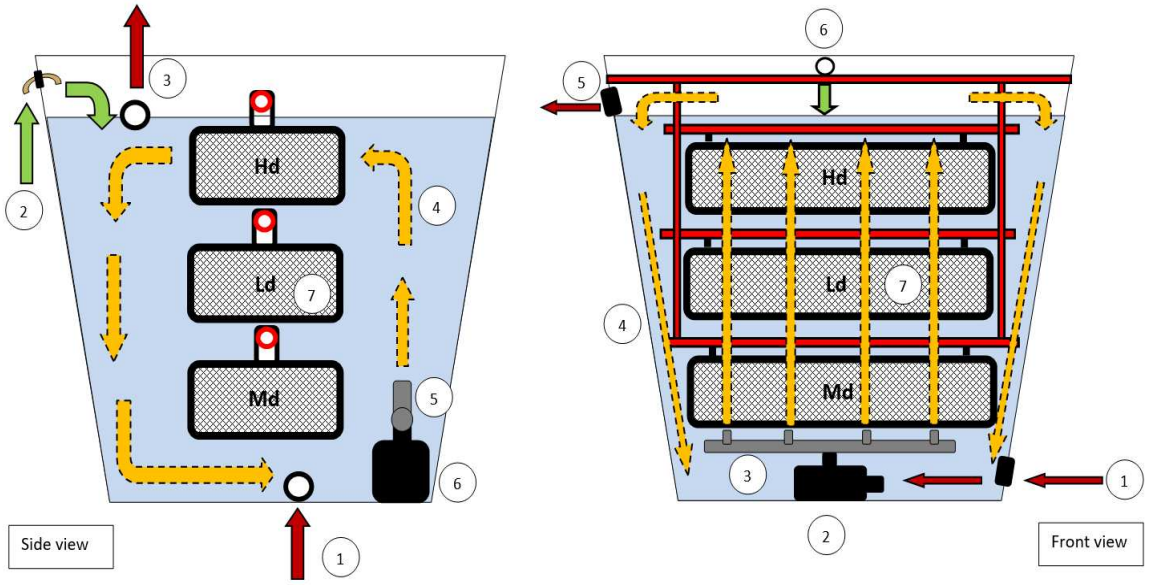

Figure 4. Description of the rearing system used at the onshore site. 1) tanks 2) support structure for oyster baskets 3) baskets 4) feeding lines 5) peristalsis pumps for diet feeding 6) 21 L carboys containing the specific diet for each tank.

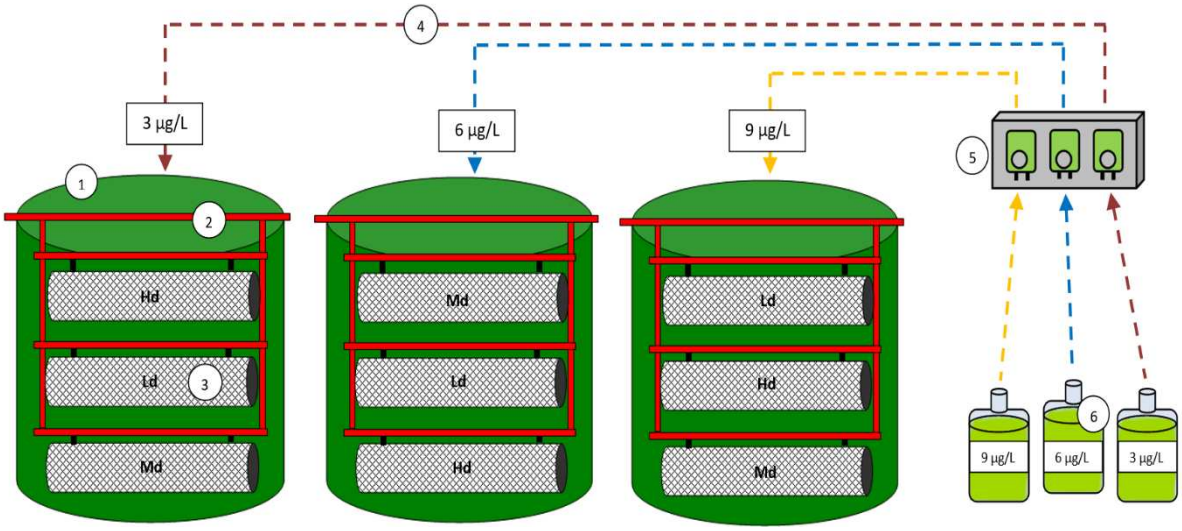

Figure 5. Representative diagram of tank-specific details. Side view:1) water inlet point 2) feeding line and feed 3) water outlet point 4) circular water flow 5) water distribution system for recirculation 6) water recirculation pump 7) oyster basket. Front view: 1) water inlet 2) water distribution pump 3) water distribution system 4) water flow (yellow arrows) 5) water outlet point 6) pump 7) oyster basket.

4.4.2 Feed production, diets, and feeding

Each site was characterized by a different type of diet. The offshore sites had a natural type of feeding, thus influenced by the different natural phenomena. In contrast, the onshore site was characterized by the administration of three different diets, one for each tank. The values of the different diets represented a weekly average where, to compensate nights during the week and weekends days where oyster juveniles were not fed, it was slightly increased to maintain the ideal weekly concentration. To determine the concentration of algal cells, a first estimation was done using the results of Houliez et al. (2012) regarding chlorophyll fluorescence of an haptophyta, namely *Phaeocystis globosa*, were fitted regression was used, in which:

$$y = 0.000703 \cdot x$$

Where y corresponds to chlorophyll while x corresponds to algal cell count. Starting from those results, it was conducted a dilution series on *I. galbana* to obtain the calibrated values. The mean of the three diets were generated with 12,000, 24,000, 36,000 cells/mL during feeding time. In this way it was determined that:

$$y = 0.000802 \cdot x$$

In this way, it was obtained that the first diet i.e., the 3 µg Chl L^{-1} diet corresponded to 3,700 cells mL^{-1}, while the second diet, the 6 µg Chl L^{-1} corresponded instead to 7,500 cells mL^{-1}. Finally, the third diet, the 9 µg Chl L^{-1} diet corresponded to 11,000 cells mL^{-1}.

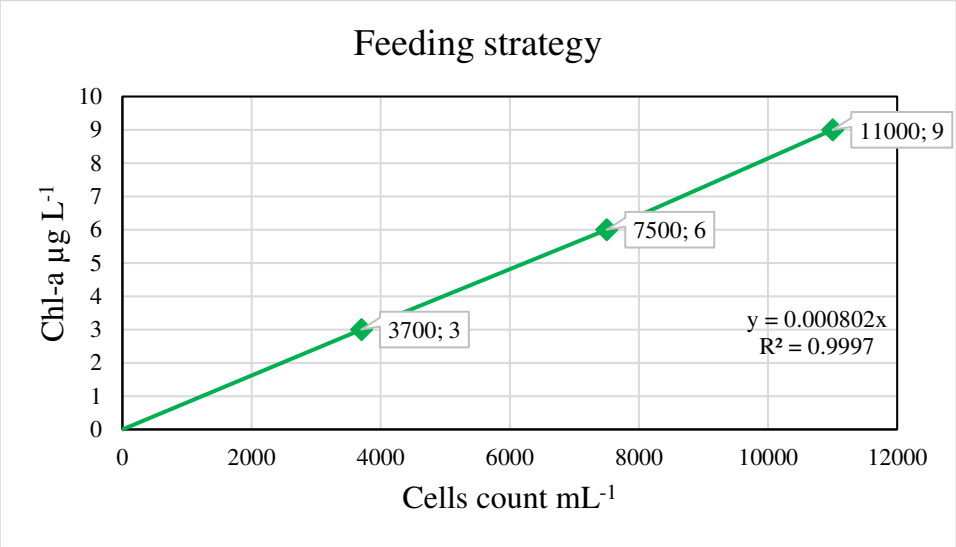

Figure 6. Relation between Chl-a and cells concentration.

The diet consisted of *I. galbana* and *C. muelleri* in a 16:10 ratio and it was administered twice a day through the use of two 21 L carboys for each tank. Therefore, feeding occurred for a total of 8 hours per day (during the first 4 hours, the first 21 L carboy was fed, during the next 4 hours, the second 21 L carboy). During nights and on weekends, no feeding occurred. In the morning, along with the first 21 L carboy, a morning bulk dose was administered directly into the tank to quickly reestablish the algal cell concentration per established diet.

In order to administer, daily and correctly, the right amount of *I. galbana* and *C. muelleri,* it became necessary to develop and use a calculator that, starting with the morning concentration of cells per ml in the different PBRs, allowed the determination of daily amounts. Each morning, samples of algae were taken from the different PBRs, samples that were analysed through the use of the Coulter counter. The results, in cells per ml, were entered at this point into the calculator, which determined the amount of *I. galbana*, *C. muelleri* and sea water to be included in each individual carboy depending on the diet. The calculator worked as follow: to determine the microalgae amount to add in each carboy, it was first necessary to determine the total cell concentration required inside each carboy per diet, by multiplying the carboy volume per the diet cell concentration. To determine the correct amount of *I. galbana* and *C. muelleri*, the 16:10 ratio was then applied to the total cell concentration inside each carboy. At this point the cells concentration per ml regarding the specific microalgae (*I. galbana* and *C. muelleri)* was divided for the cell concentration measured by the Coulter counter in the morning (for both *I. galbana* and *C. muelleri*). In this way it was possible to have the correct amount, per carboy and specific diet, to administer twice a day. The microalgae volume was then mixed with seawater until the 21 L volume of the carboy was reached. The same calculator was used to determine the bulk dose administered in the morning: *I. galbana* and *C. muelleri* 16:10 ratio was then applied on the target cell concentration required by the diet and the obtained value

was multiplied by the tank volume. The obtained value was then divided for the specific algae concentration measured in the morning. In this way the correct amount of *I. galbana* and *C. muelleri* was determined and administered early in the morning.

4.5 Biometrics

The main biometric values that were measured were condition index (CI), dry weight (DW) and the volume of the single individuals. Along with these parameters, also the clearance rate (CR) was evaluated. The CR is a test that, per unit of time, gives information regarding how a known volume of water is cleaned from the present particles (Wilson, 1983). CR tests are useful to understand the different interaction, positive and negative, between the environment and bivalves rearing practices. This kind of test is able to reflect the actual responses of the reared individuals to several physical, biological and chemical factors that are able to influence the feeding behaviour. Understanding the feeding behaviour is fundamental for aquaculture practices, since information concerning it leads to the correct site selection and better layout of the rearing system (Cranford et al., 2011).

Every month, from March to July, CR was evaluated and at the conclusion of each test, the oysters contained in the flasks corresponding to the first repetition of high,

medium and low density (H1, M1 and L1) were sacrificed to assess the CI, evaluated by using the following formula (Davenport and Chen, 1987):

$$CI = \frac{Dry\ tissue\ weight\ (g)}{Dry\ shell\ weight\ (g)}\ x\ 100$$

In order to evaluate the CI, it was first necessary to determine the dry weight of the selected individuals. Once tissues and shells samples were taken and placed in tin foil with a known weight, they were placed in an oven at 68°C for a total time of 48 hours (Lawrence and Scott, 1982). After this period, samples were taken and weighed, and with the weights obtained, the CI was evaluated. The evaluation of the CI is necessary to have information concerning health of reared bivalves, since it considers parameters related to their growth. CI also provide ecological information concerning the individual's performance in the selected culture method, and it also provides information regarding the appropriateness of the selected site for bivalves' aquaculture practices, since there is a correlation between food availability in the selected site and CI (Cotou et al., 2024).

Another biometric used to assess the individual growth was the shell volume, that represent the volume of a single individual as if it were a compact and perfect structure. The used formula was the following one:

$$(((((Length/2) * (Width) * (Height/2)) * (4/3)) * \pi)/2)/100$$

Length was measured considering the most external point on the left and on the right of the shell, while height was taken from the umbo to the highest point of the upper margin. As for width, it was measured by placing the individual between the two ends of the calliper, such that these were both positioned at the highest points of both the right and left valves. Therefore, once length, width and height were measured, the volume of each individual was assessed and an average was then calculated.

