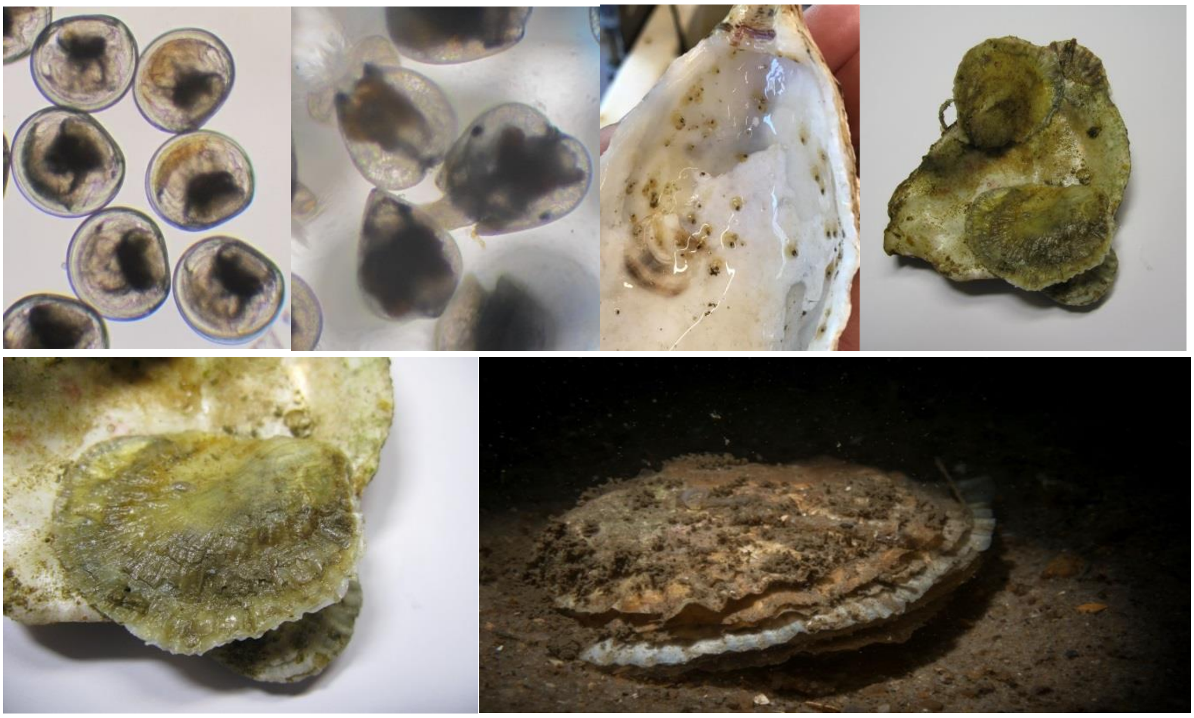
A co-operative and reliable *Bonamia*-free flat oyster production in hatcheries for nature enhancement in the North Sea



Final report 2019-2023, December 2023

The Rich North Sea

in cooperation with

Wageningen University & Research

Waardenburg Ecology

HZ University of Applied Sciences

Stichting Zeeschelp

NIOZ Sea Research

Roem van Yerseke

A co-operative and reliable *Bonamia*-free flat oyster production in hatcheries for nature enhancement in the North Sea

Final report of the Breeding Line project 2020-2023

December 2023

Editor: Marco Dubbeldam

This project has been carried out in close co-operation and open knowledge sharing. In this way progress was possible which could not be achieved by a single partner. The results are therefore a collaborative achievement.

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Summary

Introduction
Flat oyster (*Ostrea edulis*) reefs have almost disappeared out of the Dutch part of the North Sea for many decennia and there are no signs for a serious natural come back. Millions of oysters are needed for restoration purposes. The only way to provide these numbers is the use of juveniles or spat which originate from a hatchery-nursery approach.
The cultivation of flat oyster larvae and spat is erratic and has so far (up until 2019) resulted to be unpredictable. This is also a well-known fact in hatcheries-nurseries outside the Netherlands, for which there is no good explanation or solution available yet.

For the Breeding Line project of the Rich North Sea a co-operation has started with partners working in and doing research on reproduction of flat oysters. For efficiency and a (relatively) fast progress a working group called KOPON has been set up. KOPON stands for 'Kweek en Onderzoek Platte Oester in Nederland' meaning 'Culture and Research on Flat Oyster in the Netherlands'. The primary goal is to share knowledge and experience to gain insights into the strengths and challenges within the culture of flat oysters. This approach saves time and effort, allowing for a clearer focus on what works well and shows promise.. This way of working and communicating has some simple rules for participation in the KOPON working group:

- Partners which are practically active in the restoration of flat oyster populations
- Open-source exchange of knowledge and experience, advice each other when asked or not asked for. Each partner will take information for own purpose and will give information when it might be interesting to share.

An applied research project with a duration of four years was set up to answer questions about best practices and success factors for broodstock and survival, growth and spatfall of larvae. The objective of the partners in this breeding line project was to find a predictable, cost efficient and reliable method to grow *Bonamia*-free flat oyster spat, suitable for release in nature restoration projects on a large scale.

Results
The main conclusions after four years of research on three research questions are:

<u>Which factors determine the **success of conditioning the broodstock**?</u>
After anesthetising and sampling gill material for *Bonamia* analyses, a small to sometimes serious number of mortalities occur in the broodstock in the weeks thereafter. A gradual increase in temperature to 22-24 degrees at a diet of several diatoms and flagellates gives larvae production over about 10-12 weeks. Broodstock of Dutch origin can be conditioned well to the moment that it releases larvae, the quality of the released larvae is quite stable. The bacterial pressure on broodstock and released larvae is present and can cause a bad start for the larvae culture. The algae diet can be a source of bacterial pressure as well, but seems nutritional sufficient. Focus on 'clean' algae cultures and hygiene in broodstock seems to lower the overall bacterial pressure in the culture systems.

<u>Which factors determine the **growth and survival of the produced larvae**?</u>
Broodstock originating from Lake Grevelingen and the Eastern Scheldt performs well, enough numbers of released larvae are produced. Parental stress, due to Bonamia testing can affect the release and condition of larvae. The first week of larvae culture goes well in general,

with growth and development. But the second half of the larvae culture is critical to bacterial pressure (*Vibrio*) and nutrition. In this phase, diatoms seem an important part of the diet. In general, one third of the larvae becomes competent to settle and one fourth of this becomes spat, depending on condition of the larvae and substrates to settle on.

Which factors determine the **growth and survival of the spat**?
Condition of the larvae seems to be the most critical issue for success in metamorphosis to spat. Most related in this seems bacterial load and nutritional value of the diet. Once the larvae become spat and are in good hygiene, the survival is good. In weeks after spatfall the size of several mm is achieved and in several months it can grow to several cm in size.

Co-operative approach is valuable.

The expectation of open co-operation and knowledge sharing between a small group of project members has turned out to be efficient and valuable. Meetings have been periodically, every 6 to 8 weeks, to keep up with everyone's progress and to focus on the things to do in the next months. After each year, results were evaluated and a new plan with ongoing insights for the next year was made. Periodic meetings with all project partners involved, discussing ongoing matter helps to understand the role and needs of each other. Ongoing insight and adaptation to this made important steps possible to achieve a quite stable culture of flat oyster spat in the end.

The result of four years of applied co-operative research is that *Bonamia*-free spat production is now reliable and that suitable material for nature enhancement projects can be sourced in the Netherlands.
Outplacement of spat in a nearshore environment resulted in survival and growth and a better understanding of relevant success factors for this has been gained. This has been achieved due to constructive cooperation between NGOs, companies, foundations and research institutes.

Recommendations
With these conclusions, there are several recommendations to make. These are important for upscaling of the Dutch hatchery-nursery technique for flat oysters, so a suitable quantity of flat oyster spat (hundreds of millions) will become available for outplacement on the sea floor:

- Connect supply and demand of flat oyster spat for good planning. The hatchery-nursery has serious (background) costs over the year. This is only feasible when there is a serious demand for outplacement of flat oyster spat. Also for planning of delivery of spat it can take up to one year on forehand for preparation to meet production and demand.
- It is recommended to use a disease free broodstock from a *Bonamia* area, Bonamia tolerant spat is then produced and will secure survival once a clean area becomes infected compared to the situation when *Bonamia* free spat out of a *Bonamia* free area will be outplaced, as in this case there will be low(er) tolerance for this parasite.
- Scaling up flat oyster spat production needs development and attention in the current Dutch hatcheries and will take time before it will be fully operational. Efficiency in scaling up will be achieved by growing demand and therefore cost efficiency will come in time. Scaling up techniques need to get operational as spat on substrate requires a much bigger volume than larvae or single spat. This will increase the water

treatment and -volume for ongoing *Bonamia* free production. These facilities need to be built or organised.

- Settle substrate for flat oyster spat should be tested in offshore practice. Spat on shell is a good way in sheltered areas, but more exposed locations in open sea (currents, wave actions) will probably need more robust substrates such as artificial reef structures or stone structures, to remain in place. A method should be found for spatfall on these substrates, or connect single spat to them later. Single spat can be produced more efficiently than spat on shell, which requires much more tank volume. Therefore implementation of substrates for off-shore use from hatchery on is recommended.
- Agreement on and implementation of a certified or permitted hatchery-nursery procedure for outplacement of flat oyster spat offshore for nature enhancement. In the Netherlands these regulations are not active yet. Therefore, certification is not possible, although there is already a *Bonamia* free spat production in the last four years. For ongoing improvement of hatchery-nursery-outplacement this procedure should be put in practice as soon as possible, otherwise a great gap of 3 years will appear before any of the earlier recommendations can be put in practice.

Content table

Introduction and background

The Rich North Sea has a dream, being a healthy North Sea, source of sustainable energy, rich in nature and full of life. The programme started in February 2019 with a duration of 5 years. Part of The Rich North Sea programme is to enhance reef building organisms to contribute to restore the original biodiversity, like reef structures of flat oyster communities.

The flat oyster has almost disappeared out of the Dutch part of the North Sea for many decennia and there are no signs for a serious natural come back. Introduction in areas with suitable conditions is one of the goals in this project. With the use of adult oysters for reintroduction there are several drawbacks to mention: scarce availability, withdraw from areas where standing stock is also low, disease control and the possible introduction of exotic (invasive) species.

For restoration purposes millions of oysters are needed and the only way to provide these numbers is with the use of juveniles or spat which originate from a hatchery-nursery approach. The flat oyster is one of the most difficult and unpredictable shellfish species to culture, compared to Pacific oysters, cockles, mussels and clams. However controlled reproduction seems the only way to produce enough numbers for rewilding this species.

Description of the Breeding Line project

The Rich North Sea combined forces to focus on the improvement of reproduction of flat oysters in hatcheries. A so called 'breeding line' would perform the procedure to establish a predictable and reliable production of flat oyster spat.
In cooperation with research institutions and SME's the 'breeding line' part focussed on finding a predictable and reliable method to grow *Bonamia*-free and in the long run possibly *Bonamia*-resistant flat oysters, suitable for the large-scale introduction for nature restoration and cost-efficiency purposes.

Challenges, goals, objectives and research questions

The cultivation of flat oyster larvae and spat is erratic and has so far resulted to be unpredictable. The first larvae in season are promising in breeding, later in season larvae cultures show little to no success at different participants. This is also a well-known fact in hatcheries-nurseries outside the Netherlands, for which there is no good explanation or solution available yet.