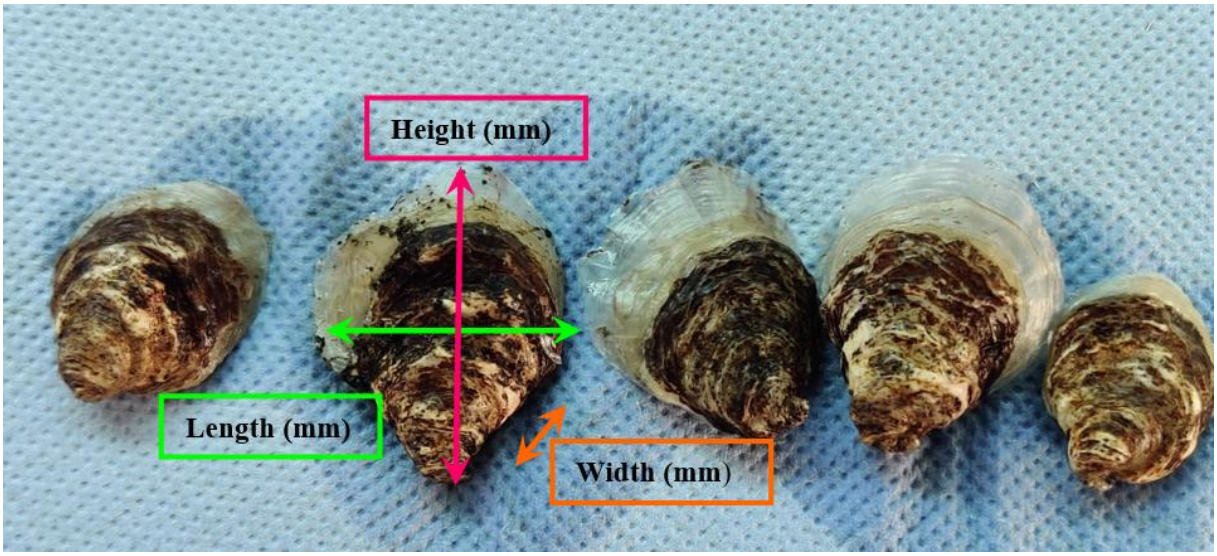

Figure 7. Explanatory diagram of biometric parameters taken with a digital calliper.

4.6 Clearance rate assessment

In the preparation of each clearance rate test, a total of 25 oysters per density were sampled for each diet and/or offshore deep and offshore shallow site, thus, during the execution of the test, there was a total of 75 oysters comprising the three different densities (Hd, Md, Ld). In the preparatory stage, biometric parameters

as height, length, width (all measured in mm) and wet weight (g), were evaluated. In groups of five individuals, oysters were placed inside replicate flasks containing 500 ml of filtered seawater.

Figure 8. Oyster juveniles, in group of five, located in replicate flask with 500 ml of seawater for the CR test.

Flasks were placed inside a water bath that had a constant water flow in order to keep the temperature stable. Above the basin there was a rigid structure composed, on the left and right extremities, of two rigid vertical bars. Attached to these bars there were other three horizontal rigid bars. Thanks to this structure it was possible, through the use of labels, to create a 3x6 grid system where to locate the total 18 bottles, where the horizontal lines were named A, B, C while the vertical ones were numbered from 1 to 6. In addition, on this rigid structure, small

silicon tubes necessary for the aeration of the flasks were positioned: therefore, starting from the main lines located along the three horizontal bars, small silicon tubes (six per line) were positioned perpendicularly, equipped with valves to regulate the air flow and attached to a glass cannula that entered the flask providing aeration. Once the oyster juveniles were sampled and prepared for the test in five groups of five per density, all the flasks were labelled and located in the correct position on the grid.

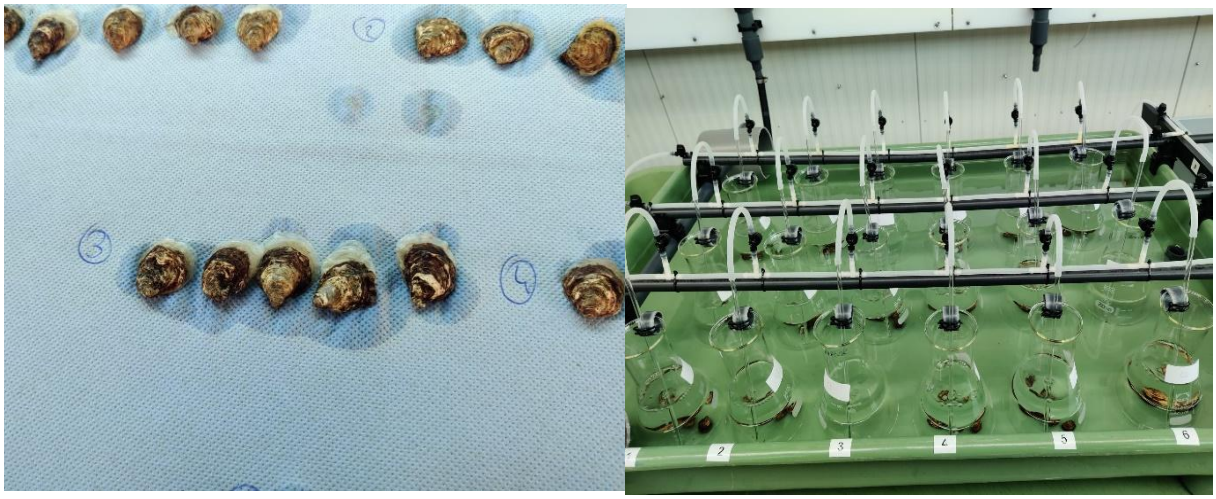

Figure 9. Left image: a group of five individuals being blot dried and measured prior to being placed in a flask for the CR test. Right image: overview of the water bath and CR experiment system, showing the flasks in position.

Through the use of a random number generator (from 1 to 18) the groups of juveniles were randomly located inside the flasks. One blank (clean control; CC) and two further controls (algae control; AC 1 and AC 2) were included in the design.

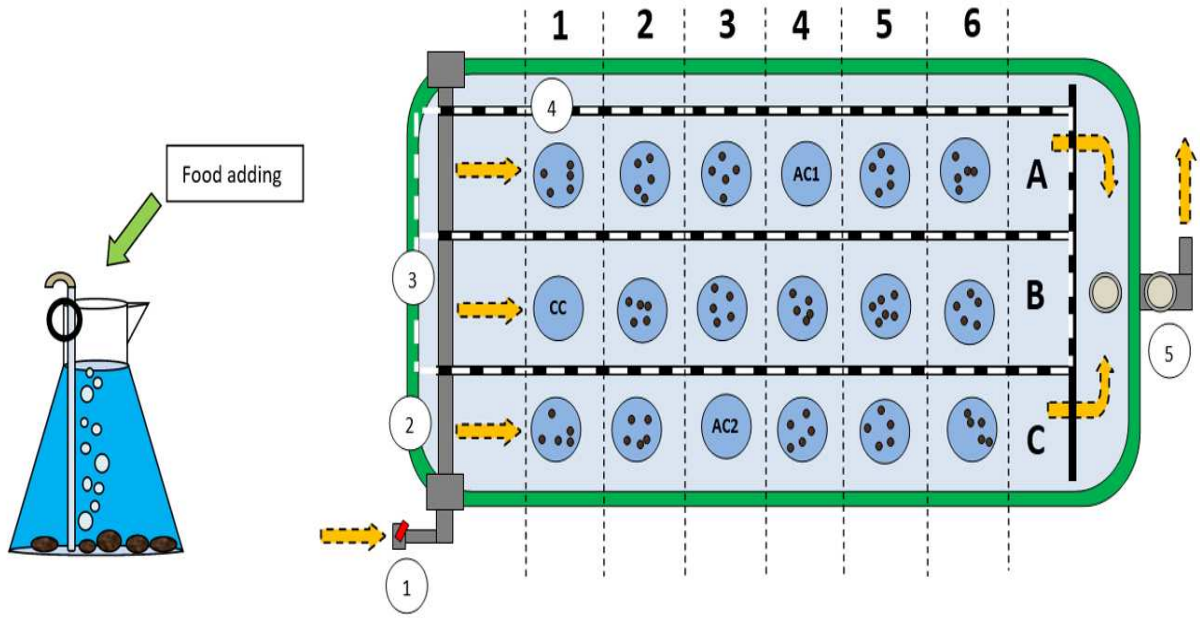

Figure 10. Schematic representation of the components of the clearance rate. Left: flask containing the 5 specimens (belonging to a specific density) tested, in each flask was inserted a glass cannula connected to the aeration system in order to have a constant flow of bubbles in the flask. Right: structure in which the CR was carried out: 1) water inlet point inside a tube where through the 2) holes the water flowed out inside the bath 3) primary silicon lines of the aeration system associated with 4) a support structure for the lines themselves from which the secondary lines entered the flask 5) water outlet point.

Before performing the clearance rate test, an acclimatization period (> 8 h) was provided. During this period, oysters were maintained in the flasks, placed inside the bath with an aeration system, in addition to an initial feeding represented by *I. galbana*. The amount of the feed given to every flask, depended on the morning cell count of the algae contained in the PBRs. In each flask, considering a volume

of 500 ml, the aim was to obtain a concentration of ~11,000 cell per ml. In order to do that, it was used a calculator where, by entering the morning algae cell count, the volume to administer in every flask was determined. This process, however, was not done for the flask representing the blank (CC).

After the acclimatization time had elapsed, before starting the experiment, two additional hours were scheduled in which the feed was added twice. After this time had elapsed, the experiment could begin. At the start of the experiment, so at 0 min, the feed was administered and, immediately afterwards, a 20 ml sample was taken from each flask and then processed through the Coulter counter to assess the presence of algae expressed as cells per ml. After one hour had elapsed, a further sample was taken, in this case without administering any feed, where the algae count was lower due to the oyster's filtration activity. At 120 min, following sampling, the flasks were restored to their original volume of 500 ml by adding the subtracted 40 ml to the present volume of 460 ml. The feed was then administered and then the sample was taken to determine, once again, the algal cell count. At 180 min, the last sample was taken and analysed without adding the feed and, also in this case, the algae count was lower due to the oyster's filtration activity.

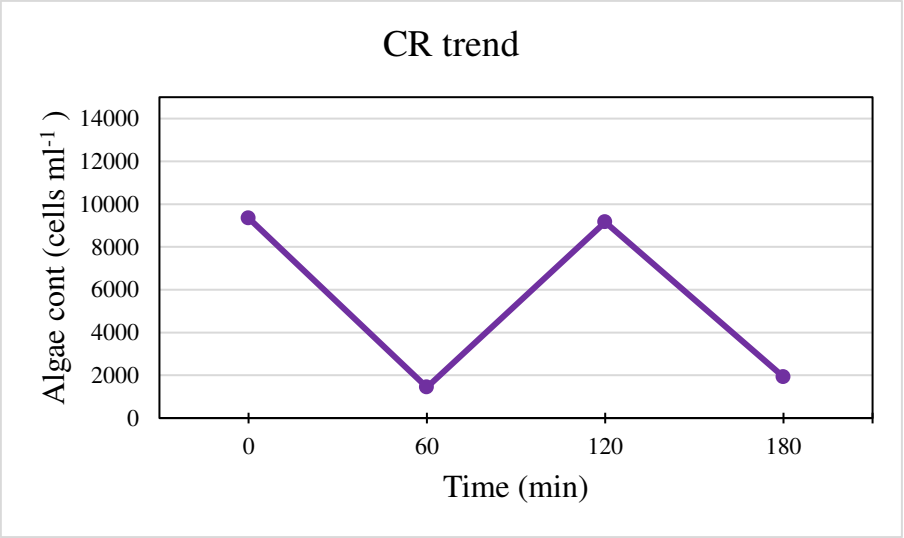

Figure 11. Example of a classic CR trend (specifically CR performed in May). The peaks in the graph represent the interval when the food was added (0 minutes and 120 minutes) while the lower points represent the intervals where the food was not added (60 minutes and 180 min).

At each time interval, the values obtained were then transcribed in order to perform the different statistical evaluations. The following formulas were used to evaluate the values for each CR:

$$CR = \frac{CR_5}{5}$$

Where CR_5 was determined in the following way:

$$CR5 = \frac{(((lnC_0 \; - \; lnC_1) - \; Cc \;) \cdot \; V)}{t}$$

CR_5 represent the clearance rate, expressed in L/h, of the 5 spat in one flask, C_0 represent the cell concentration at t_0 (first time interval), C_1 represent the cell concentration at t_1 (second time interval), C_c in the change in term of cell

concentration in control (C_1- C_0), V represent the water volume expressed in litres and t represent the time in hours.

$$CRw = CR \cdot (\frac{Wstd}{Wexp}) \cdot b$$

Where CR_w represent the weight corrected clearance rate (L/h/g dw), CR in the clearance rate (L/h), W_{std} represent the standardised tissue weight (1 g·dw), W_{std} represent the tissue weight of experimental animal (g·dw), b corresponds to 0.62 which is an allometric coefficient for *O. edulis* (Haure et al. 1998).

4.7 Experiment overview and timeline

The experiment started in March and it ended in July. During the whole period, every month by following the temperature trend, growth (shell length, shell volume and CI) and fitness (CR) were evaluated. The experiments, therefore, were carried out as shown below:

- 1st sampling point in March to determine the baseline at 0 days of treatment (30/03/2023)
- 2nd sampling point in April after 25 days of treatment (24/04/2023)
- 3rd sampling point in May after 53 days of treatment (22/05/2023)
- 4th sampling point in June after 74 days of treatment (12/06/2023)
- 5th sampling point in July after 96 days of treatment (4/07/2023)

The first step of the experimentation was the determination of the baseline values, related to biometric indices, of oyster juveniles. Therefore, a total of 85 juvenile individuals were selected and sampled. For each individual, the different biometric parameters were measured. The wet weight was evaluated after a cleaning with water to remove dirt, algae and any sediment present. Individuals were then blot dried with tissue paper to not distort the measurements with the excessive presence of residual water and dirt. The first data related to shell length dates back to March, when baseline data were evaluated for a total of 85 individuals.

In this way, it was possible to show that, at the beginning of the experiment, the average measurement concerning shell length was 17.12 ± 1.7 mm. Similarly, wet weight was also assessed on all individuals evaluated during base line preparation. It was evidenced that the average weight was 1.09 ± 0.3 g.

Using protocols outlined by Lawrence and Scott (1982), each individual was dissected to divide the soft tissue from the shell. These two samples were placed inside previously weighed pieces of foil, and then oven dried (48h, 68 °C) in order to correctly establish the condition index (CI). The relationship between shell and tissue was determined by measuring the weight once they were dry, with the CI being the percentage of the dry shell weight represented by the dry tissue weight (Davenport and Chen, 1987*).*

During the whole process, out of a total of 85 randomly selected juvenile oyster individuals, 7 individuals were found to be dead and therefore it was impossible to determine the CI. After this process, it was possible to assess in an accurate way the baseline values for the experimentation.

The baseline tests were performed starting from the 30/03/2023 on the tank with the 3 µg Chl L^{-1} diet and, in the next days, the analyses were conducted on the 6 µg Chl L^{-1} and 9 µg Chl L^{-1} diet. During all individual tests, different biometric parameters were measured and evaluated on the total of 75 individual pre-sampled individuals (25 individuals for each density present in the tank under study, i.e., Hd, Md and Ld).

The second round of analyses started after 25 days of treatment with the tank with the diet 3 µg Chl L^{-1} and in the days after, they were conducted on the other tanks with the 6 µg Chl L^{-1} and 9 µg Chl L^{-1} diet.

The third set of tests were performed after 53 days of treatment. The analyses started with the juveniles coming from the offshore shallow site. Therefore, with the support of the divers of the AWI Centre for Scientific Diving, the bags containing 100 g of spat prepared and placed inside the baskets at the beginning of the experiment, were retrieved. The bags were manufactured with a design that allowed easy retrieval by the divers. These, in fact, were built with a tightly woven plastic net whose closures were made with a rope. In one of the corners of the bag,

a loop was made with the rope so that the divers, despite wearing gloves on their hands given the low temperatures, were able to retrieve them easily. After that, the tests concerning the tanks located in the onshore site were conducted. In this set of tests, samples related to the offshore deep site were also analysed on the 1/06/2023. Again, with the support of the AWI Centre for Scientific Diving, the bags containing the 100 g spat located in the baskets were retrieved.

The fourth set of analyses took place after 74 days of treatment, starting with the 3 µg Chl L^{-1} diet tank. After that the different analyses were done on the remaining tanks.

The fifth and last set of analyses were carried out after 96 days of treatment. The first test was performed on the deep site baskets. In this case, all the baskets were retrieved as the pouches were used for conducting the tests related to the third sampling point. Then the analyses were conducted on the tank with diet 3 µg Chl L^{-1} and after it the tests were done on the shallow site too, where all baskets were retrieved with the support of the divers of the AWI Centre for Scientific Diving. Finally, all the analyses concerning the tank with 6 µg Chl L^{-1} diet 9 µg Chl L^{-1} diet were carried out.

At the end of the experiment, it was also possible to determine the degree days (also indicated as DD), a method that allow to assign to every single day a value in term of heat (Miller et al; 2018). From the definition, is clear that DD derive

from a relationship between the detected temperature and the growth rate (Bonhomme, 2000). The degree days, defined also as temperature sum because in this case it corresponds to the total sum of degrees per day over 7 ◦C (Chapman et al; 2021), were calculated using the following formula (Wilson and Simons; 1986):

$$\sum_{n=1}^{n} (T° - T°0)$$

Where n represents the totality of days, while n=1 represents the initial day. T° represents the daily temperature, while T°0 the limiting temperature below which no development occurs (in this case 7°C) (Wilson and Simons, 1986; Maathuis et al., 2023). The result, therefore, is expressed as C°D (Maathuis et al., 2023).

4.8 Statistics

The different statistical analyses were carried out using the Analysis of Variance (ANOVA). To determine significant differences in growth performance, evaluated as a shell volume increase, across the treatments, it was used a two factor ANOVA with replication since the analysis required the evaluation of the interaction between different factors. ANOVA was then applied to handle the multifactorial experimental design, in order to exacerbate the influences of diet, site characteristics and stocking density on oyster growth. To analyse the data

coming from the CR tests it was then used a two factor ANOVA without replication, in this way it was possible to evaluate how diets, densities and sites influenced the CR during the time.

Fifth chapter - Results

5.1 Growth rates

The growth of the individuals was evaluated through the collection of the different biometric parameters taken during the monthly samplings. Therefore, starting from height, width and length, the growth was evaluated in term of shell volume.

In the next graphs, regarding every sampling point, the values are grouped in terms of density where 1.20 $g \cdot cm^{-2}$ corresponds to low density (Ld), 1.60 $g \cdot cm^{-2}$ to medium density (Md) and 2.00 $g \cdot cm^{-2}$ to high density (Hd).

At the first sampling point, there was no significant interaction between diet and density ($p=0.77$), although the p-value for diet was 0.01. The within-group variance was reduced (0.08), so the within-group data were similar to the group mean, likewise the between-group variance was (0.04). Considering Hd, the best results in terms of growth came from diet 9 µg Chl L^{-1}, while in Ld, the best diet was 6 µg Chl L^{-1}. Finally, in Md, the best results were in diet 9 µg Chl L^{-1}. The variance value was the same for all. The obtained results aligned with the expected ones for the initial state of the experiment.

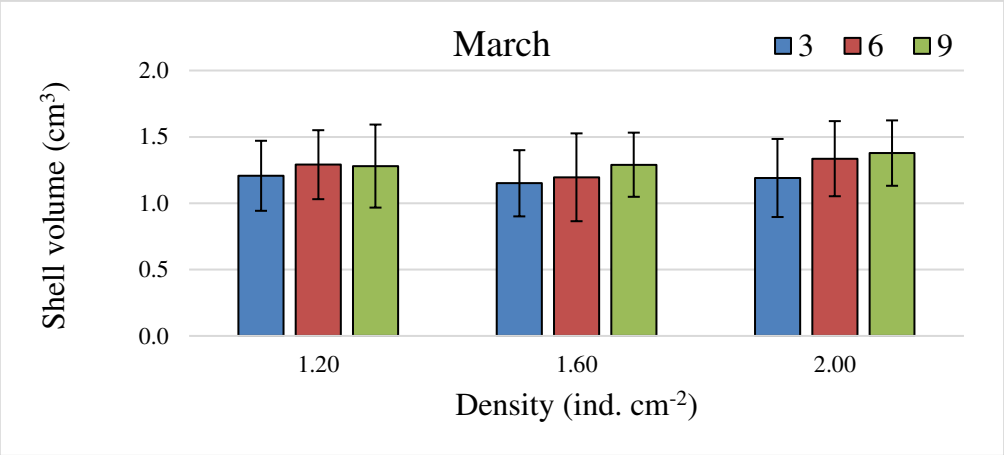

Figure 12. The chart reports the average shell volume values for the first sampling point.

At the second sampling point, there was no significant interaction between diet and density (p=0.53). The averages were very similar and the variance between and within groups was reduced (0.07 and 0.09, respectively). In this case, in Hd, the best results came from diet 6 µg Chl L^{-1}, where the growth was 1.31 cm with a very reduced variance. The highest growth occurred in diet 3 µg Chl L^{-1}, where, however, the variance was high. In Ld, all three diets reported the same effects in terms of growth. Finally, the best results were in Md with the 3 µg Chl L^{-1} and 6 µg Chl L^{-1} diets, where the results were extremely similar (1.35 cm in diet 3 µg Chl L^{-1}, while in diet 6 µg Chl L^{-1} it was 1.32 cm; the variances were 0.05 and 0.06, respectively).

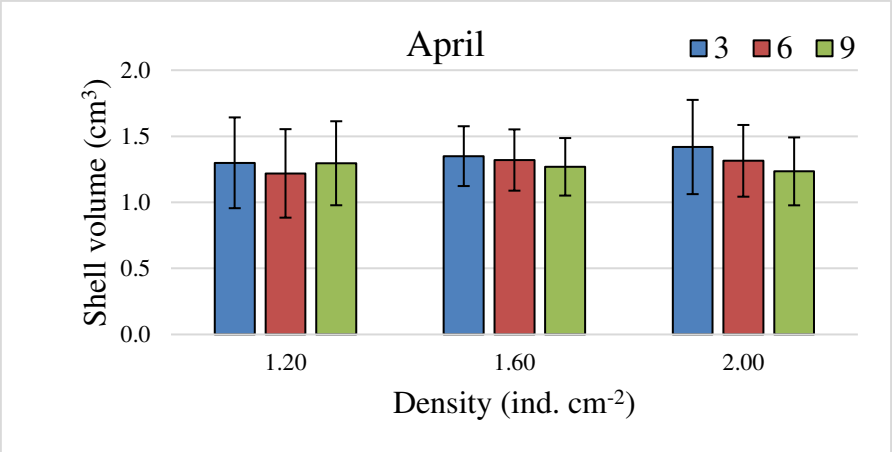

Figure 13. The chart reports the average shell volume values for the second sampling point.

At the third sampling point, there was no significant interaction between diet and density, although diet had a $p<0.05$. The variances between and within groups were reduced and similar to each other (0.11 and 0.18, respectively). Considering the Hd for the deep and shallow sites, the shallow site had better growth results than the deep site (1.18 cm and 1.16 cm, respectively), although the variance for the shallow site was higher than for the deep site (0.15 and 0.05). At the same density, diet 9 µg Chl L^{-1} had the best growth values (1.45 cm) with a variance of 0.18. Diet 6 µg Chl L^{-1}, on the other hand, presented slightly reduced growth (1.41 cm), where, however, the variance was reduced too (0.06). For Ld, the shallow site performed significantly better than the deep one, with a growth of 1.23 cm and a variance of 0.15. The diet with the highest growth was diet 9 µg Chl L^{-1} (1.65 cm), where, however, the variance was high (0.65), while diet 6 µg Chl L^{-1} showed a growth of 1.56 cm with a reduced variance (0.14). For Md, the deep site performed better than the shallow site (1.14 cm) with a variance of 0.07, while the

diet that had the best results was diet 3 µg Chl L^{-1} (1.58 cm) with a variance of 0.13.

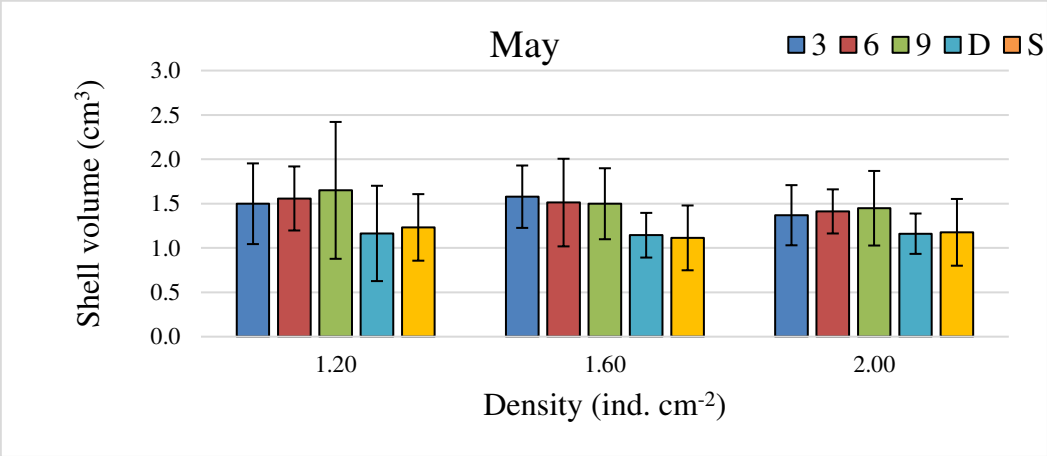

Figure 14. The chart reports the average shell volume values for the third sampling point.

At the fourth sampling point, there was no significant interaction between diet and density, although diet had a $p<0.05$. The between and within group variances were 0.16 and 0.42, respectively. For all three densities (H, L, M), the best results in terms of growth were in diet 9 µg Chl L^{-1} (2.22 cm, 2.29 cm, and 2.36 cm, respectively), where, however, the variances were very high. Compared to the previous results, diet 3 µg Chl L^{-1}, for all three densities, had very reduced variances and growth (1.79 cm, 1.74 cm, and 1.71 cm, respectively).

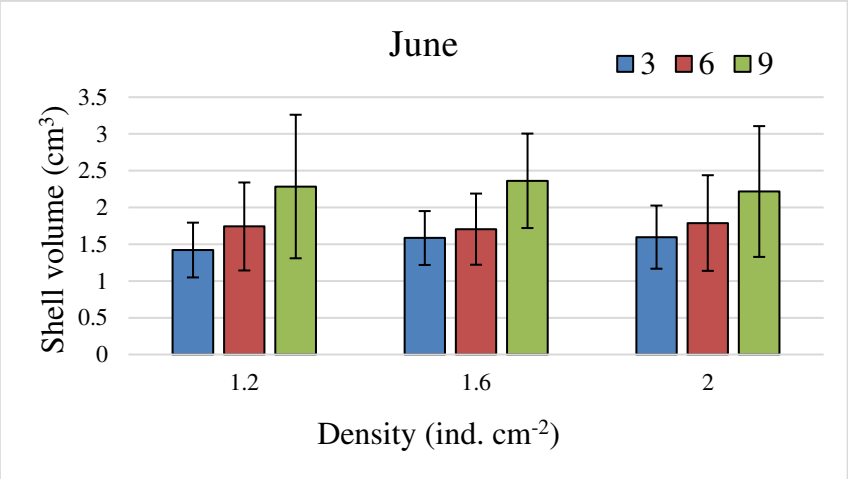

Figure 15. The chart reports the average shell volume values for the fourth sampling point.