In addition to a productive breeding technique, the quality of the oyster spat is also important. In particular, the material released into the North Sea must be demonstrably free of infection with the oyster parasite *Bonamia ostreae*. This is to prevent that the release of flat oysters will lead to the introduction of *Bonamia* in an area where it may not yet occur. The oyster spat must also survive and in time become reproductive in the North Sea environment.

When flat oyster beds are restored in areas where *Ostrea edulis* is functionally extinct it is advised to treat these as disease-free areas. Restoration pilots need oysters in order to start a bed. In the Dutch Delta area a local flat oyster population exists and is farmed. This can be a source of flat oyster spat. However, the population is infected with *Bonamia ostreae* since the 1980s (Engelsma et al., 2010). Bonamiosis is a disease caused by the intracellular protistan parasite *Bonamia* sp. and can result in mortality of flat oysters (Engelsma et al., 2010, 2014). *B. ostreae* was first observed in France in 1979 (Pichot et al., 1980) and is now present in most countries surrounding the North Sea, except for areas in Ireland, Scotland, Norway and Sweden (Sas et al., 2020). In the Netherlands, the parasite was introduced in 1980 by import of oyster stocks from France into the Oosterschelde area (Van Banning, 1982) and subsequently spread of the parasite to the adjacent Lake Grevelingen (Van Banning, 1991).

Seed from *Bonamia* infected areas cannot be used in restoration projects because of the risk of spreading the disease (Pogoda et al, 2019). Seed from *Bonamia*-free hatcheries has the disadvantage that the seed is vulnerable to the disease (Culloty et al., 2004; Lynch et al., 2014). If, for some reason, *Bonamia* will reach the restoration site, the oysters may suffer from high mortality. Therefore, the use of disease-free seed produced from disease-tolerant parents would solve this problem if tolerance is inheritable. This offers opportunities to produce oysters that are not infected with *Bonamia*, despite the parents having been exposed to *Bonamia*, potentially creating offspring with some tolerance to the disease.

Recent research by WMR has shown that it is possible to grow *Bonamia*-free young oysters from *Bonamia*-contaminated areas. This line of research deserves further attention.
In the Netherlands, the population of flat oysters seems to become more tolerant to *Bonamia*, a part of the population is infected, but does not show the high mortalities as before. Another part shows no infection and might have become even resistant?
It is probably possible to test adult oysters for tolerance to *Bonamia*. The advantage is that resistant oysters will not die if the disease unexpectedly reaches the area of expansion. Whether there is resistance is currently still under investigation and needs to be further elaborated.

In the Netherlands we want to focus on the cultivation of juvenile flat oysters for release for nature restoration, in the way of hatcheries or ponds ('spatting ponds'). However, none of these ways have a recognised or certified procedure for growing *Bonamia* free oysters.

In order to be able to release flat oyster juveniles in the North Sea, a reliable cultivation method is therefore required, which is (in the long term) also EU certifiable (as a *Bonamia* free production site). To be eligible for certification, disease-free production must be demonstrated for three years under EU-protocol. In practice, this will probably be longer, because the cultivation technique also needs to be improved. Currently, there is a lack of clear insight into the requirements and procedure to initiate the certification. The development or application of this - in consultation with the competent authority in this matter - is part of the present plan.

The intention of the plan for this Breeding Line project is intending to bring producing and purchasing partners together and co-operate, so that substantial quantities of flat oyster brood will be produced in a reliable, responsible and affordable way for nature restoration and population recovery.
If new scientific insights are gained, these will also be published through scientific publications (scientific publishing requires extra attention and requirements, which means that it takes longer for knowledge to be published in this way).

The goals of the partners in this breeding line project are, in summary, to find a predictable, cost efficient and reliable method to grow *Bonamia*-free and in the long term perhaps *Bonamia*-resistant flat oysters, suitable for release for nature restoration on a large scale.

This must also be done in such a way that the necessary certification for the status '*Bonamia* free production site' is possible and that risks such as the introduction of invasive species into the North Sea are mitigated. So a clear procedure should become available for licensing the introduction of flat oyster spat for nature restoration at sea.

The objectives of the Breeding Line project are:

1. A predictable and reliable production method for *Bonamia*-free flat oyster larvae and juvenile oysters ('spat')
   - To encourage oysters to produce larvae, they must be conditioned. This is done by changing temperature and/or food quality/quantity. There is still little control over the best way to do this. Various conditioning protocols should therefore be tested. The origin of the parent animals can also play a role in the quality of the offspring, so this aspect is also tested.
   - In practice it appears that there is a big difference in numbers and health of the larvae produced during the breeding season and/or during the year. The mechanisms behind this need to be unrevealed in order to make production more reliable throughout the year. In relation to this also serves:
     - Gain better insight into optimal growth conditions for oyster larvae and spat.
     - Control or prevent outbreaks of (bacterial) infections and diseases that can harm the larval population.
     - The method to be used towards the production of *Bonamia*-free oyster larvae will be tested further in practice.
2. Cost efficient production of larvae and spat including a start for upscaling.
   - Minimizing mortality during production is the most important hurdle to be taken in order to be able to produce larvae and spat in a cost-efficiently way and on a larger scale. This is being worked on in practice within Objective 1.
   - In addition, important objectives are:
     - Minimizing production costs, especially the important items of food, manpower and culture space.
     - In addition to hatcheries, use natural production conditions ('spatting ponds', algae production in ponds, etc.).
   - Experiments will also being carried out to determine the optimal way of scaling up production.
3. Provide suitable material for (large-scale) introduction for nature restoration
   - Determining the optimal age and condition of spat for successful outplacement in coastal water.
   - Gaining insight into the most optimal type of settlement material (substrate) for the spatfall and the optimum density of oyster spat on this substrate.
   - Gaining insight into the most optimal method of releasing oyster spat into the open water.
   - Coordinate the certification procedure of disease-free material with the authorities and, if necessary, further develop the necessary protocol.
   - Besides nature restoration, spat produced can also be useful for oyster farmers since flat oyster spat is less abundant in nature last years.

Project partners

For the Breeding Line project The Rich North Sea has set up a co-operation with partners working in and doing research on reproduction of flat oysters. For efficiency and a (relatively) fast progress a working group called KOPON has been set up. KOPON stands for 'Kweek en Onderzoek Platte Oester in Nederland' meaning 'Culture and Research on Flat Oyster in the Netherlands'. Main goal is sharing knowledge and experience to learn about the good and bad things happening in the culture of flat oysters. Especially sharing the bad things will help to understand how to proceed and to avoid same mistakes by other partners. This saves time and effort and will help to focus to items which go well or are promising. This way of working and communicating has some simple rules.

Starting points in efficient co-operation

Starting points for participation in the KOPON working group are:

- Partners which are practically active in the restoration of flat oyster populations
- Open-source exchange of knowledge and experience, advice each other when asked or not asked for. Each partner will take information for own purpose and will give information when it might be interesting to share.

Partners and roles

The partners and their initial role in the breeding line project are:

Stichting Zeeschelp – Stichting Zeeschelp is coordinator and applied research partner in this project. The objective of this foundation is the development of and innovation in marine aquaculture, using an experimental field station with facilities for shellfish, flatfish, seaweed and algae cultivation.

In principle, previous cultures of flat oysters gave a good production of larvae, which were initially active and feeding, but which gradually faded after a few days to weeks. Our starting point is that the larvae are well developed when released by the parent animal, but that they will then lack something or become bothered by something. This phenomenon becomes stronger as the breeding season progresses. The various treatments are aimed at distinctiveness, in order to find out the direction of the unpredictability of larval development points.

Specific activities:
• Collect various brood stocks, condition them in different ways (2-3 treatments), brood stocks must be free of *Bonamia*.
• Larvae culture in 10 tanks, 5 treatments in duplicate, or 3 in triplicate.
• Larvae will settle on various substrates, growing spat.
• Support with stocking and outside water monitoring

Treatments consist of various ways of filtration/cleaning of sea water to be supplied, algae (species and whether or not centrifuged for 'cleaner' nutrition), oyster larvae (housing in high-low density systems) and broodstock (origin and conditioning with or without season). The working method will be coordinated with WMR and HZ University of Applied Sciences.

Produced spat will be released into open seawater.
Work is also in progress, including consultation with the competent authority in this regard, on the certification procedure for the supply of oyster spat for restocking in the North Sea.

Roem van Yerseke - Roem van Yerseke is a shellfish processing company with its own shellfish hatchery. The hatchery focuses on the cultivation of flat oysters directly from the field, which are then kept in different ways in the hatchery. These oysters are treated partly in the same way and partly in a different way and compared to the conditioning at the Zeeschelp. In addition, small-scale experiments by WMR & NIOZ can be carried out on a large scale by Roem van Yerseke.

Roem van Yerseke has already started an additional project for the production of flat oysters, in co-operation with WMR on specifying bacteria and how they affect the quality and quantity of the flat oysters, both broodstock and larvae. For this purpose, both broodstock and larvae are kept and reared in different ways (in duplicate or triplicate).

In addition, different periods in the year are examined and treatments are performed several times a year to get a better overall picture. The various treatments in terms of broodstock origin, temperature, water treatment and nutrition are carried out in close contact with Zeeschelp in order to compare the same or different factors. The larvae will be settled as spat-on-shell for the recovery of shellfish beds. It is then investigated to what size the spat-on-shell should be kept in the hatchery before it can be released, to achieve the highest survival and growth in the field. Single seed production will be investigated as well, which can be supplied to nature or oyster farmers.

NIOZ Texel - NIOZ Texel is a marine research institute with a breeding facility for flat oysters. They also have an advisory role for the applied partners.

For the advice, NIOZ carries out small-scale research in a climate chamber for:
• Optimization of the conditioning of the brood stock (temperature, food quality/quantity), differences in conditioning between origin of the broodstock (Wadden Sea and Delta waters)
• Detect differences in numbers and quality of larvae produced during the breeding season
• Test optimal growth conditions for oyster larvae and spat (different diets).
• Identify factors that increase success in spatfall.
• Provide advice on breeding success to Edmelja, Zeeschelp and Roem van Yerseke
• Testing the survival of spat at sites in open water (Wadden Sea): in which constructions, with which substrate and at what size are the oyster spats relatively resistant to predation?

Oyster spat produced in this way is in principle used in open water (Wadden Sea and North Sea).

WMR Yerseke - WMR Yerseke is a shellfish research institute. They focus on scientific research and will advise the applied partners on the conditioning of brood stock and the cultivation of larvae and spat.

- WMR contributes to solving the bottlenecks in the hatchery production of flat oysters with research and analyses. To this end, brood stock is screened for the absence of *Bonamia* with a non-destructive method so that the hatcheries can produce *Bonamia* free spat in an *Bonamia* infected area, so the spat can be released in the North Sea or coastal waters. Optionally (depending on the available budget and the results of ongoing research), the spat is tested for the presence of the resistance gene. This guarantees that the oysters are not susceptible to *Bonamia* after spawning, should this occur in the North Sea in the future.
- In addition, a comparison with brood stock from the field is used to investigate whether a possible lower quality of hatchery broodstock (conditioned out of season)

or larvae is the cause of regularly occurring larval deaths. The hatchery management is adjusted on the basis of the insight obtained.

Specific activities:

- Selection of *Bonamia* free broodstock for use in hatcheries of Roem van Yerseke, Zeeschelp and NIOZ, and possibly in ponds of Edmelja
- Testing spat produced by hatcheries for the absence of *Bonamia.*
- Testing spat produced by hatcheries for the presence of a resistance gene.
- Research on broodstock conditioning by comparing the quality of broodstock and larvae from hatchery versus field and origin of the broodstock (eDNA and fatty acid analyses for diet quality and bacteria analyses for the presence or absence of pathogens)
- Research into the differences in numbers and quality of the larvae produced during the breeding season (eDNA and fatty acid analyses for diet quality and bacteria analyses for the presence or absence of pathogens)
- Research into factors that increase the spawning success of flat oyster larvae (e.g. chemical cues) by means of a student topic.