Finally, at the fifth sampling point, there were significant interactions between diet and density ($p<0.05$). The variance between groups had a value of 1.06, much higher than the within-group variance, which had a value of 0.44. At Hd, the diet that performed better was 6 µg Chl L^{-1} (growth of 2.63 cm, variance 0.52), while between deep and shallow sites, the deep site performed better (1.35 cm with a variance of 0.08). At Ld, diet 9 µg Chl L^{-1} performed better (2.27 cm) but had an extremely high variance of 1.02, while diet 6 µg Chl L^{-1} performed similarly (2.02 cm) but with a lower variance (0.35). Among deep and shallow sites, the shallow site presented the best growth (1.57 cm) with, however, a variance of 0.20, while the deep site had a growth of 1.30 cm with a variance of 0.07. At density M, the best performing diet was diet 9 µg Chl L^{-1} (2.76 cm with, however, a variance of 0.93), while diet 6 µg Chl L^{-1} had a growth of 2.42 cm with a variance reduced to 0.42. The shallow site had a growth of 1.75 cm with a variance of 0.46, while the deep site showed a growth of 1.24 cm with a variance of 0.07.

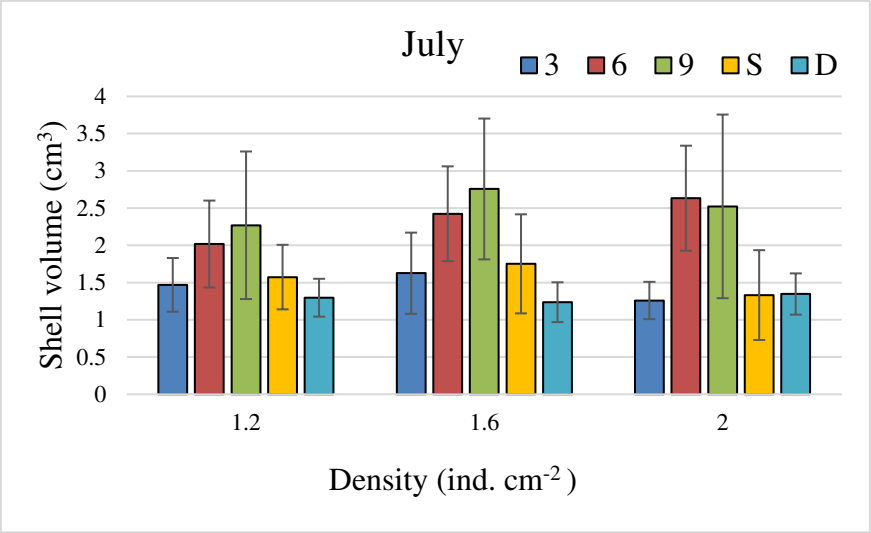

Figure 16. The chart reports the average shell volume values for the fifth sampling point.

5.2 Temperature

For the duration of the experiment, from March to July, temperature was measured daily with the support of the multimeter, but also thanks to temperature loggers associated with the cages. With the data collected, it was possible to see how temperatures affected biometrics and performances of the oyster individuals. In the following graphs, the measured temperatures are shown and the data are organized and grouped according to densities.

Thanks to the temperature loggers, it was also possible to determine the degree days. The temperature loggers, from the beginning of the experiment, collected temperatures every hour on a daily basis. A daily average temperature was therefore calculated and displayed in graphs. Once the daily average temperature was determined, it was possible to calculate the degree days for each site.

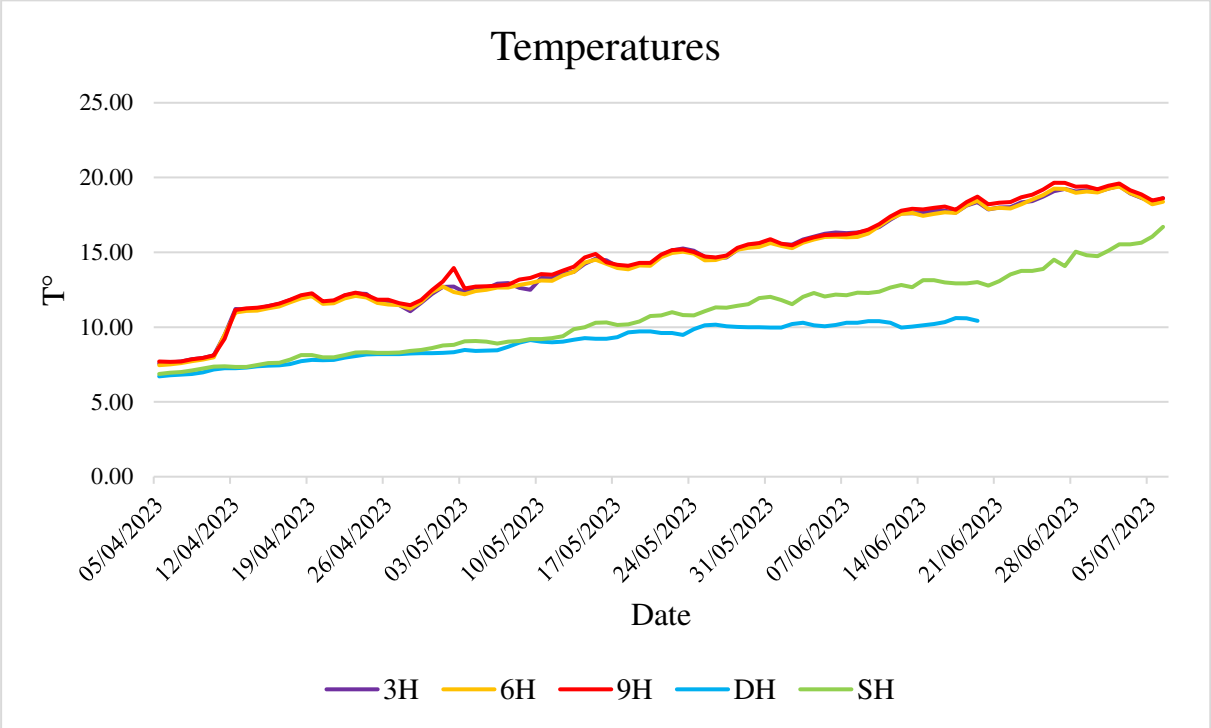

Figure 17. The graph provides a summary of temperatures measured from April to June by temperature loggers located at the onshore site and the offshore deep and shallow sites.

Therefore, considering the different sites and the different beginnings of data transmission by temperatures loggers, for the onshore site the beginning can be considered as 5/04/2023 while the end is on 12/07/2023. For the offshore deep site, the beginning can be considered as 30/03/2023 while the end is to be located

on 20/06/2023. Finally, for the offshore shallow site the beginning can be considered to be on 8/03/2023 while the end is on 6/07/2023.

Therefore, given the measured days and temperatures, the total degree days for the onshore site are 781.1, while for the offshore shallow site they are 348 while, for the offshore deep site, they are 155.7.

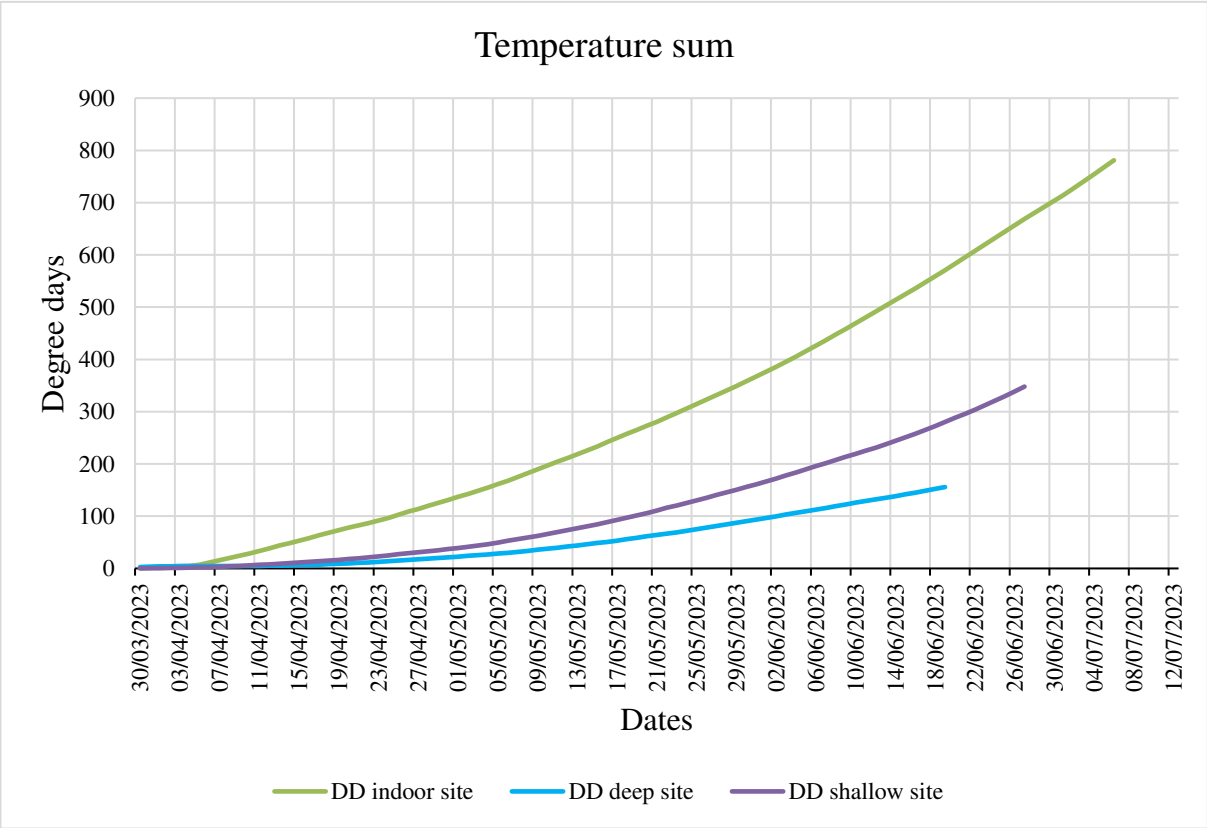

Figure 18. Chart showing the total degree days evaluated for all sites (onshore, offshore shallow and offshore deep site)

5.3 Feed availability

Considering what was stated in chapter 4.4.2 regarding the administration of the diet, it was necessary to evaluate if, during the experimentation, the real quantity of algae fed daily to the tanks corresponded to the theoretical one. In order to verify it, a water sample was taken weekly from each tank, and it was then analysed through the use of the Coulter counter. Regarding the offshore sites, it was possible to obtain information regarding their feeding thanks to the collected data, concerning Chl-a expressed in μg/L, by the AWI-COSYNA Helgoland Underwater Observatory.

Therefore, using the dates on which water samples were taken from the tanks at the onshore site, Chl-a data regarding the offshore site were selected for each day during the hours of midnight, 6 a.m., midday, and 6 p.m. After that, an average of these values was calculated in order to establish a single Chl-a value for the day. From the data collected, a graph was then derived comparing the values of the three diets administered in the onshore site with the Chl-a present in the offshore deep and shallow sites.

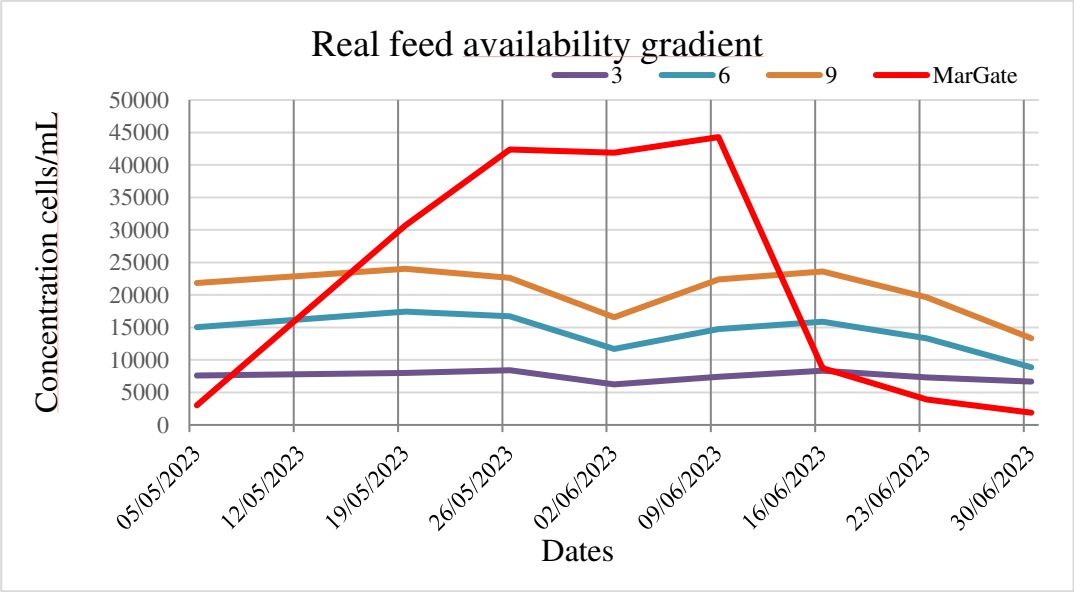

Figure 19. The chart shows the comparison between the feeding in offshore and onshore sites.

Thanks to the data analysis with ANOVA, it is possible to make some observations regarding the administered diets and their effect on the reared juveniles. Starting with the nursery site and the applied diets, the diet consisting of 3µg Chl L^{-1}, had the lowest average growth rate compared to the other diets, associated with a low variance, indicating that all individuals are similarly affected by the diet. The second diet, the 6µg Chl L^{-1}, shows a significant increase in average growth rate with a general higher variance compared to the previous diet. The last diet, the 9µg Chl L^{-1}, had the highest average growth rate, performing from time to time better than the 6 µg Chl L^{-1} diet. However, the variance is the highest among the three diets, probably due to overfeeding.

Concerning the offshore sites, the microalgae concentration expressed in cells/ml started to increase from May, reaching the maximum around the 16/06/2023. This

situation is common in natural marine environment, where nutrient and phytoplankton levels vary depending on seasonal conditions such as water temperature, light availability and nutrient cycling. After the peak, it is possible to observe a decrease in the microalgae concentration, suggesting that environmental conditions changed, probably due to physical and chemical variables such as water stratification or turbulence. Oyster juveniles, then, could benefit from a natural peak in feed abundance, but natural food availability could be unpredictable and limited at other times of the season, with possible effects on health and growth rate.

5.4 Fitness

As previously reported, the clearance rate test can be described as a test that, considering a specific time interval, is able to provide information and results regarding the removal of particles present in a specific volume of water (Wilson, 1983).

Starting from the first sampling point (March), the 3 µg Chl L^{-1} diet recorded the highest average filtration rate with a value of 0.169 L/h, followed by the 6 µg Chl L^{-1} diet and the 9 µg Chl L^{-1} diet, that had the lowest average clearance rate of 0.099 L/h. In terms of stocking density, the Ld group showed the highest average filtration rate, with a value of 0.1386 L/h, while the Md group recorded the lowest average filtration rate of 0.1036 L/h. The results revealed that neither diet type

(p=0.132) nor stocking density (p=0.486) had a statistically significant effect on filtration rate in March.

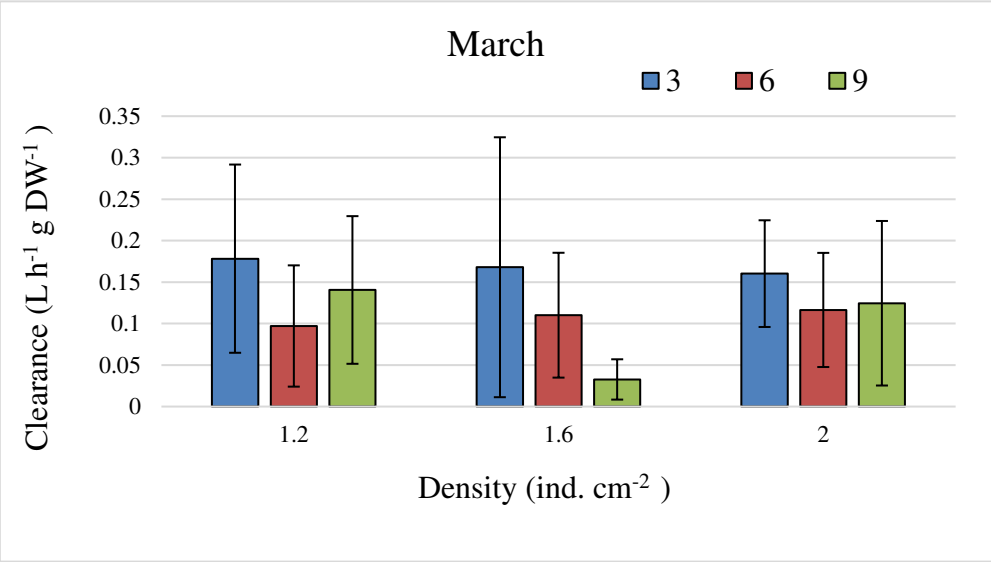

Figure 20. The chart reports the average CR values for the first sampling point.

At the second sampling point (April), the 9 µg Chl L^{-1} diet showed the highest mean clearance rate of 0.495 L/h, followed by the 6 µg Chl L^{-1} diet and finally the 3 µg Chl L^{-1} diet. In this case p=0.039 suggesting then a significant impact of diets in clearance rate. Concerning densities, the Hd group showed the highest mean clearance rate of 0.400 L/h, while the Md group showed the lowest mean value of 0.296 L/h. In this case the p-value corresponded to 0.429 indicating that the

stocking density did not have a statistically significant effect on the clearance rate in April.

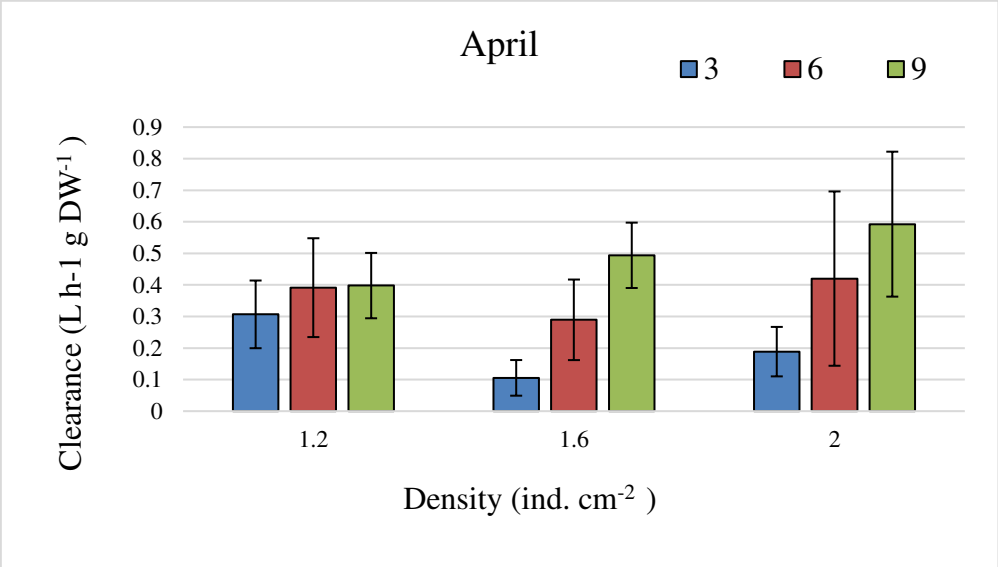

Figure 21. The chart reports the average CR values for the second sampling point.

At the third sampling point (May), starting from the diet in the onshore site, the 6 µg Chl L^{-1} diet presented the highest average clearance rate of 1.621 L/h, followed by the 9 µg Chl L^{-1} diet and the 3 µg Chl L^{-1} diet. The ANOVA showed that the effect of the diets was statistically significant, with a p-value of 0.013. Considering density, the Md presented the highest clearance rate (1.511 L/h). The ANOVA revealed a p-value of 0.198, indicating no statistical significance concerning stocking densities.

Regarding the offshore sites, the shallow one had the highest average clearance rate (0.522 L/h) compared to the deep one (0.480 L/h). However, the ANOVA showed that the effect of the sites was not statistically significant, with a p-value of 0.446. Concerning the density effect at the offshore sites, the Md showed the highest clearance rate (0.541 L/h). The ANOVA revealed a $p>0.05$ determining the impossibility to confirm a statistically significant impact of the different densities to the CR values of the month.

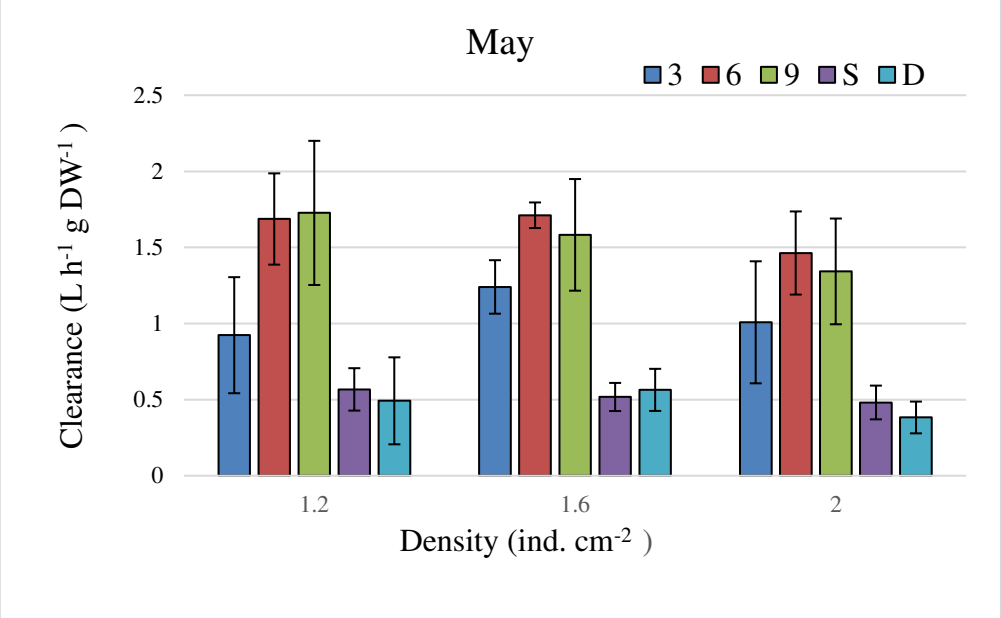

Figure 22. The chart reports the average CR values for the third sampling point.

At the fourth sampling point (June), the 9 µg Chl L^{-1} diet had the highest average clearance rate, with a value of 3.042 L/h, followed by the 6 µg Chl L^{-1} diet and finally the 3 µg Chl L^{-1} diet. For diets, $p<0.05$ indicating that there are significant

differences between the filtration rates resulting from the different diets administered.

Regarding densities, the Ld showed a slight tendency towards higher filtration rates (1.977 L/h) followed immediately by the Md (1.944 L/h). In this case p=0.882, suggesting that there are no statistically significant differences in filtration rates between the different stocking densities. Although there is a slight difference in the mean values observed, no substantial variation was found to indicate a significant impact of density on clearance rate.

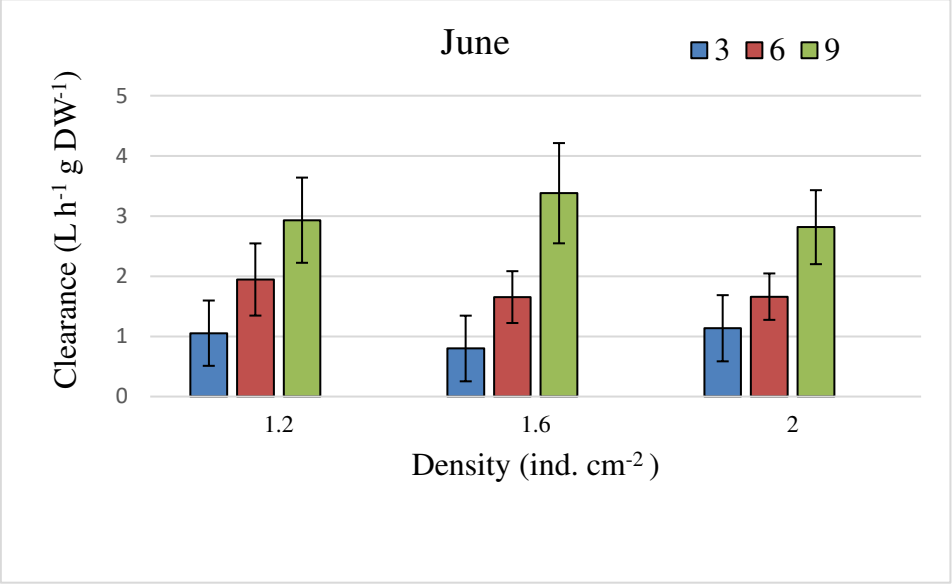

Figure 23. The chart reports the average CR values for the fourth sampling point.

For the fifth sampling point (July), between the three tested diets at the onshore site, the diet 3 µg Chl L^{-1} had the highest clearance rate (2.35 L/h) while diet 6 µg Chl L^{-1} had the lowest. The p-value corresponded to 0.859 indicating that there

were no statistically significant differences between the clearance rates for the different diets. For the different stocking densities, the highest clearance rate was observed in the Hd (2.85 L/h), while the lowest was recorded at the Md (1.69 L/h). Considering p=0.167, the stocking density did not significantly influence the clearance rates. Concerning the offshore sites, the oysters at the shallow site had an average clearance rate of 3.176 L/h, while those at the deep site had a significantly lower average clearance rate of 0.829 L/h. Even if the p-value was 0.063, slightly above 0.05, it is evident that depth have an effect on clearance rate even if it does not quite reach statistical significance. Concerning the stocking density effect at the offshore site, the Ld (2.46 L/h) and Md (2.15 L/h) performed better than Hd (1.39 L/h). In this case p>0.05, indicating that the differences in clearance rates across the different stocking densities were not statistically significant.