HZ University - The HZ University of Applied Sciences is an HBO education and applied research institution. HZ is active in the field of shellfish cultivation through the research group Aquaculture in Delta Areas. This research group conducts practice-oriented research in collaboration with SME's. In this capacity, HZ has built up a great deal of expertise and practical knowledge in the field of oyster farming in recent years. The relevant educational programs at HZ are: Water management and Civil engineering.
Research has been done in projects on the quantity and quality of microalgae and how these influence the quality of oysters, the cultivation and monitoring of algae breeding ponds. The HZ is currently participating in two projects in which the combination of nature development/aquaculture with offshore wind farms is investigated.

Within this project, HZ has substantive input in the following areas:

- Conditioning broodstock
- Advice on microalgae cultivation
- Fatty acid analysis of microalgae. Applied research in the spatting ponds (measurement of biotic and abiotic factors)

Waardenburg Ecology- Waardenburg Ecology is a research and consultancy for the environment. It conducts oyster surveys in the field with both theoretical and practical knowledge and skills. They can also provide and guide the application for the associated laws and regulations.

- Waardenburg Ecology contributes to the cultivation with advice based on knowledge about natural oyster beds and results of oyster recovery projects in the Netherlands. The intended field research is aimed at learning how the restocking in the field can be as successful as possible and the survival in the field as large as possible.

Relevant research questions are:

- What is the best time and method of stocking to achieve a high survival rate?
- How does this compare with the costs of keeping the spats longer or shorter in the nursery?
- To answer these questions, a quantitative trial is being designed in which different treatments are compared with each other. When the opportunity arises, monitoring

by diving shows how the released spat and juveniles are doing to evaluate the success of the various treatments.

- In addition to monitoring in the field, legislation and regulations on restocking are one of Waardenburg's specialisms. Waardenburg Ecology applies for permits to carry out field experiments.

Knowledge partners

Edmelja – Edmelja was initially involved in the Breeding Line project as a private company researching the cultivation of flat oysters in open ponds, using the natural growth of algae as food. Cultivation in ponds is an extensive and, in principle, cost-efficient method. Edmelja shared knowledge on this topic with the KOPON group.

ARK Rewilding NL – Ark Rewilding focusses on rewilding areas from land to sea. One of their priorities is rewilding the seafloor by introducing flat oysters to create a reef building community in time. Ark Rewilding joined the KOPON-group in 2020 and took part in the discussions about outplacement of hatchery cultured flat oyster spat in near and off-shore areas. Ark Rewilding provided space for outplacement and monitoring of spat produced by the breeding line project of The Rich North Sea project. The experiments with outplacement of 'DRN' spat took place in their experimental plot in the Voordelta, where Ark Rewilding has own ongoing outplacements of flat oysters and monitoring.

Project set up and methods

The three partners that have hatchery facilities are NIOZ on Texel (NIOZ), Roem van Yerseke in Yerseke (RvY) and Zeeschelp in Kamperland (ZS). These three locations differ in circumstances (different water source, different pre-treatment of this water, different cultivation method of algae, different brood stock, different tanks, etc.).

In addition, Wageningen Marine Research (WMR) and HZ University of Applied Sciences (HZ) have breeding knowledge and analysis capacity. By setting up comparable experiments at the three locations, more insight can be gained into factors that determine the success of the culture. To this end, systematic experiments are carried out in which different conditioning protocols are used and experiments in which oyster larvae and spat are kept under different conditions. These experiments are then analysed for best results. An integrated approach to monitoring and data compilation during execution ensures that comparisons between batches and locations are possible. WMR and NIOZ are responsible for the content of the monitoring plan, the implementation of the experiments lies with the cultivating parties.

Research questions

In summary the main research questions are:

- Which factors determine the success of conditioning the broodstock (period, temperature, origin of the broodstock, food quality/quantity, bacterial pressure)?
- Which factors determine the growth and survival of the produced larvae (conditioning brood stock, origin, food quality/quantity, bacterial pressure)?
- Which factors determine the growth and survival of the spat (brood stock conditioning, origin, food quality/quantity, bacterial pressure)?

Method

The reproduction of flat oysters anno 2019 was still erratic and unpredictable. A research program over a period of 4 years should be open for adjustments based on ongoing insights, evaluation and discussion, trial and error, murphy and some luck.

From the beginning we have started to work from an interactive working group perspective, with for everyone a moment for ongoing matter with good and bad things happened. In response, comments by others could be given, trying to get improvement in culture or make decisions with best info possible. These meetings have been periodically, every 6 to 8 weeks, to keep up with everyone’s progress and to focus on the things to do in the next months. Also it was important to keep track of costs, when work packages change. After each year, results were evaluated and a new plan with ongoing insights for the next year was made.

In this way, all partners thought that best results could be found, by using everyone’s knowledge and experience for the common goal of the project: predictable flat oyster spat.

Summary of results in 2020

Bonamia analysis method, *Bonamia* free brood stock and spat

For routine monitoring of *B. ostreae* in the flat oyster population in the Netherlands a pool of oyster tissue was collected from gill, mantle and digestive diverticulum (up to a total of ~25 mg tissue per individual oyster), below referred to as “standard method”. In order to investigate whether a new non-destructive method of sampling (gill tissue of approximately 4 mm^2) is providing a representative status of the presence of *Bonamia* in the oyster, both methods were compared with real-time PCR using 190 oysters collected in 2018, 2019 and 2021 during the routine monitoring for shellfish diseases from Lake Grevelingen.

Bonamia genetic material was detected in 40 individuals out of the 190 oysters for each method: the standard sampling method and the non-destructive sampling method (Table 1). However, both methods had positive samples which were not detected by the other method. It should be noted that in all positive oysters only low amounts of *Bonamia* genetic material was detected (real time PCR Ct values ≥ 32), which might have caused the false negatives. It can be concluded that the non-destructive sampling is comparable for detecting a *Bonamia* infection.

Table 1. Comparison of the standard method with the non-destructive method for detection of *Bonamia* by PCR.

		Standard method		
		positive	Negative	Total
Non-destructive method	Positive	40	1	41
	Negative	11	138	149
	Total	51	139	190

In 2020, a selection of brood stock took place for Zeeschelp. Gills were cut from 150 oysters that were sampled from Lake Grevelingen. Of these, 98 oysters were *Bonamia* free. These oysters have been used as brood stock by Zeeschelp and have produced larvae and spat. Of these, 9 larva batches and 130 spats have been tested for *Bonamia*. Due to the size of spat and the minimum required biomass, 3 spats were pooled to one sample. All samples were analysed free of *Bonamia*. The 7 oysters that died in the brood stock were tested for *Bonamia* and were found to be *Bonamia* free.

Photo 1 and 2. Stunning flat oysters and gill sampling

This indicates that the selection method has worked properly. There is now a *Bonamia*-free brood stock present at Zeeschelp that has produced *Bonamia*-free spat.

Survival larvae to spat

In 2020, research has focused on larval survival to spat, which may be affected by several factors. This is shown schematically in Figure 1. A hatchery consists of different compartments (broodstock tanks, larvae tanks, spatting tanks). Each tank is supplied with water and algae. The quality of the algae and the water, but also the amount of algae are important parameters. In addition, the treatment of the brood stock and the larvae is important. This concerns e.g. the temperature or the frequency of cleaning.

Photo's 3,4,5. Culture systems NIOZ, Roem van Yerseke and Zeeschelp.

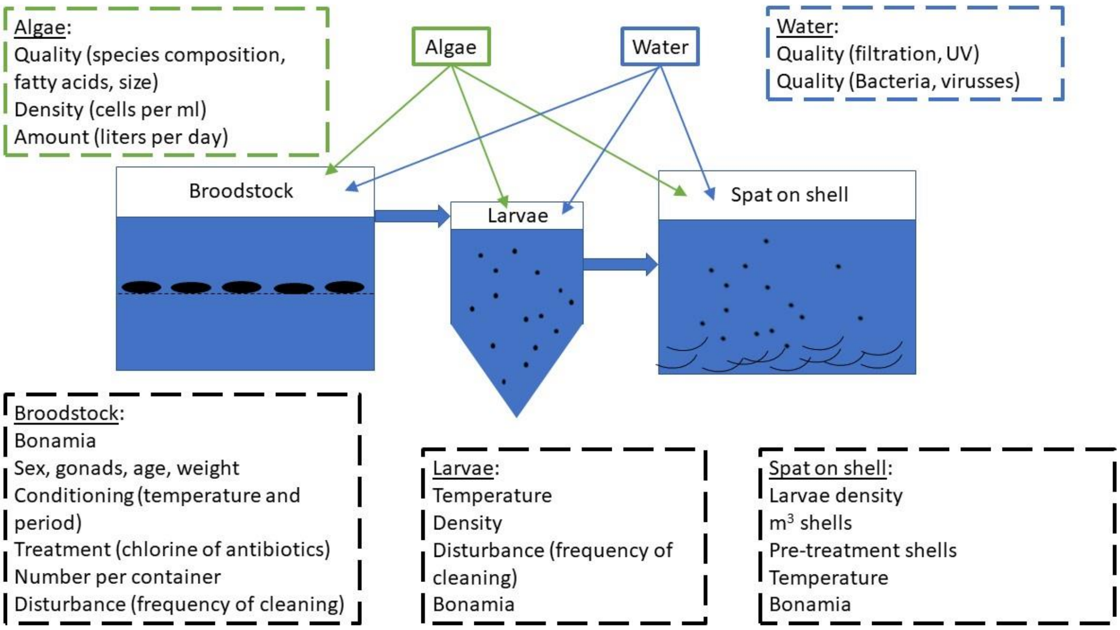


Figure 1. Factors that may influence larval to brood survival are shown in the dotted box.

In 2020, the brood stock showed some dead oysters in the first weeks, probably as a result of the cutting treatment for the *Bonamia* analysis. After that, no more oysters died.

The brood stock regularly produced larvae, which were on average smaller than 200 micron. After initial growth, this slowed down and larvae began to drop out after a few days to a week. In a few cultures, small numbers of larvae reached the competent stage and developed into spat. One larval culture showed good growth and development and produced spat on a variety of substrates.

The expectation that sexually mature oysters from the field and that working with the season would be a good starting point did not improve the result of culture. The larval cultures were mostly problematic. During the culture, the flow rate, water and food quality, temperature and culture volume were varied. There is no treatment that showed a clear positive effect.

The results of Zeeschelp, Roem van Yerseke and NIOZ are summarised as follows:

In total, brood stock oysters released larvae 31 times. An average of 2.7 million larvae at each release. This has led to spat for 18 times. The size of the larvae at release does not appear to have an effect on the likelihood of spat establishment. The period of time of the brood stock in the hatchery does not appear to be of influence, as successful rearing from larvae to spat took place both at the beginning and at the end of the rearing period. The temperature at which the brood stock was kept resulted in larvae from 18 °C onwards.

In the end, some spat was produced on shells of scallop, oysters, mussels, limed shells and a small sized reef cube. The average number of spat per type of shell is given in the figure below. Total number of produced spat on various shell types is 32.000. This spat is used for field experiments in the Voordelta.