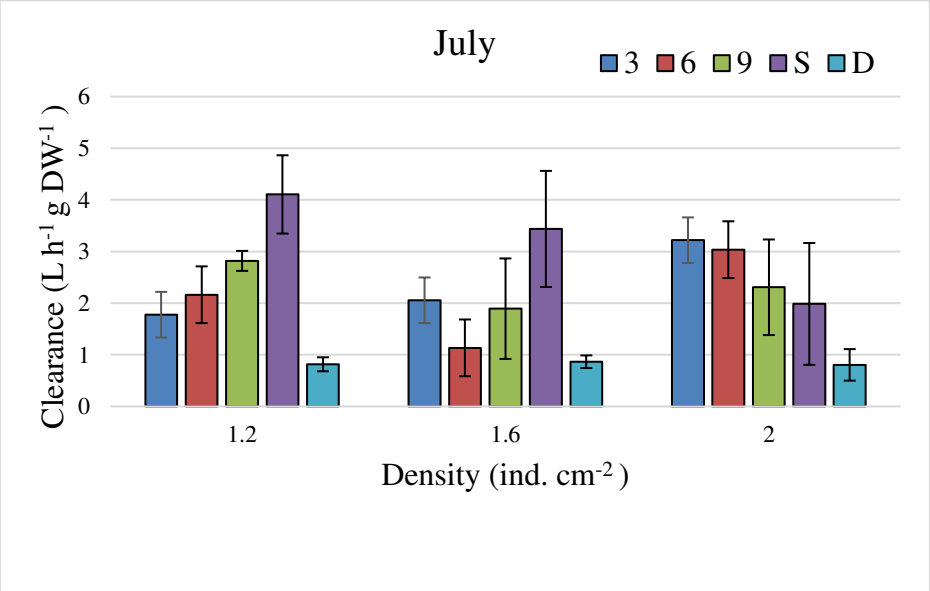

Figure 24. The chart reports the average CR values for the fifth sampling point.

Sixth chapter - Discussion

6.1 Site suitability

The trial showed a marked difference in the performance of oyster individuals at the different sites. When we consider the results coming from the onshore site as a comparison point, between the results coming from shallow and deep offshore sites, the site that showed the most promising results was the shallow offshore site. The evaluation of the reasons behind the better performance of this site compared to the deep one considers various environmental parameters, including water quality, temperature, salinity, food availability, and potential threats from predators and/or human activities.

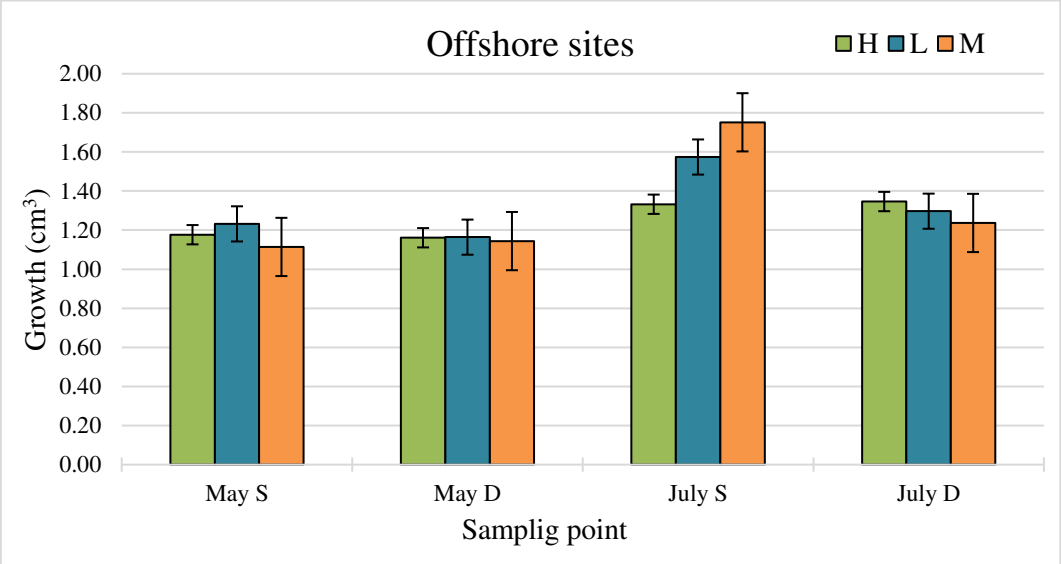

Figure 25. The chart shows a comparison of the growth at the shallow (S) and deep (D) site at the third sampling point (May) and fifth sampling point (July).

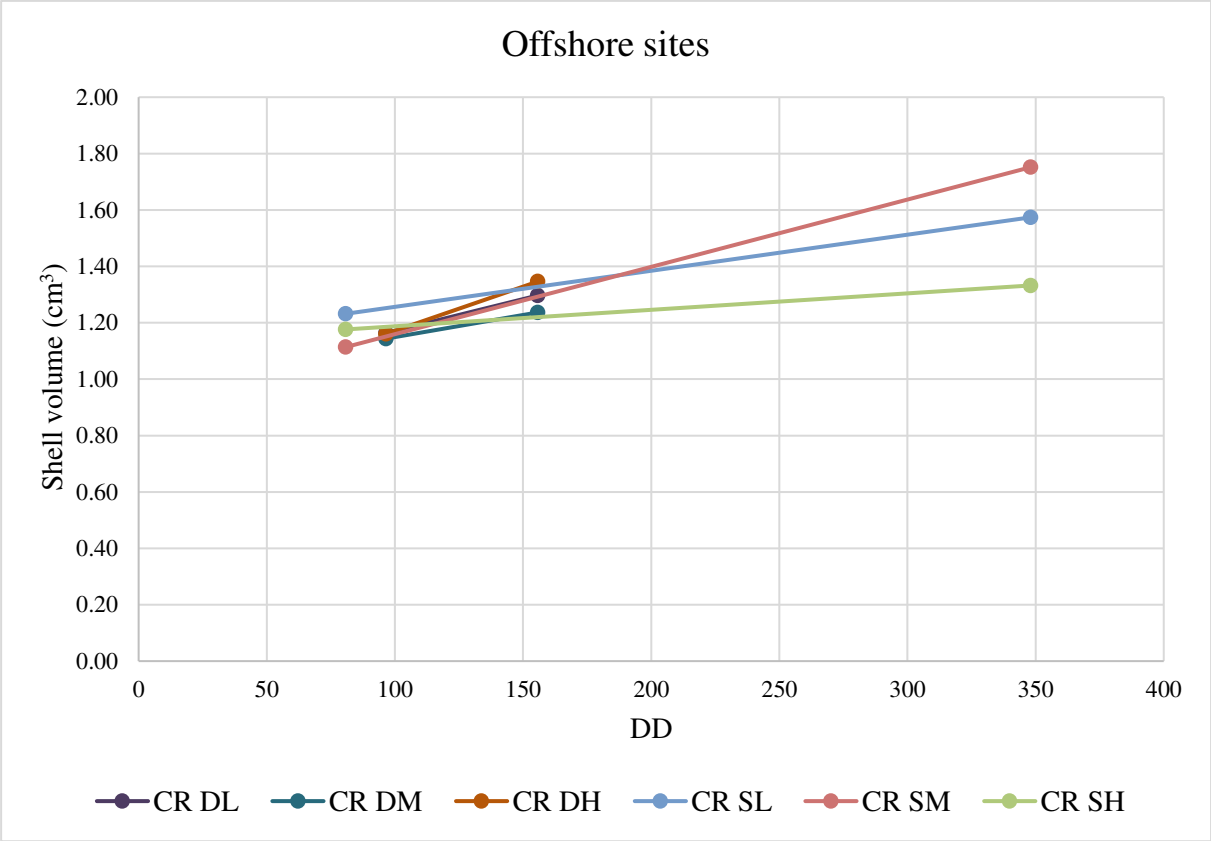

Figure 26. The chart presents a summary of the shell volume data of the offshore sites, concerning all the months of the experiment, related to the degree days.

Water quality is extremely important for the survival and growth of juvenile oysters, where low levels of contaminants (as for example heavy metals and organic pollutants) is crucial for preventing bioaccumulation and ensuring the health of the oysters. In general, at shallow sites, waters may experience higher levels of turbidity due to sediment resuspension from wave action and human activities. However, if the water remains relatively clear, the high levels of light and nutrient input can support robust phytoplankton communities (Xu et al., 2020). Even if at deep sites water is usually less affected by turbidity from

sediment resuspension, a huge problem is represented by reduced light since it affects the primary production. To ensure adequate feed supply and prevent hypoxic conditions at these depths, water quality must be carefully monitored (Xu et al., 2020; Stechele et al., 2023).

Temperature significantly influences metabolism and growth of young oyster individuals. The waters around Helgoland experience seasonal temperature variations, ranging from approximately 4°C in winter to 20°C in summer. These temperatures fall within the tolerable range for *O. edulis* (Bartsch et al., 2013; Kamermans and Saurel, 2022). The warmer summer temperatures support rapid growth and development, while the cooler winter temperatures are within the oysters' tolerance range but may slow growth rates. The shallow site (8-11 meters) is subject to greater temperature fluctuations compared to the deep one. During the summer months, the temperature can rise significantly, promoting rapid growth of juvenile oysters. However, this site may also experience more extreme temperatures in the winter, which can stress the oysters if temperatures drop too low. The deep site (28-30 meters) offers more stable temperature conditions, typically cooler and less variable than shallow waters, slowing in this way metabolic rates.

Salinity levels are another critical factor for oyster nurseries. *O. edulis* is euryhaline, tolerating a wide range of salinities but preferring stable conditions

between 25 and 35 psu (Pogoda et al., 2023). The North Sea around Helgoland maintains relatively stable salinity levels, averaging around 30 psu. These conditions are ideal for the growth and survival of juvenile oysters, providing a stable osmotic environment necessary for physiological processes (Stechele et al., 2023).

Food availability, primarily in the form of phytoplankton, is crucial for the growth of juvenile oysters. The waters around Helgoland support a productive phytoplankton community, driven by nutrient inputs and seasonal dynamics. The availability of phytoplankton peaks during the spring and summer months, aligning with the optimal growth periods for oysters (Käse et al., 2020). At the shallow site, light penetration is generally high, enhancing primary production and phytoplankton availability, which are crucial food sources for oysters. This increased light can lead to higher rates of photosynthesis, benefiting the overall food web and supporting oyster growth (Stechele et al., 2023). On the other hand, at the deep site, light penetration is significantly reduced at this depth, leading to lower primary production and phytoplankton availability (Käse et al., 2020).

Considering these factors, the shallow offers better conditions for oyster growth compared to the deep site, however, the moderate variance in the analysed results indicates that not all oysters benefit equally, possibly due to localized differences in microhabitat conditions or competition. On the other hand, the deep site shows

a more consistent but poor growth performance probably due to the less favourable environmental conditions The low variance suggests that these conditions uniformly affect all oysters, leading to uniformly poor growth. By using the degree days calculated on the basis of the temperatures measured during the experiment, and the oysters' weight data gained during the experiment, it was possible to estimate the weight of the individuals after one year at the shallow site at density Md. Considering as growth months all the months with a temperature above 7°C, by using averages temperatures of the waters surrounding Helgoland, and also considering a final weight in July of 1.46 ± 0.47 gr, it is possible to estimate a weight of 2.40 ± 0.45 gr of the individuals, in July, in the following year.

The comparison between shallow and deep sites highlights the significant impact of environmental conditions on oyster growth. These findings emphasize the importance of site selection in restoration projects, as the environmental conditions at a site can greatly influence the success of oyster populations.

From the obtained results, Helgoland represent a suitable location for a nursery of juvenile *O. edulis*, particularly the offshore shallow site. The site offers favourable environmental conditions, including good water quality, temperature, salinity, and food availability. However, potential threats from predators and human activities must be managed effectively to ensure the success of the nursery. Strategic site

selection and management practices will be crucial in supporting the restoration of *O. edulis* populations in the North Sea.

6.2 Density effects

The selection of the correct stocking density has the potential to mitigate mortalities and enhance growth. At the same time, wrong stocking densities (e.g. too high or too low) goes to promote several bad outcomes (Cowan et al., 2024).

Starting from high density, the data suggest that high densities may exacerbate competition, leading to higher variance in growth rates due to uneven resource distribution. High densities determine reduced growth rates because it leads to increased competition for feed, since the present individuals must share the available nutrients. This competition can result in inadequate nutrition, slowing their growth and development (Kamermans et al., 2004; Domínguez-Machín et al., 2024). A study by Cranford et al. (2009) demonstrated that oysters stocked at higher densities exhibited reduced growth rates compared to those at lower densities. The limited availability of feed resources in densely populated areas forces oysters to use more energy towards survival rather than growth. High stocking densities can also lead to increased mortality rates in the oyster population. The crowded conditions can create stressful environments that weaken the oysters' immune systems, making them more susceptible to diseases and parasites (Buestel et al., 2009). Additionally, high densities can exacerbate

the spread of pathogens, as diseases can quickly transmit from one oyster to another in close proximity (Bishop et al., 2023). Research by Culloty et al. (2001) found that high stocking densities were associated with higher incidences of diseases such as *B. ostreae*. The increased stress and disease prevalence in densely populated nurseries highlight the importance of maintaining appropriate stocking densities to ensure the health and survival of the oysters. Another issue associated with high stocking densities is the deterioration of water quality within the nursery environment. High densities of oysters produce large amounts of waste, which can accumulate and degrade water quality (Cranford et al., 2007). The buildup of organic matter can lead to eutrophication, characterized by low oxygen levels and the proliferation of harmful algal blooms. It has been observed that using high density in oyster farming can result in localized hypoxia, negatively affecting both the oysters and other marine organisms in the vicinity (Newell, 2004; Burkholder and Shumway, 2011). Moreover, the high-density farming of oysters can lead to physical alterations of the seafloor, such as sediment compaction and erosion, which can further affect benthic communities (Mazouni et al., 2001).

It is then clear that even if high stocking densities can maximize yields, they come with a significant number of negative effects. Reduced growth rates, increased mortality, poor water quality, behavioural changes, and negative environmental impacts highlight the challenges associated with high-density oyster farming (Zorita et al., 2021).

Concerning low densities, the data show varied growth outcomes, possibly due to underutilization of food and space, leading to lower averages and higher variances. At low densities, oysters may not effectively filter the available plankton, resulting in wasted food resources. Additionally, low densities might reduce the benefits of positive interactions among oysters, such as enhanced water flow and nutrient availability due to collective filtration activities.

Low stocking densities are often promoted as a way to increase growth and health of oyster individuals. At the same time, there are several negative effects that this stocking density can determine. Juvenile oysters grown at lower densities may be exposed to a high predation risk (Guy et al., 2019). Predators are more likely to target isolated individuals at low densities, leading to higher mortality rates. This vulnerability can be easily observed in sites with big predator populations, such as crabs and starfish, that usually feed on juvenile oysters (Tedford and Castorani, 2022). Another problematic of the application of low stocking densities is the inefficiency coming from the bad use of the available space at the selected site. Low stocking density then may leave significant part of the farming area not used which is not an efficient use of resources. This inefficiency can be particularly problematic in regions where aquaculture space is limited or where competition for suitable farming sites is high. Optimizing space utilization is essential for sustainable aquaculture practices and can be challenging to achieve with low-density cultivation strategies (Miossec et al., 2009).

Even if low stocking density offers certain advantages, it is not without its drawbacks. The increased predation risk, limited habitat complexity and inefficient space utilization highlight the challenges associated with this practice. Balancing the benefits and drawbacks requires a gradual approach that considers both economic and ecological factors.

Medium densities, then, typically strike a balance between resource availability and competition. The data indicate that medium densities support more stable growth rates, with lower variance compared to high densities. Considering what was stated previously, medium stocking densities have been shown to provide a balance between maximizing yield and maintaining optimal conditions for oyster health and growth. At medium densities, oysters experience less competition for food compared to high density conditions, ensuring adequate access to nutrients. This balance allows oysters to allocate more energy towards growth rather than merely survival. Research by Laing et al. (2006) demonstrated that oysters reared at medium densities exhibited significantly higher growth rates compared to those at high densities. The study found that medium densities allowed for efficient filtration of phytoplankton, ensuring a steady supply of feed for the oysters. Malham et al. (2009) found that oysters reared at medium densities had lower mortality rates compared to those at high densities. The reduced stress levels in medium density nurseries help maintain a healthier immune system in oysters, making them more resilient to environmental fluctuations and pathogen exposure.

At the same time, a medium stocking density helps in preserving optimal water quality within the nursery environment. In fact, this density allows a better waste dispersion and assimilation, maintaining a healthier environment for the oysters. Cranford et al. (2007) highlighted that medium stocking densities are less likely to cause eutrophication and harmful algal blooms. This is because the moderate biomass of oysters can efficiently filter and process the available nutrients without overwhelming the system. As a result, the water remains clearer and more oxygenated, which is beneficial for both the oysters and the surrounding marine ecosystem.

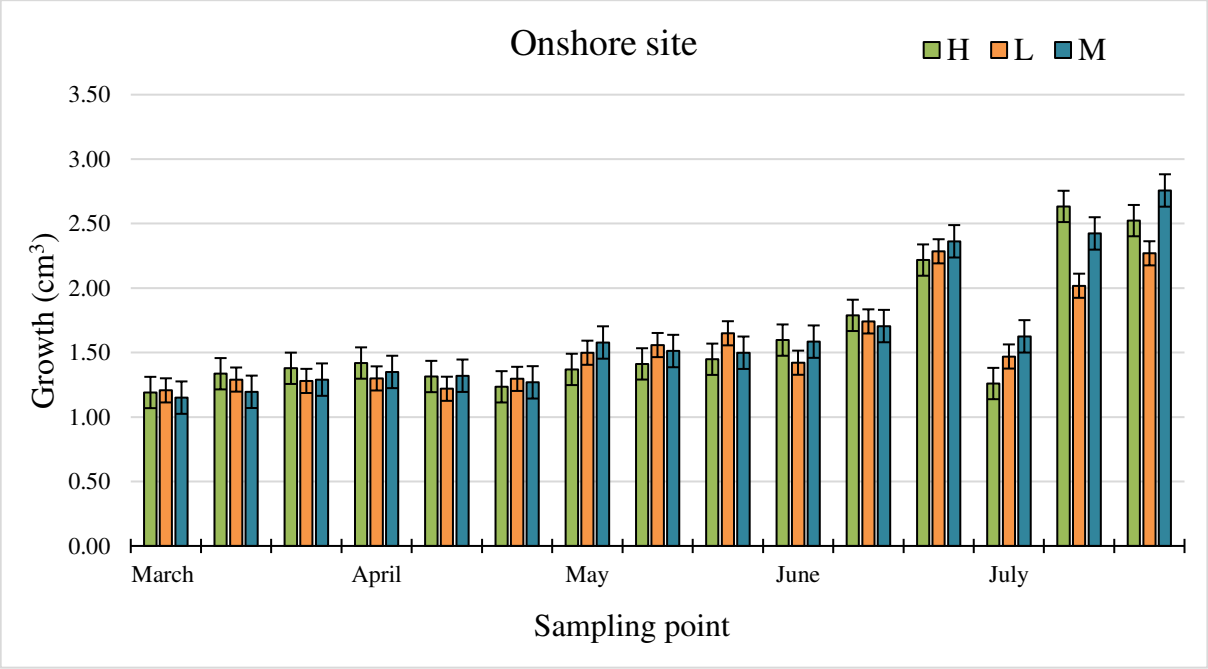

Figure 27. The chart shows a comparison of the growth at the onshore site during the months. The data, per sampling point, are divided per diet (3, 6, 9 µg Chl L^{-1}) and density. In association with Figure 25 concerning the offshore sites, it confirms that M density has the best results in terms of growth.

In general, the effects of stocking density on clearance rates seems to be not really consistent. The results show that within the three different densities tested, oysters' clearance rates were not strongly impacted by rearing density. In the different sampling points, there is a high variability concerning density effects, and because of that, it is necessary to further assess how stocking density interacts with clearance rate with replicating these tests over longer period of time.

Another important aspect is the impact that this stocking density can play towards the surrounding environment. As proved by the study conducted by Newell (2004) the application medium densities can enhance the ecosystem services provided by oysters, such as water filtration and nutrient cycling. These services contribute to the health and productivity of the surrounding marine environment, making oyster farming an environmentally beneficial activity. From an economic point of view, medium stocking densities offer a balance between maximizing yield and minimizing costs associated with disease management and water quality maintenance. The study carried out by Laing et al. (2006) noted that medium densities are economically advantageous as they reduce the need for intensive monitoring and intervention compared to high-density systems. This reduction in management costs, combined with the higher survival and growth rates, makes medium density nurseries a cost-effective choice.

Considering the different stoking densities options, it is clear that medium stocking densities can offer a balance between the different drawbacks and benefits. This stability is essential for the overall health and well-being of the oysters.

6.3 Optimisation of field nursery

Optimizing an offshore field nursery requires a multifactorial approach that addresses site selection, nursery design, stocking densities, feeding regimes, environmental monitoring and disease management. By considering these factors, it is possible to enhance the survival, growth and health of oyster juveniles, leading to more sustainable and productive oyster farming operations. Continued research and the application of advanced technologies will further improve the efficiency and success of offshore nurseries for this valuable species. Future research should continue to explore the interactions between environmental factors, nutrient availability, and stocking densities to further optimize oyster restoration and aquaculture practices. Monitoring and adapting these factors will be fundamental to the successful restoration and sustainable management of flat oyster populations.

Starting from the site selection, it is important to improve the evaluation of the environmental conditions and biological factors. The location should have optimal water quality, with suitable temperature, salinity, and oxygen levels

necessary for oyster growth and health. The chosen site should be free from pollutants and not prone to harmful algal blooms, which can significantly impact oyster health (May et al., 2010). Also water currents must be considered since they are involved in the movements of phytoplankton and help to remove waste. Areas with stable sediments are also preferable as they minimize the risk of resuspended sediments that can clog oyster gills and inhibit feeding (Laing et al., 2006). Biological factors must be considered too, as for example the presence of natural predators and competitors. Sites should be selected to minimize these risks, potentially using predator-exclusion devices or by choosing locations less frequented by predators (Cranford et al., 2007).

To optimize a nursery offshore, also its design must be carefully evaluated, especially the structure and the used material, as well as the spatial arrangement. Nursery design plays a central role in optimizing the growth and health of juvenile oysters. Various designs have distinct advantages and challenges to face. For example, floating rafts offer mobility and easy access for maintenance and monitoring, while suspended cages provide better protection from benthic predators and allow adjustment for optimal depth based on environmental conditions (Helm et al., 2006). Concerning the spatial arrangement, it is necessary to provide proper spacing and density for oysters. As shown from this study and by many others, the rearing density has an evident impact on the growth and performance of the reared oyster.

Feeding regime plays a key role too. The administered diets at the onshore site during the five months of the experiment provided some important results and information. The first diet, 3 µg Chl L^{-1}, as mentioned earlier, had the lowest average growth rate associated with a low variance, suggesting then that the diet do not provide sufficient nutrients to support optimal growth, leading to lower, but consistent, growth rates among the juvenile oysters. The CR values concerning this diet were the highest in the months of March, where considering the early stage of the experiment the type of diet probably did not substantially alter the feeding performances, and July, where an explanation could be a seasonal shift in the physiological condition of the oysters, or also eventual changes in environmental factors, which could have altered their feeding preferences. The 6 µg Chl L^{-1} diet present a higher increase in term of growth, with a variance that is higher compared to the 3 µg Chl L^{-1} diet, suggesting that this diet provides a more balanced nutrient profile, even if there is variability in how individual oysters respond to it. A possible explanation about this variability could come from micro-environmental conditions within the nursery. For this diet, the highest CR value was detected in May, indicating that intermediate concentrations of chlorophyll may offer an optimal balance between food availability and the oysters' ability to process it efficiently. The last diet, the 9 µg Chl L^{-1}, had the highest average growth rate, performing from time to time better than the 6 µg Chl L^{-1} diet. In this case, the variance is the highest, suggesting a wide range of

responses to this diet, possibly due to overfeeding. Overfeeding can lead to increased metabolic waste, which may negatively affect some oysters more than others. This diet may also contain excess nutrients that some oysters cannot process efficiently, leading to greater variability in growth rates. The CR values for this diet where the highest in the months of April and June. A possible explanation for this trend in April, is that there could be an increased metabolic demand of the growing oysters, requiring a higher concentration of phytoplankton to sustain their development. In June, an explanation of this trend, could come from the fact that juvenile oysters may have reached a stage where their energy demands, and filtration capabilities are maximized when exposed to abundant food resources. From all these data, is possible to state that the diet 6 µg Chl L^{-1} offers the best balance of nutrients for consistent and optimal growth of juvenile oysters. Even if the diet 9 µg Chl L^{-1} support higher growth for some individuals, it may not be ideal due to the higher variability and potential negative effects on some oysters. These results are then able to give indication concerning the correct site selection, in order to choose a site that have the correct food availability for the reared oysters. In fact, in offshore locations, oysters rely on natural phytoplankton for their nutrition, so ensuring a steady supply of phytoplankton is crucial and site selection plays a significant role in this (Newell & Langdon, 1996). In the case of areas with limited natural food supply, supplemental feeding can enhance growth. However, it is crucial to monitor water quality closely when

using supplemental feeds to avoid nutrient overloading and subsequent eutrophication (Cranford et al., 2007).

In order to take correct decision in the hatchery management and to fulfil the necessity of the reared oyster, it is also necessary to closely monitor the environmental conditions. Parameters such as temperature, salinity, oxygen levels and plankton concentrations should be regularly measured to ensure optimal conditions for oyster growth (Gazeau et al., 2007). An accurate monitoring allows a quick detection of eventual problematics, leading to fast decision to correct them.