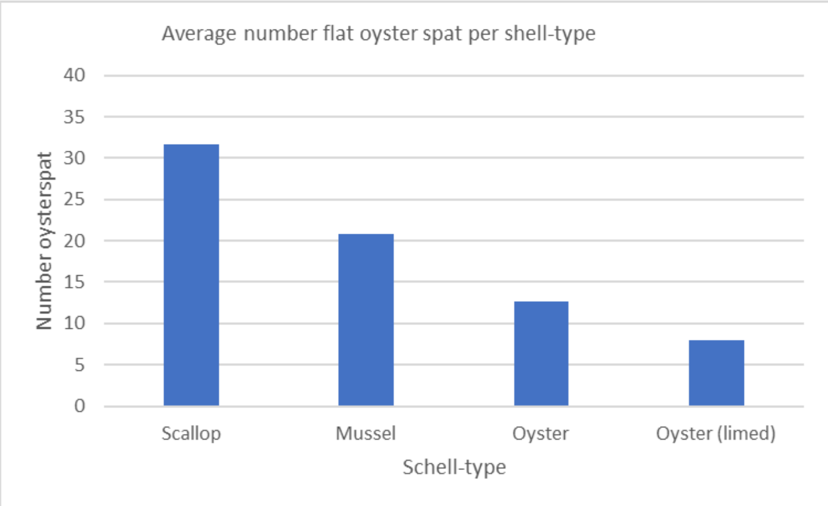


Figure 2. Settlement of flat oyster spat on various shell-types

Photo's 6,7,8. Flat oyster spat on mussel-, oyster- and scallop shells.

Bacterial pressure

Of the bacterial species identified so far in two flat oyster hatcheries, only *Vibrio splendidus* and *Vibrio ostreicida* are described as pathogenic for *O. edulis*. *V. ostreicida* was found in the culture water of larval batches that eventually died before settlement, but also in one of the batches that did settle on shells. No cause-effect relation was found with the bacteria analyses.

Food quality

An analysis of the nutritional value of the algae diet used at Zeeschelp indicates that the protein content and fatty acid content is low compared to values from the literature. The carbohydrate content is actually high. Proteins and fatty acids are, as a rule, more important for the development of larvae than carbohydrates.

Spatting ponds

Spatting ponds were tried in already existing ponds left over by Zeeland Aquaculture. Edmelja started up water supply, restored filtration and cleaned the ponds for making them operational for spatting oysters on mussel shells. *Bonamia* free broodstock from Norway was introduced and put in recirculation with the ponds. In the end, and due to several practical reasons (i.e. a hot summer season), most broodstock did not survive and no spat was found on the spat collectors. A lot of needed adjustment on the current pond system and uncertainty of licence procedures for the next season made it to decide to stop this way of trying to get oyster spat from ponds.

Outplacement in the Voordelta

Several types of substrates are available with oyster spat. These will be outplaced in 2021.

Photo's 9, 10, 11. Spat of flat oysters on a reef cube.

Conclusions and recommendations 2020

There is a *Bonamia*-free brood stock at Zeeschelp (from 2020) and Roem van Yerseke (from 2019) available that has produced *Bonamia*-free spat. This result can be a start to the procedure of certification to produce oyster spat free of *Bonamia*.

A start has been made on constructive insight into factors that determine the success of brood stock conditioning and the quality and quantity of larvae, and thus a start has been made towards a (more) predictable breeding procedure. Both bacterial pressure and nutritional value of the algae cannot yet be ruled out as the cause of the larval mortality.

Evaluation of these results and the experiences of the partners involved resulted in a list of points for further research.

1. The influence of diet on brood stock and larvae

What is the composition of the most commonly used algae species (fatty acid, protein), which combination of algae species gives better growth and survival of the larvae, which algae are eaten by the larvae. What influence does the algae cultivation process have on the quality of the algae.

2. The relationship between bacterial pressure and larval survival

Which types of bacteria are present during culture, which types are dominant and what is the proportion of (potentially) harmful bacteria (*Vibrio* species). Is there a change in the bacterial population during the breeding season, what is the effect of ultrafiltration and are positive effects to be expected from probiotics?

3. The effect of stress on the brood stock

Changing temperatures, the presence of people, noise and frequent handling of the oysters can cause stress. Stress seems to have consequences for filtration and the (time of) release of larvae.

Summary of results in 2021

In both April (RvY) and May (ZS) 180 oysters were collected from the field (Grevelingen, Eastern Scheldt) and analysed for *Bonamia*. About 85% of these oysters was free of *Bonamia* and taken in for broodstock.

In summary, the way of culture at Zeeschelp is described, to demonstrate the volatility of the ongoing larvae culture. The temperature of the broodstock was increased by one degree every 3-4 days to 22 degrees, after which it was kept constant. The brood stock from 2020 survived the winter period without loss. The brood stock from 2021 showed a low mortality rate of a few oysters in the first weeks after delivery. It was striking that after this, a steady dropout began to occur in both groups, which were housed separately. Every week a number of (approximately 2 to 6) oysters per group. Checking the system, seawater, water quality, nutrition and temperature showed no remarkable things. After the loss had persisted for several weeks, it was decided to adjust the algae dosage to a lower level. After this, the loss gradually decreased and eventually stopped. Apparently, the flat oysters had an excessive supply of food, although according to literature this was in the optimal range for larval yield (3,5% dw). However, some loss of oysters was also reported there. There were also pseudo faeces present, as a sign that not all algae were consumed. After lowering the algae dose to 2% dw, pseudo faeces were also no longer visible.

About 3 weeks after the temperature was set to 22 degrees, the first oyster larvae were released. The quality was initially good, little to no loss and the first spat was produced. The following cultures were cultures with total failure, with partial failure and some of the larvae became competent and became spat, and there were cultures that were moderately successful. Initially, only batches of larvae considered promising (dark in colour and with an average size of 200 mu) were taken into culture. Batches with a light colour and on average less size were not cultured. Because cultures that passed this selection also showed failure over time, it was decided to culture all batches after all.

The strategy now was to see if a pattern could be discovered with a large number of batches. A total of 21 batches of oyster larvae were cultured in the period June-August. There was little regularity in the release of 'successful' batches of oyster larvae. It was impossible to estimate in advance what chance the larvae had of reaching spatfall. On average it took 10 days until a large part of the oyster larvae became competent and could be transferred to the spatfall tank.

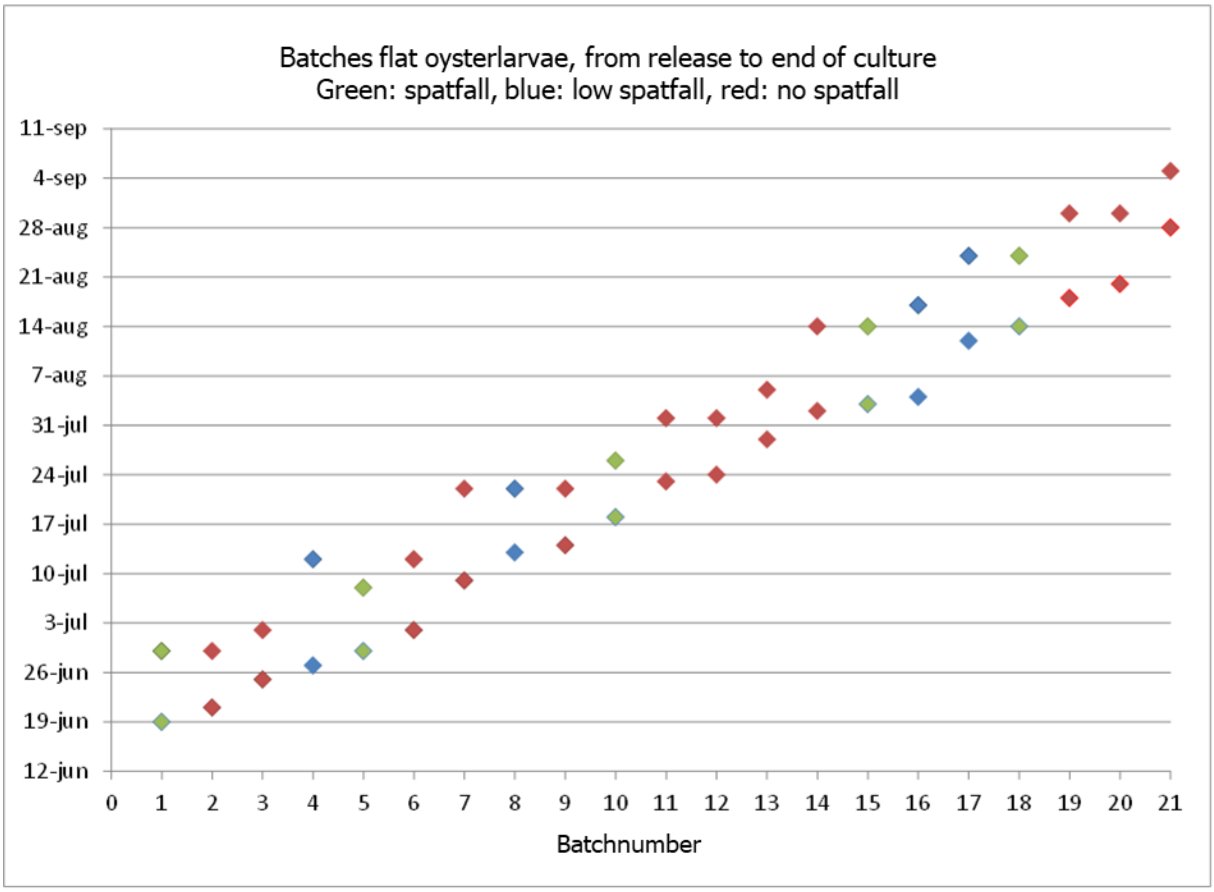


Figure 3. The follow up of larvae batches which were not, a bit or quite successful.

To distinguish between larvae quality, experiments were carried out with a larval and broodstock diet of algae on the oyster larvae, splitting a batch of a few days old by sieving them over 150 and 180 mu, after which they were further cultured (to see if the faster growing larvae have a better chance) and increasing the flow to 18 times the volume per day (from 9th July). None of these treatments showed a clear difference in survival or growth. The graph shows that batches of oyster larvae often occur simultaneously. These groups are housed in the same room, receive the same seawater and algae, and the same degree of refreshment/cleaning/treatment. There are then batches that go well and batches that go worse at the same time, with the observation that it is often the younger batches that go well and the older batches that go worse. This points in the direction of (an (increasing) lack of) food quality. The algae used in 2021 are *Isochrysis* T-strain, *Pavlova lutheri*, *Nannochloropsis oceanica*, *Rhodomonas salina*, *Chaetoceros calcitrans*, *Thalassiosira pseudonana*, *Thalassiosira weisfloggi*, *Skeletonema costatum*, *Skeletonema marinoi*.

The general insight or hypothesis is that the oyster larvae apparently have sufficient nutrition for growth and development in the first week, but that a lack or deficiency occurs after this week. The hypothesis is that the oyster larvae have a need that cannot be obtained from the algae given. The occurrence of a harmful bacterial bloom is considered as unlikely, given the high flow rate (but the bacterial analyses must confirm this).

Competent oyster larvae, with visible eyes and a foot, were transferred to tanks containing substrate in the form of empty oyster shells in onion bags and plastic crates. The volume of empty shells was 0.75 m3. The density of spat turned out 1 to 2 spats per shell.

Photo's 12, 13. Spat on shell tank with 0,75 m^3 oyster shells and spat 2021.

At Roem van Yerseke broodstock was maintained in the same way to 22°C. Here also mortality of adult oysters occurred, but no explanation could be found . Mainly in 2020, the brood stock tanks were cleaned as little as possible because it was thought that stress on the parent stock would have a negative effect. However a more frequent cleaning of the parent animals in combination with a lower feeding and replacement rate increases the quantity of the larvae in 2021. A similar result as shown at the hatchery of Zeeschelp. The larvae yield of this broodstock in 2021 was high, so maintenance protocol looks like an important tool for productivity.

The algae species used for the larval diets are; *Rhodomonas salina, Skeletonema costatum, Isochrysis galbana* (T-ISO), *Pavlova lutheri, Chaetoceros muelleri* and *Chaetoceros calcitrans.* A total of 7 diets were tested. Often a standard diet with an addition after a few days.

The larvae cultures were also quite problematic. Many diets are used, but mortality (assumed) due to presence of *Vibrio* played an important role. The algae which performed growth at the larvae in 2021 were *Isochrysis* T-strain, *Pavlova lutheri, Rhodomonas salina* and *Chaetoceros calcitrans*. Competent larvae were retrieved, after settlement a small amount of spat survived. The use of a probiotic resulted in a longer lifetime of larvae, but so far not in a (higher rate of) spatfall.

For experiments at NIOZ with sensor technology 8 adult individuals were equipped with heart rate sensors and valve gape sensors, before the water temperature was increased to induce spawning. Two cameras were installed above the broodstock tank that took images throughout the spawning period. The aim was to determine if changes in heart rate and valve gape opening can be detected before or during spawning. Although the data is not analysed yet, it is assumed that the heart rate sensors were giving false readings while the valve sensors worked fine and also spawning events on the images taken are captured so that a match of valve gape openings with spawning activities can be investigated.

After conditioning, the broodstock spawned for 14 days at temperatures between 18 and 20°C. Experiments were carried out on effects of different thermal regimes (low "stable" (20-21°C) versus high "fluctuating" (20-24°C), n=3 tanks for each treatment) on survival, growth, settlement, oxygen consumption rates, swimming speed and direction, thermal preference and critical thermal temperature.
Larval survival was low (10%) and development until settlement was 30 days in the fluctuating treatment and 37 days in the stable treatment. Temperature did not affect survival, larval size at life-stage and the critical thermal maximum which was between 27 and 30C for all three life stages (D-shape, umbo, pediveliger).

However, when given the choice of temperatures between 17 and 30°C, all life-stages chose the critical maximum as their preferred temperature. The critical thermal maximum was determined via oxygen consumption rates, swimming speeds and swimming directionality. All three parameters resulted in similar critical thermal maxima. Oxygen consumption rates increased from D-shape to umbo to pediveligers while swimming speeds and directionality of swimming remained similar throughout the larvae development.
In the fluctuating treatment 5.5-fold more larvae settled (20%) compared to the stable treatment (3.5%) In the fluctuating treatment, pediveligers consumed 2.2-fold more oxygen and swam 1.4-fold faster compared to pediveligers in the stable treatment.

Conclusion: The lower oxygen consumption rate of pediveligers experiencing a stable temperature regime was not sufficient enough to supply metabolic demands for the highly energetically costly settlement and metamorphosis leading to lower settlement success in this treatment. Further, higher swimming speeds in pediveligers experiencing fluctuating temperatures during development may be beneficial for larvae to find the most suitable habitat for settlement quicker.

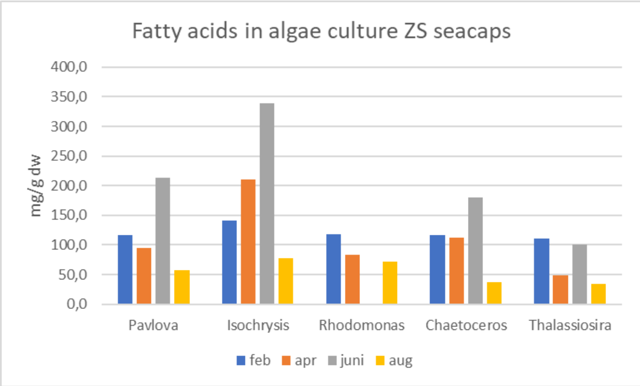
Food quality

The nutritional value of the mentioned algae species is better, compared to other used species. HZ analysed the fatty acid profiles of the 13 species microalgae that are produced at Zeeschelp and 7 species cultured at Roem van Yerseke. The highest fatty acid concentration was observed in *Isochrysis galbana*, *Skeletonema marinoi* and *T-iso lutea*. The highest PUFA concentration was demonstrated by *Isochrysis galbana*, *T-iso lutea* and *Rhodomonas salina*. Due to their high DHA but also low EPA concentration, *Isochrysis galbana* and *T-iso lutea were the only strains that* showed a DHA to EPA ratio higher than 1, which is said to be favourable for larvae development. The highest EPA concentration was observed in *Chaetoceros calcitrans* and *Pavlova lutheri*. *Pavlova lutheri* presented the highest sum of DHA and EPA concentration.

However, the fatty acid content seems to vary over season. At Zeeschelp the algae are grown in Seacaps using natural daylight conditions during May to September, in the period Octobre to April the natural photoperiod is low. Therefore additional TL light is used in order to increase the photoperiod to 24h during these months. At first sight, the photoperiod seems to influence especially the EPA and DHA content.

At Roem van Yerseke, the algae were cultured under continuous TL-light and stable temperature. In general, the fatty acid content was lower compared to Zeeschelp, EPA and DHA were quite comparable. In June the EPA and DHA with continuous light was also lower compared to February, the reason for this difference in a temperature and light controlled culture room is unclear.

In conclusion: the fatty acid content and composition is variable in time and in system. Whether this variability might influence the performance of the larvae culture is unknown. The larvae culture shows serious variability as well, in terms of outcome of spat. Both Seacaps systems show quite stable algae densities over the days and weeks (quantity), but if this is also the case for the fatty acids (quality) is a question for further research.



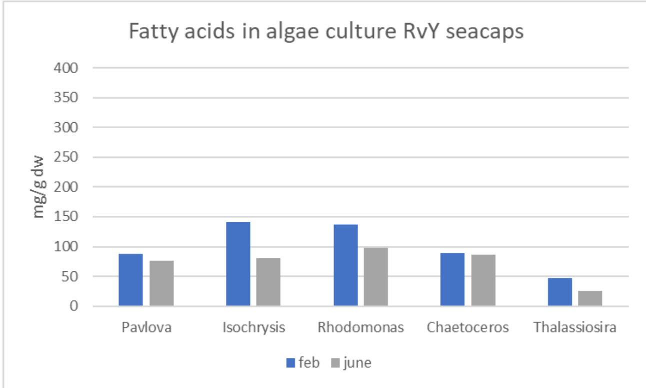
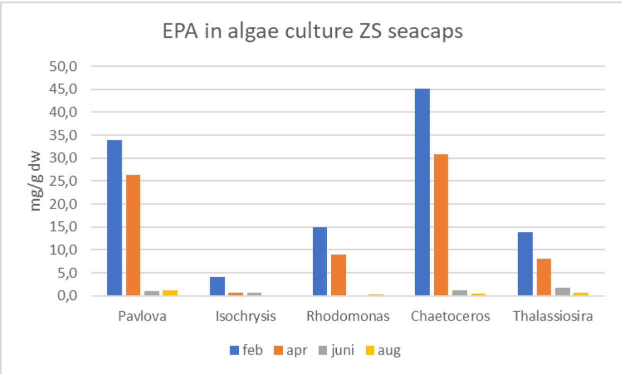


Figure 4 and 5. Total fatty acid content in mg/g dw of several algae species cultured in Seacaps in daylight (Zeeschelp) and TL-light (Roem van Yerseke) over several months in the year.



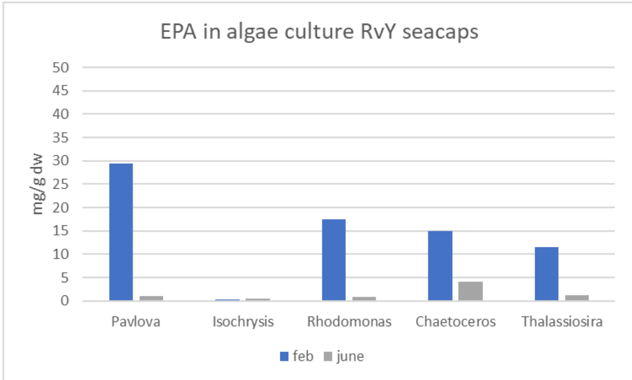
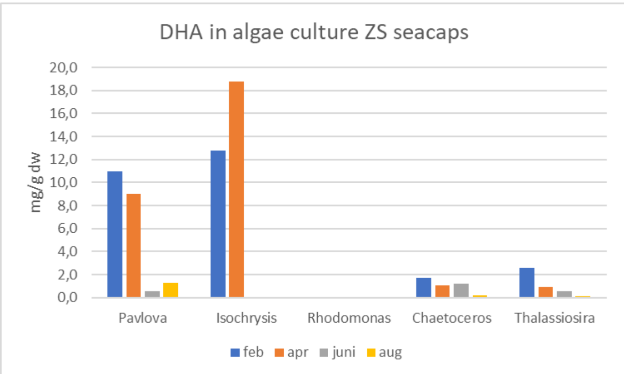


Figure 6 and 7. EPA-content in mg/g dw of several algae species cultured in Seacaps in daylight (Zeeschelp) and TL-light (Roem van Yerseke) over several months in the year.



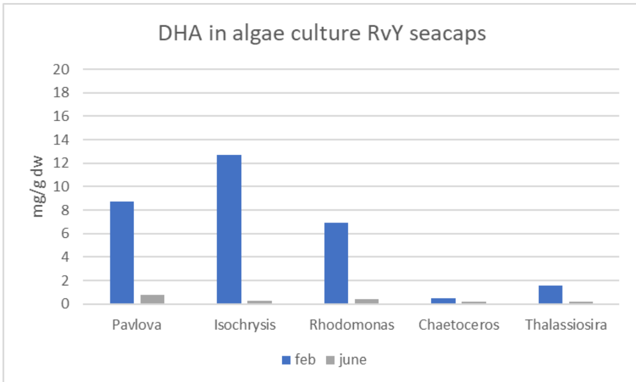


Figure 8 and 9. DHA-content in mg/g dw of several algae species cultured in Seacaps in daylight (Zeeschelp) and TL-light (Roem van Yerseke) over several months in the year.

Bacterial pressure

Larvae samples were collected for the different culture tanks by sieving them. Larvae were collected from the sieve with a plastic Pasteur pipette and transferred into 1.5-2 ml Eppendorf tubes with the minimum amount of water possible. Samples when then stored at -80ºC until DNA extraction took place. DNA was extracted from a total of 73 larvae samples (43 samples Hatchery Stichting Zeeschelp and 30 samples Hatchery Roem van Yerseke). These larvae samples correspond to both larvae batches that made it to settlement and to larvae batches with low survival percentages. Because the bacterial samples have not yet been analysed yet, no conclusion can be drawn yet about the role of pathogenic bacteria in larval mortality.

Bonamia analysis of spat 2021

In April and May 2021 a batch of 182 and 180 flat oysters were tested for the presence of *Bonamia*, 163 and 149 flat oysters were free of this parasite respectively and taken in as broodstock. From both hatcheries (RvY and ZS) spat was produced and showed no presence of *Bonamia.* Again, a broodstock from a Bonamia infected area which was analysed *Bonamia* free by gill sampling, showed no presence of *Bonamia* in the produced spat.