Also technology improvements are necessary**.** Advanced technologies as remote sensing and automated monitoring systems, that are able to provide real-time data, allow fast correction interventions (Bates et al., 2021). These technologies can optimize nursery management by providing detailed insights into environmental conditions and oyster health. It is anyway important to underline the fact that oyster rearing practices present a limited potential concerning the use of industrial machinery and automation (Barillé et al., 2020)

Disease management is a critical component for optimizing an offshore nursery since in these kinds of sites it is extremely complicated. Juvenile oysters are susceptible to various pathogens, including bacteria, viruses and parasites (Helmer et al., 2019). Implementing biosecurity measures, such as regular health

checks, quarantine procedures for new individuals and maintaining clean equipment, can help to prevent disease outbreaks. To face the disease problematic, probiotic treatments and the use of disease resistant oyster strains are emerging as effective strategies for enhancing disease resistance (Taky et al., 2024).

At last, also the socioeconomic aspect must be considered. Sustainable practices that enhance the productivity and health of oyster populations can lead to increased economic benefits for coastal communities. Furthermore, sustainable oyster farming practices can support ecosystem services, such as water filtration and habitat provision, which have broader environmental and economic implications (Beck et al., 2011).

6.4 Nursery to restoration site deployment

There are different strategies and considerations to take in account to successfully moving oyster juveniles from nurseries to restoration sites. Key aspects include site selection, preparation, transportation methods, acclimatization processes and long-term monitoring. As previously discussed, the characteristics of the restoration site are fundamental for the success of a restoration plan. It is anyway important to highlight the fact that restoration efforts should prioritize historically significant sites for *O. edulis*. Reintroducing oysters to their former habitats enhances the likelihood of successful restoration due to the suitability of these sites (Gercken and Schmidt, 2014).

Concerning transportation methods, oyster juveniles must be handled and transported without causing stress and mortality during the transfer from nurseries to restoration sites. According to guidelines by the Native Oyster Network (2018), oysters should be packed in aerated seawater and kept cool during transportation. In general, humidity must be maintained high to avoid desiccation. Also transportation logistics should be carefully planned to ensure a smooth transfer process, where short transport times are preferable to minimize stress. Acclimatation to the sea condition is necessary to improve the performances. Once at the restoration site, a gradual acclimatization it is helpful for the juvenile oysters to adjust to the new environmental conditions. This involves slowly acclimatizing the oysters to the site's water temperature, salinity and other conditions. During acclimatization, it is important to monitor oyster health and behaviour. Indicators such as shell gaping, respiration rates and feeding activity can provide insights into the oysters' acclimatization progress (Thompson et al., 2012; Scanes et al., 2023). After the acclimatation, it is possible to proceed with the deployment. In this experiment, the deployment was done by the divers of the Centre for Scientific Diving (AWI-CSD) that placed the oyster baskets both at the shallow and deep site. During the experimentation the basket were monitored at the third and fifth sapling points. Thanks to the temperature loggers it was possible to monitor the temperature during the months and with AWI-COSYNA

Helgoland Underwater Observatory it was possible to have information concerning food availability.

Adaptive management practices are extremely important too. These practices involve the use of the data collected from monitoring actions to make informed decisions and adjustments to the restoration strategy. This includes altering deployment techniques, adjusting site conditions, or implementing additional habitat enhancements. The success of oyster restoration relies on a dynamic approach that responds to changing environmental conditions and oyster health status.

At last, to reduce the disturbance level at the restoration site, it would be optimal to establishing protected areas and working with local stakeholders. Public awareness campaigns can also help gaining support for oyster restoration projects and reduce human-induced threats.

Seventh chapter - Conclusions and Outlook

7.1 Summary of main findings

The primary focus of the study was to evaluate the effects of stocking densities and environmental factors at different sites (shallow and deep) on the growth performance of juvenile individuals of *O. edulis*, where the growth performance was evaluated in terms of shell volume increase. ANOVA was used to identify significant differences in growth across different treatments, including diet, stocking density, and site characteristics.

The analysis of diets at the onshore site revealed significant differences in growth performance across the three different diets. The best diet was the 6 µg Chl L^{-1}, where the increased growth indicates a more balanced nutrient profile, supporting better overall growth, although the higher variance suggests variability in individual responses. Although diet 9 µg Chl L^{-1} supports high growth in some individuals, its high variability and potential negative effects suggest it may not be ideal for all oysters.

The study compared growth performance at different sites, specifically focusing on the shallow and deep sites, alongside the inside nursery. The shallow offshore site had the best growth, and the possible explanations are linked to higher temperatures and better light penetration, which enhance phytoplankton

production and food availability. However, the moderate variance indicates that not all oysters benefited equally of it, possibly due to localized differences in microhabitat conditions or competition. The comparison between shallow and deep sites highlights the significant impact of environmental conditions on oyster growth.

At last, the study also examined the impact of different stocking densities on growth performance, where the medium stocking density results to be the best one. Medium densities typically strike a balance between resource availability and competition. The data proved that this density support more stable growth rates optimizing the utilization of available resources while minimizing competition.

This study provides valuable insights into the optimal conditions for the growth and survival of flat oyster juvenile, where the environmental factors play a key role. During the site selection phase, areas with favourable temperature and light conditions should be prioritized to maximize growth and survival rates. Overall, the study highlights the importance of carefully managing environmental conditions and stocking densities to optimize the growth and survival of oysters in restoration projects.

7.2 Potential for oyster restoration using the Helgoland field nursery

Oyster restoration is gaining attention as a crucial environmental initiative aimed at reversing the decline of the species and restoring the ecological balance of marine environments (Beck et al., 2011). Considering the importance of the *O. edulis* in the North Sea, particularly in Helgoland, a field nursery represents a promising opportunity for the restoration of these vital ecosystems.

Considering the experience gained from the onshore nursery, there is significant potential for establishing an offshore nursery that would provide a larger and a more natural habitat for oyster growth, reducing the logistical constraints of land-based operations and enhancing restoration efforts.

An offshore nursery offers numerous advantages, including the ability to support larger oyster populations, reduced impacts from land-based pollution and enhanced integration with natural marine ecosystems. Additionally, the offshore location can provide greater protection from human interference, allowing for more natural oyster behaviours and interactions, which are critical for their long-term survival and ecological function (Beck et al., 2011). Also, offshore nurseries can be scaled up to accommodate large restoration projects. The vast open waters provide space for the expansion of nursery operations, enabling the cultivation of more individuals. By creating suitable habitats for oysters, offshore nurseries also support other marine organisms, promoting the overall biodiversity. The presence

of oyster reefs can attract a variety of species, enhancing the ecological richness of the area (Thomas et al., 2022).

The key to the success of the offshore nursery will rely on accurate monitoring plan. Advanced monitoring techniques, such as underwater drones and remote sensing, could be employed to gather data efficiently and accurately. This information is crucial for adapting and refining restoration strategies to ensure their effectiveness and sustainability (Helm et al., 2006).

A technology that could be implemented, especially in an offshore nursery, could be the Biorock®. This technology greatly improves oyster growth by using low voltage currents that flow through an anode (made of titanium) and a cathode (a large structure made of steel). The Biorock® technology is then based on the electrolysis that happens between these two metals components, determining the formation of limestone (where several organisms will settle and grow) on the cathode while the anode progressively get consumed. The used electrical currents are safe for both humans and marine organisms, and there are no limits in the size and shape of the structure itself (Bakti et al., 2010)

Based on the amperage, it is possible to have different type of material deposition. Usually, is possible to observe the presence of calcite (primarily used by molluscs) and aragonite (used mainly by corals), but in case of an extremely high amperage

it will be possible to observe brucite, a material not able to create stable structures (Allen and Snyder, 2016).

The explanation behind the success of this technology application towards oysters is that individuals use large amount of their metabolic energy to grow shell and, to do so, the organism must create specific chemical conditions inside their cells to speed up the formation of calcium carbonate crystals from the surrounding waters. This requires the active, and energetically dispendious, pumping of protons and calcium ions across membranes and cells to create the high pH conditions that are essential. Biorock technology solves this limitation thanks to the electrolysis process, resulting in precipitation of calcium carbonate and magnesium hydroxide, raising the pH within the precipitate and directly at the surface of the growing calcium carbonate crystals, not in the water. Because they are getting the high pH they need for free, more metabolic energy is available for growth, reproduction, and for resisting environmental stresses (Goreau and Hilbertz, 2005).

Even if most of the experiments with the Biorock were conducted in the tropics, this technology was also applied to restore oyster reefs in cold areas with promising results. To apply the technology in cold areas, the used voltage has to be higher than in tropics because of the great difference between the two areas in term of salinity and temperature. It is anyway important to consider that the

reaction is 100% efficient at 1.23 volts, which is the minimum for effective deposition. As proved by several study, the application of this this technology will determine a fast growth (in term of length, width, and volume), great wellness and survivance in oyster too (Goreau & Hilbertz, 2005; Berger et al., 2012; Allen & Snyder, 2016).

7.3 Recommendations for future studies

Future studies should focus on understanding the genetic diversity and population structure of European oysters in the North Sea. Genetic diversity is crucial for the resilience of restored populations to environmental changes and diseases. Researchers should employ genomic tools to assess the genetic health of both wild and hatchery-reared populations. Understanding the genetic variability within and between populations can guide selective breeding programs and ensure the long-term sustainability of restoration efforts (Lallias et al, 2010).

To do that, a possibility is to use the next-generation sequencing (NGS) techniques to monitor genetic diversity and ensure the maintenance of robust gene pools in restored populations. Genetic studies can reveal historical population bottlenecks, levels of inbreeding and connectivity between populations, which are essential for designing effective restoration strategies (Gutierrez et al., 2017). Studies such as those by Bierne et al. (2016) highlight the importance of genetic monitoring in bivalve restoration.

Research should also focus on assessing environmental parameters to determine the best sites for restoration. Habitat suitability models can integrate these parameters to predict areas where oysters are likely to thrive. Additionally, long-term monitoring of environmental conditions is essential to adaptively manage restoration sites and ensure their suitability over time (Hirzel et al., 2001). In order to do that, GIS tools and remote sensing technologies to map and monitor these areas are a suitable solution. Such tools can provide spatially explicit data on environmental conditions, which is crucial for pinpointing the best locations for oyster beds (Liu et al., 2024).

Austin and Newaj-Fyzul (2017) discussed the potential for disease management in shellfish aquaculture. Implementing stringent biosecurity measures, such as regular health screenings and the use of disease-free broodstock, is essential to prevent the spread of pathogens. Collaboration with veterinary and biosecurity experts can enhance the effectiveness of these measures.

Future nurseries can benefit of new technologies to improve efficiency and sustainability. Automation and remote monitoring systems can significantly enhance nursery operations. These technologies can reduce labour costs, improve precision in oyster management, and facilitate real time responses to environmental changes (Schnurawa et al., 2024). Also, the integration of artificial

intelligence (AI) can provide several advantages as optimizing feeding regimes and detecting early signs of disease (Rather et al. 2024).

7.4 Conclusion

The experimentation emphasised the importance of several variables, such as stocking density and environmental characteristics, in the rearing process of juvenile *O. edulis* individuals in the North Sea. The results confirmed the feasibility of an offshore nursery in the island of Helgoland at depths between 8 and 11 m. At these depths, the environmental parameters were optimal for healthy growth of the reared individuals. Associated with the environmental characteristics, also rearing density plays a central role in the development and growth of the individuals. The results obtained in the experiment confirmed that a medium stocking density is optimal and advised for this purpose since it avoids the excessive competition that develops at high stocking intensity, avoiding at the same time the waste of space that occurs at low stocking densities. Considering the site characteristics necessary for an optimal growth, there are different possible locations in Helgoland, or close to it, that can be suggested for the establishment of an offshore nursey. A potential site could be in the North part of Helgoland, near the Lange Anna, which is a sheltered area where it is possible to find location with depth of around 10 m, with sea conditions that are adequate to support an oyster nursery. Another possibility could be the South-West area of

Helgoland since it is located near a rocky slope, providing an optimal substrate for the growth of juveniles. However, in this location, it is important to assess the characteristics of the sea currents to ensure that they are not too turbulent. Also the northern or eastern area of the Düne Island could be considered a possibility, as they are hardly disturbed by human activities and have depths of around 10m. Here too, the presence and strength of the currents, as well as the stability of the seabed, must be carefully assessed. Disadvantages, however, include the high presence of seals that could damage the structures necessary for rearing oyster juveniles, but also the higher logistical costs as the island is located in front of Helgoland, where the main AWI buildings are located.

Before the actual establishment of the offshore nursery, it will be necessary to conduct a longer experiment in order to assess the impact and variation of environmental parameters during the year with the change of seasons, particularly in the colder months, on the reared individuals. Future research should continue to explore the interactions between environmental factors, nutrient availability and stocking densities to further optimize oyster restoration and aquaculture practices. Monitoring and adapting these factors will be a key to the successful restoration and sustainable management of *O. edulis* populations.

In the light of these findings, establishing a field nursery on Helgoland represents an important step towards the restoration of the European flat oyster in the North

Sea. With the application of advanced aquaculture techniques, along with addressing logistical and environmental challenges, there is the potential for the development of an offshore nursery with the aim to obtain a large-scale oyster restoration. Continued research, monitoring and collaboration will be essential to ensure success in the long term.

Through the implementation of these recommendations, researchers can enhance the effectiveness of restoration projects, ensuring the sustainability and resilience of *O. edulis* populations in the North Sea. The knowledge gained from these efforts will not only benefit oyster restoration but also contribute to broader marine conservation and sustainable aquaculture practices.

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