Photo 14, 15. Produced single spat at Zeeschelp for *Bonamia* analysis.

Bonamia protocol

The culture of flat oysters is based on the principles of draft 060421 'Biosecurity protocol for Dutch hatcheries surrounded by *Bonamia*-infected water' by Pauline Kamermans.

This contains guidelines that culture rooms must comply with and how staff must observe hygiene. From April 2021, work has been done according to the guidelines of this draft protocol. In short, compliance with this draft protocol at Zeeschelp comes down to the following:

-The culture rooms are separated from other areas; access is restricted through doors. These culture rooms have their own seawater supply and own equipment (no exchange with materials from other rooms). Ultra-filtered seawater (0.02 µm) is used in all phases of cultivation (brood stock, larvae, brood) to keep *Bonamia* away. The ultrafilter is located outside these areas and the ultra-filtered seawater is brought into the respective cultivation areas via pressure pipes.

-Only clean dry materials are used at the start, because dry materials and seawater do not contain viable *Bonamia* parasites after ultrafiltration or after a standing time for at least 2 days.

-Access is restricted to only those personnel who maintain the culture. There are disinfection baths for footwear at the entrance. Disinfection and cleaning of culture material is done with thin bleach and rinsed with fresh water.

-Only *Bonamia*-free brood stock is brought into these areas.

- With 'spat on shell', shells are used that have been stored on land for at least 12 months.

Roem van Yerseke followed partly the protocol by Kamermans, and performed the hatchery after Zu Ermgassen *et al* (2020). Table 2 below indicates the measures that have been followed by the RvY-hatchery. However, the hatchery does not have ultrafiltration to remove particles down to 0.02 µm from the water. The 'raw' seawater is filtered in the hatchery to particles of at least 1 µm, this water is also continuously passed through a UV filter, which, according to the same concept protocol, leads to a reduction of the *Bonamia* parasite.

Table 2. Levels at which spat contamination with *Bonamia* can occur and recommended measures to minimize the risk. The starting point is the biosecurity protocol of zu Ermgassen et al., (2020).

LEVEL OF TRANSMISSION	MEANS OF TRANSMISSION	ROUTES OF TRANSMISSION
Entry-level	Livestock	e.g. import of wild broodstock.
	Feed/algae	e.g. purchase of algal paste or starter cultures from external suppliers.
	Water	e.g. intake water.
	Equipment	e.g. admission of gear from outside the hatchery.
	People	e.g. entry to the hatchery by staff and visitors.
	Settlement substrates	e.g. transfer of shells.
Internal-level	Livestock	e.g. movement of broodstock, larvae or spat between production areas.
	Feed/algae	e.g. algal cultures.
	Equipment	e.g. sharing of gear between production areas.
	People	e.g. movement of staff between different production areas.
Exit-level	Livestock	e.g. discard of mortalities.
	Water	e.g. discard of water.
	Equipment	e.g. disposal of wastes.
	People	e.g. exit of the hatchery by visitors.

Outplacement of spat in the Voordelta

At the beginning of January 2021, spat on shell originating from august 2020 was placed at the Brouwersdam, within the permit area of ARK Rewilding NL. Both on the open bottom (enclosed by crates, see photo) and in closed crates. A reef cube with spat has also been installed. 3 months later the setup was checked and still intact.

Photo's 16,17. Experimental setup and several shell types with spat after deployment in the Voordelta.

The shells with spat have become scattered over the bottom, due to wave actions and currents. The shells in the crates, on the other hand, are intact. There is little loss (15%), the spat showed clear growth margins.

Scallops and oyster shells show less dispersal due to weight and shape of the shells as substrate. The mussel shells begin to disintegrate and scatter with the spat in the environment. The reef cube is still in place, with oyster spats on it.

Larger oyster spat (approx. 20-25 mm) seems to survive better than smaller oyster spat (approx. 5-10 mm).

Conclusions and recommendations 2021

- Larvae cultures showed good growth and survival in the first week, after which growth decreased and losses started to occur. The cause seemed to be both food quality and the presence of harmful bacteria, however these parameters are different over the hatchery locations.
-Various batches of larvae eventually yielded oyster spat, as 'single spat' and as 'spat on shell'. It remains difficult to predict in advance the spawning success and thus the amount of spat on shell to be delivered. This is directly related to the success of the larva culture and remains the bottleneck in the breeding process for the time being.
-Several species of algae seem to provide a better growth to the oyster larvae. These species are analysed relatively high in PUFA, DHA and EPA, which confirms the general experience with culturing shellfish larvae
- There is a difference in spat success between 'single spat' and 'spat on shell'.
-The *Bonamia* protocol concept seems to work well and can be integrated in the hatchery-nursery culture. Still application to a *Bonamia* free certifying procedure is not available.
-After outplacement of spat out of 2020 in the Voordelta in January 2021, there is little loss (15%) and the spat showed clear growth margins in March. Larger oyster spat (approx. 20-25 mm) seems to survive better than smaller oyster spat (approx. 5-10 mm).

-The 'spat on shell' and an amount of 'single spat' produced in 2021 was delivered on November 24th 2021 for restocking in the Voordelta.

Recommendations for topics in 2022 are:
- spring-summer-autumn monitoring of released spat in the Voordelta
- Retrieve spat in 2022 for *Bonamia* analysis
- larval diet on fatty acid composition
- try spatfall initiators (epi-gaba) for increase in metamorphosis success
- try probiotics for lowering bacterial pressure in larval cultures

Summary of results in 2022

In 2022 the results of the experiments in the hatcheries went different ways.

RvY experienced a high mortality of newly taken in broodstock and effects of *Vibrio* infection in the larvae culture. After harvesting adult flat oysters, anesthetising and sampling gill material for *Bonamia* analyses a small to sometimes serious number of mortalities occur in the broodstock in the weeks thereafter. Symptoms of *Vibrio* were soon seen in the larvae by the overactive vibrating of the velum. After this, growth slowed down or stopped completely and mortality quickly occurred. With the use of a probiotic, the lifespan could be slightly extended, but not fully completed until settling. Unfortunately no spat was produced and research on recommended topics not possible.

At Zeeschelp the culture has been started with a reconditioned and joined broodstock of flat oysters from 2020 and 2021. Both stocks have been analysed free of *Bonamia* and *Bonamia*-free oyster spat has already been produced in 2020 and 2021.

From the beginning of May, the brood stock of flat oysters has been gradually warmed up to 24 degrees. About 4 weeks after the temperature was set to 24 degrees, the first oyster larvae were released. In the period from 29 May to 11 August, eleven batches of larvae were cultured with a total of 22 million larvae.

The algae used in 2022 are *Isochrysis* T-strain, *Pavlova lutheri, Nannochloropsis oceanica, Rhodomonas salina, Chaetoceros neogracile, Thalassiosira weisfloggi, Skeletonema costatum, Skeletonema marinoi*.

The batches of 29th and 30th of May and 1st of June were not successful, after a week a dropout started to occur. This was the same phenomenon as in 2021. After these batches, monitoring for algae, refreshment, food supply and larvae has been increased, as has the breeding temperature from 22 to 24-25 degrees. From June 4, the larval cultures became more successful, with the percentages of competent larvae increasing, see also the table below.

Table 3. Cultured batches of flat oyster larvae, number and % competent.

Date release	10^6 larvae T0	% competent
4-jun	0,8	38
11-jun	0,2	20
18-jun	0,2	2
17-jun	2,7	8
20-jul	6,0	27
26-jul	4,4	49
1-aug	3,2	34
11-aug	4,2	70

The growth of the larvae was good in general, with an average of 11 µm per day. The period until a competent larva (with visible eyes and feet, searching behaviour) was 8 to 14 days, see also the graph below. Over the cultures from June 4th, 31% of the larvae reached the competent stage, which is quite decent.

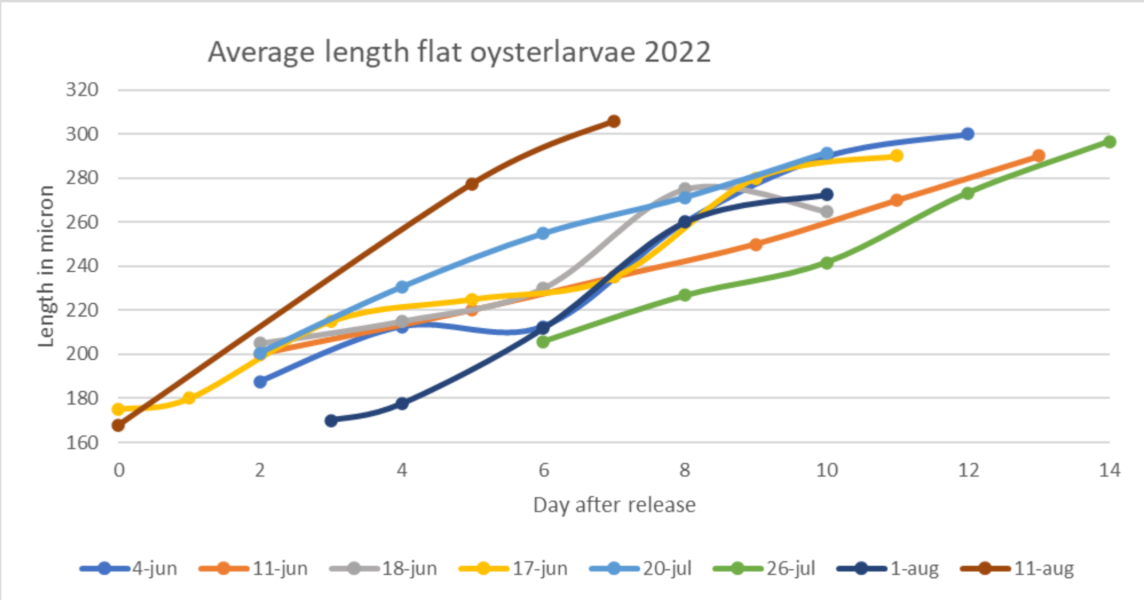


Figure 10. Average lengths during the various larval cultures.

The batch of July 20th (6.0 million larvae) was split into 2 groups, each with 3 replicas, in collaboration with NIOZ-Texel. A group at 25-26 degrees and a group at 29 degrees. The development of the larvae was monitored, as well as the success of spatfall. The growth data is shown in the chart below.

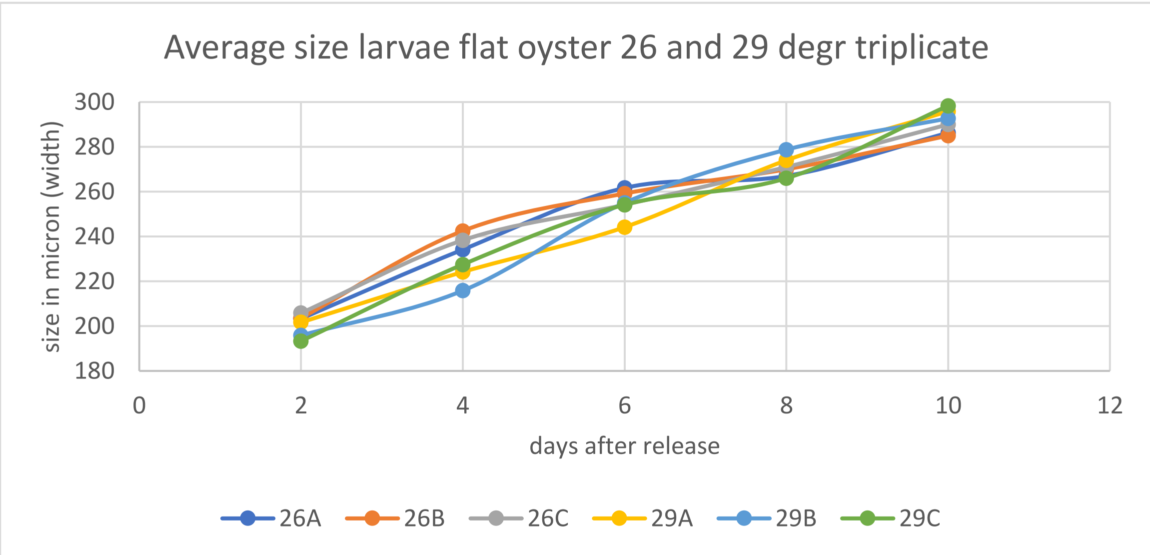


Figure 11. Average lengths during temperature experiment.

The graph shows that the growth of the larvae in the groups and between the replicates was almost identical. Meaning no difference for practical cultivation. This temperature difference apparently does not result in a difference in growth. The percentages of competent larvae between temperatures seem to fall within a normal range and are also equivalent on average, see table below.

Table 4. Percentage of competent larvae at two temperatures and 3 replicates.

		average-%
Batch 20 juli 6,0*10^6 larvae		27
26C		36%
26B		21%
26A		28%
29C		30%
29B		21%
29A		26%

On average, 35% of the competent larvae from this experiment reached the spat stage (single spat 2 weeks after spatfall), with a range of 32 to 39%. These values are comparable to other single spat cultures (see also Table 3.)

Further results of this collaboration between Zeeschelp and NIOZ are published in Aquaculture, see Alter et al (2022).

Batches of competent larvae from the culture of 17th and 20th June were used for spat on shell. A total of 2.0 m^3 oyster shells were used for 1.75 million competent larvae. The oyster shells are stacked in plastic crates in tanks of 2 m^3 each. The tanks are aerated, with a flow of 1 time the volume per day. In October, this material was transferred to the Voordelta and counts were made of the number of spat on oyster shells.
More than 2 months after spatfall, the average number of spat per oyster shell was 6.5. The total number of spat on 2 m^3 oyster shells is estimated at over 163.000.

In addition to spat on shell, single spat has also been prepared by housing competent larvae in so-called down-wellers with a layer of lime powder. Larvae prefer a chalky substrate, so they prefer to attach themselves to the lime scales and not to the mesh or wall. Just like last year, there is a clear difference in hatching success between single spat and spat on shell. Where this was not yet clearly quantified last year, it has been recorded in 2022. This is shown in the table below.

Table 5. Averages of larvae rearing and spawning on various substrates.

		average-%
To competent larvae		31
(all cultures 2022)		
Competent larvae to spat		
Spat on shell		12
Single spat		39
Average larvae->spat 2022		9

On average, 12% of competent larvae become spat in a tank with empty oyster shells (spat on shell), while 39% of the same batches settle in down-wellers (single spat). The cause of this clear difference is not known, but the systems are clearly different in volume, water movement and renewal. It is suspected that different conditions arise in the 2m^3 tank with approximately 0.67 m^3 empty oyster shells than in the down-well of 0.02 m^3.

As mentioned, the larvae came from the same batches, flow through of the same water and algae. Perhaps the oyster shells, which are not always spotlessly clean, cause different conditions in terms of water movement, food supply or hygiene. In any case, a clear difference occurs repeatedly and reason enough to take this into account in future spatting.

The production of single spat was 1.92 million (2 weeks after hatching).

A total of 2.09 million spats have been raised from more than 22 million released flat oyster larvae. In 2022, an average of 9% of the larvae released from the flat oysters have become spat.

Photo's 18, 19, 20, 21. Single spat produced at Zeeschelp in summer 2021 grown till end of 2022.

These latest results show the work carried out at the Zeeschelp for various other projects in 2022. In this way, an overall summary can be given of the progress in the cultivation of the flat oyster, in which the insights and experiences gained are expressed. Also in this way, the partners involved benefit from knowledge sharing for predictable cultivation, so that larger quantities can be placed at sea in the long term.

Besides the ongoing production of spat on shell, trials were carried out with single spat. In 2022 a co-operation started between Zeeschelp, Meromar Seafoods and Klink International Seafoods, both based in Yerseke. In autumn 10.000 single spats were delivered and placed in baskets on culture plots by the farmers.

Food quality

Samples of algae were taken at Zeeschelp and Roem van Yerseke, but due to technical problems during storage, samples were lost. New samples will be taken in 2023.

Bacterial pressure

Because the bacterial samples have not yet been analysed with an appropriate method, no conclusion can be drawn yet about the role of pathogenic bacteria in larval mortality.

Bonamia analysis of spat 2022

In 2022 the produced spat was kept in the ZS-hatchery for several months, *Bonamia* analysis was done in 2023. At the hatchery of RvY, no spat was produced.

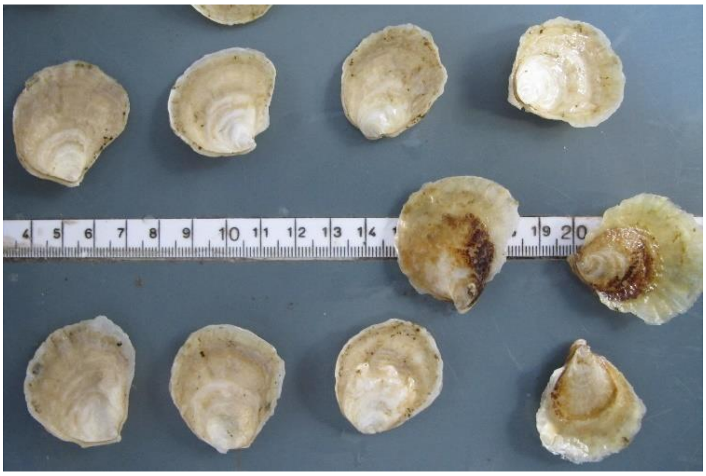


Photo 22. In august 2022 produced single spat, raised until January 2023 in the hatchery for *Bonamia* analysis.

Bonamia protocol

Followed as in 2021. In practice, no progress was made in the procedure towards certification. We invited ministry LNV and NVWA to start visiting the hatchery of Zeeschelp and take samples of produced spat for *Bonamia* analysis. The invitation was not accepted, because there was no active policy for certification yet. Therefore *Bonamia* analysis was done by WMR again.

Outplacement of spat in the Voordelta

In august 2022 the experimental structure of 2021 was gone, likely due to three storms (in February 2022: Dudley, Eunice, and Franklin). Remaining shells of scallop and oyster were present on the bottom, with small flat oysters still attached. The juvenile oysters grew from average 2,6 cm wide in March 2021 to 5,5 cm wide in August 2022. These juveniles were scattered over the plot in time.

The reef cube was still present and an Edible crab (*Cancer pagurus*) was present inside. There were no flat oysters visible on the outside of the reef cube anymore.

In August and September, a new pilot with spat on shell from 2022 started. This research aimed to gain insight into factors that underly the outplacement success of spat from the nursery into the natural environment. Earlier conducted experiments (2022) showed that flat oyster released as "spat on shell" was a suitable method of introducing spat in the Dutch Voordelta. In order to further improve outplacement success, we need to gain insight into how factors, such as methodology, timing and optimal age influence survival and growth of spat in the field.
Firstly, the age of the spat at release might impact subsequent survival and growth in nature, with a potential trade-off between a size-advantage and loss of adaptation to natural conditions. Additionally, a longer hatchery time yields increased production costs and thus reduces cost-efficiency. Secondly, timing the moment of outplacement might be essential, as environmental conditions and predator abundance in the habitat vary temporally. Lastly, the mesh size of the cage effects local conditions, through sedimentation, water exchange, food availability and predator ingression.

By conducting a field experiment in the Voordelta, we aim to explore these elements in natural environment, comparing survival and growth between three different age classes, two release moments and two different cage types. To determine if keeping spat longer in the hatchery before release provides a fitness advantage (survival or growth), we used three age classes: 1-month, 2-month or 3-month-old spat at moment of release. The effect of timing and outplacement structure of spat in open seawater was tested by outplacement flat oyster spat in August and September, in either cylinder-shaped oyster baskets or square-shaped crates. These results will contribute to better understanding of best practices for future spat outplacement efforts.

Photo 23. Square-shaped grey crates with lid and cylinder-shaped oyster black baskets.

<u>In summary the main research questions for this field experiment were:</u>

1. What is the effect of increased hatchery time on survival and growth of spat?
2. What is the effect of outplacement in August versus September on survival and growth of spat?
3. What is the effect of the outplacement structure on survival and growth of spat?

Method Outplacement

For this experiment spat on shell from Stichting Zeeschelp was used. To obtain the different age-classes, two batches were started with a one-month interval (batch one: started end June and batch two started end of July. Outplacement of this spat occurred either in August (1- and 2-month-old spat) or September (2- and 3-month-old spat) (table 1). Approximately 200 flat oyster individuals were put in each cage, exact amount of spat and shell material per cage was recorded. From the two-month and tree-month old batches a t-zero size measurement was conducted to have size data at the moment of outplacement.
Oyster baskets and crates were attached to each other in a square, 3 cages each side, to fix to the sea bed. Cages were fixed to the seabed using rebar staples. The research location is at the Voordelta Brouwersdam Reef at 3-meter depth. In total 24 cages were outplaced in two squares. First monitoring was planned for march 2023.

Conclusions and recommendations 2022

- Larval cultures have become stable in the second half of the period and repeatedly show substantial spat production.
- The breeding success can be expressed in average terms as 31% of the released larvae reach the competent stage, 12% of these become spat on shell in tanks with empty shells and 39% become single spat in down-wellers.
- There is again a clear difference in spatfall success between 'single spat' and 'spat on shell', the cause is still unknown.
- There is still no clear relation between algae species nutrition (fatty acids) and larvae success. Nutrition analyses show variable results for the same species in time.
- With the current culture technique and *Bonamia* protocol spat of flat oysters is produced in 2020-2021-2022. All batches were analysed free of *Bonamia*, so certification of this procedure is possible, as soon as legislation is in operative mode.

Recommendations for 2023 are:
- Monitoring spat in Voordelta.
- *Bonamia* protocol in actual progress to operation.
- Further larvae culture experiments to confirm findings of 2022 (new budget or projects should be found).

Summary of results in the first half of 2023

Larvae culture and spat production

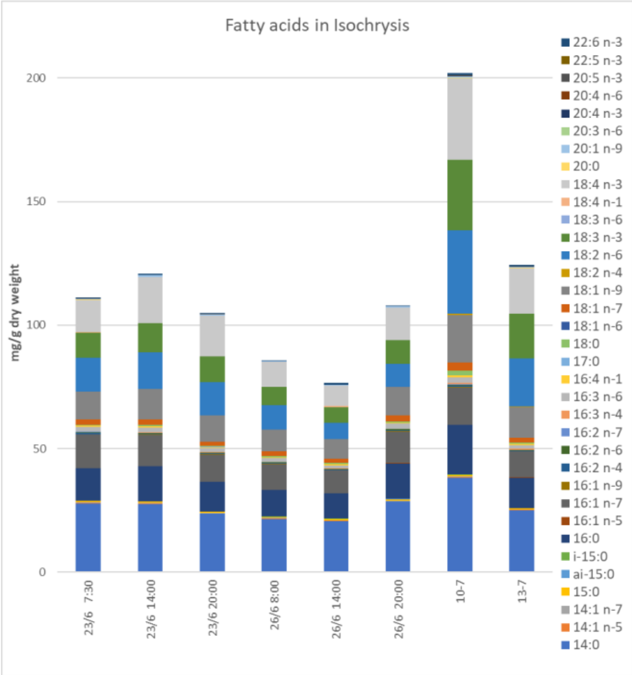
For 2023 RvY changed the whole water supply with another setup; filtration till 0,2 µm, UV and different temperatures. RvY started the culture with a new broodstock and received larvae in the beginning of June. Larvae were released from 3 different broodstock treatments (paste diet and 2 different algae species mixes).
The larvae were growing well and first results from different experiments in stocking density, water renewal and cleaning became visible. From multiple batches of larvae, single spat is produced and growing. It seems that RvY has tackled the most critical problems from the past in the larvae culture, so repeated and successful larvae culture has become operational in 2023.

At Zeeschelp the flat oyster reproduction started in April for other ongoing projects, both with restoration of reef biodiversity as the goal. The reproduction of flat oysters showed the same patterns as in the second half of 2022, and spat of flat oysters is now produced in a predictable way. Planning is to produce flat oyster spat in an ongoing way, with follow up of several conditioned broodstocks. The next step of scaling up is getting in process, but dependant on real time demand for spat for restoration (or farming) purposes and getting the status of a certified production site free of *Bonamia*.

Food quality

In 2021 a fluctuating content of total fatty acids was present in the seacaps system (ZS) with natural daylight conditions. In the seacaps system with 24h artificial light only, the total fatty acid content was more stable, but lower. In both systems, EPA and DHA were considerably lower in June compared to February. In 2023 we were curious how the fatty acids content evolved over the course of a day due to natural light conditions, while these algae were fed to larvae culture. Therefore samples were taken in the morning, afternoon and evening in the daylight seacaps system (ZS) and also in the artificial light only seacaps system (RvY). In principle, these systems are technically the same, only difference is daylight compared to artificial light. Furthermore the concentration of fatty acids of the microalgae was monitored over the course of several days in order to monitor reference variation.

The variation of fatty acid content during the day seems species specific and there is no general pattern. Also between the different days there is considerable difference in fatty acid content. Continuous artificial light shows more stable fatty acid concentrations, however between days there is also difference for *Isochrysis* and *Rhodomonas*.



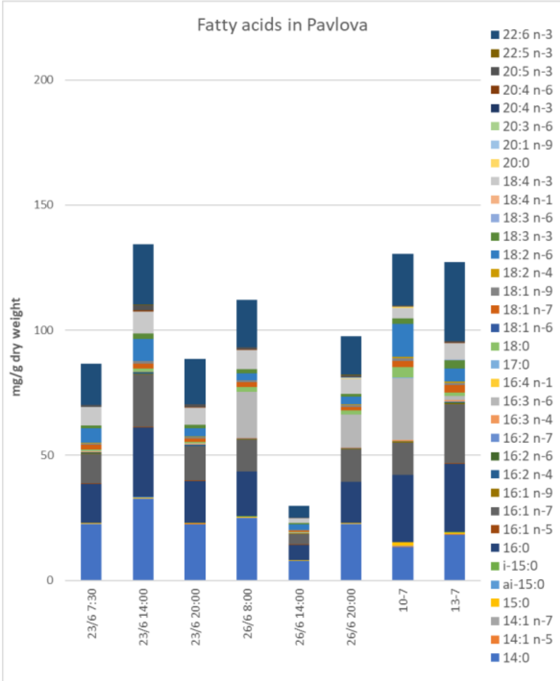
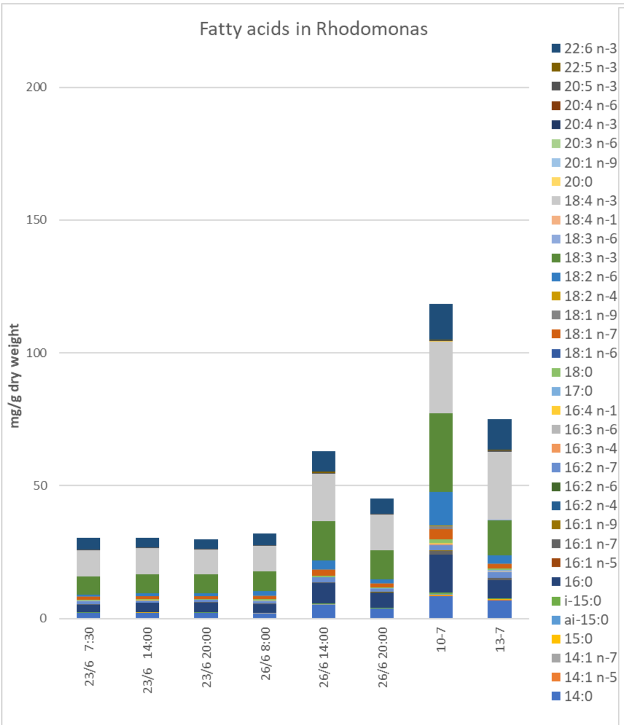


Figure 12 and 13. Total fatty acids in algae. At 23 and 26th June, samples in morning, afternoon, evening on the same day and 3 days later in daylight seacaps, at 10 and 11th July samples from artificial light only.



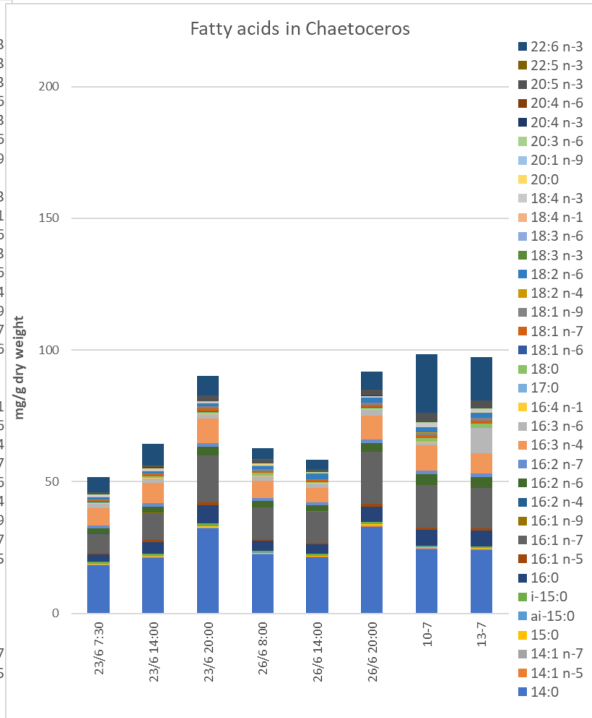
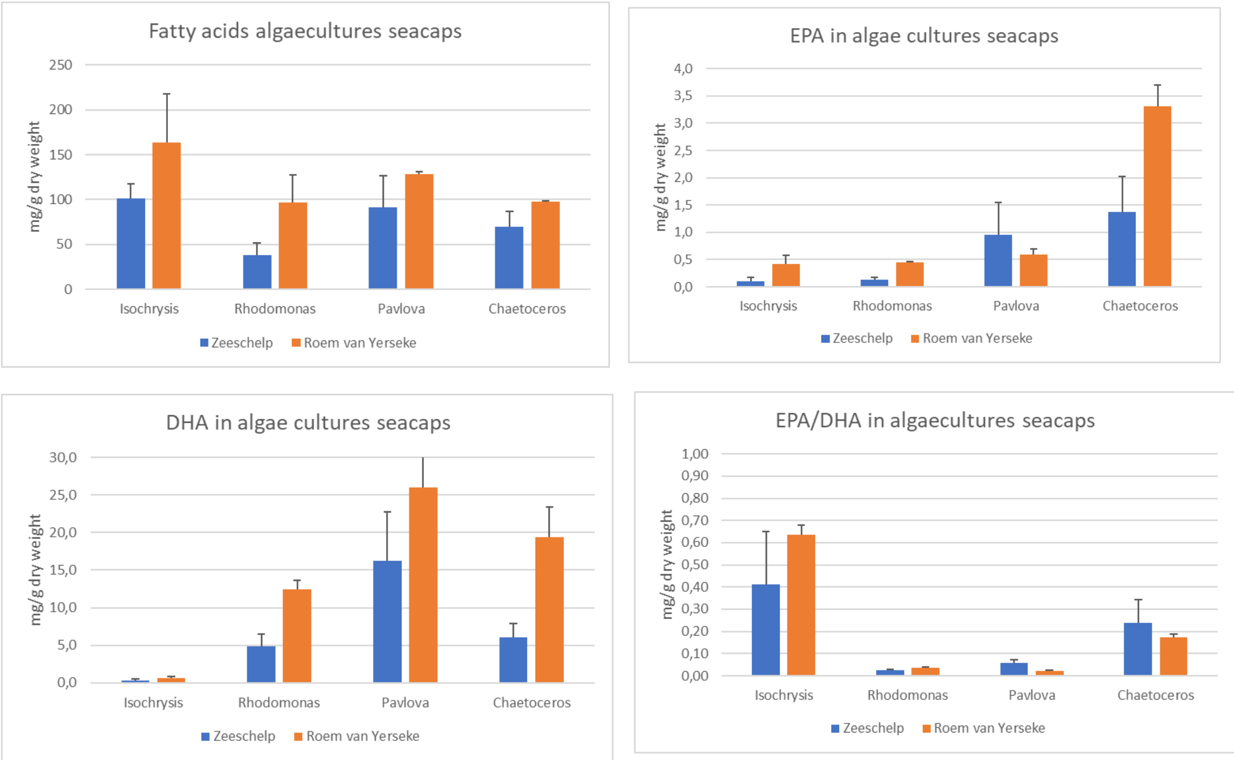


Figure 14 and 15. Total fatty acids in algae. At 23 and 26th June, samples in morning, afternoon, evening on the same day and 3 days later in daylight seacaps, at 10 and 11th July samples from artificial light only.

Although there is a different number of samples, if the averages are taken from each system then they show that the daylight system has lower fatty acid concentrations then the continuous artificial light system. The relation with light source seems present for all algae species and also for the type of fatty acid in the samples of 2023, while in 2021 this relation was the other way round.



Figures 16, 17, 18 and 19. Average fatty acid content in seacaps with daylight only (ZS) and artificial light only (RvY)

Outplaced single spat

In May 2023 the single spat delivered by Zeeschelp for farming purposes in Grevelingen in autumn 2022 was grown very well in the protected way of culture in baskets and bags, see photo's.

Photo's 24, 25, 26, 27. Spat by Zeeschelp out of 2022 in May '23 by oyster farmers in lake Grevelingen.

Also first trials are performed to induce spatfall with oyster collectors, as possible source material for oyster farmers. The trials with oyster collectors are carried out in summer-autumn 2023. The plastic collectors showed a reasonable spatfall success, but with the presence of empty shells in the same tank the larvae showed a clear preference for the last. Further experiments will be carried out to establish a good spatfall on these collectors.

It will be a good opportunity for the hatchery to supply oyster farmers with young spat, because collectors can leave the hatchery after a few weeks, making a high turnover in delivery moments possible.

Single spat produced in 2023 reached a good size in the nursery in September 2023, and Zeeschelp, Meromar Zeeland and an oyster farmer in lake Veere are preparing to start up a flat oyster culture on the bottom in the west part of the lake. So the culture of spat or juveniles for farming oysters is starting up and ongoing.
The advantage of single spat or collectors is that the spat can leave the hatchery after a few weeks and will grow out to suitable size in outside water with natural food availability. This nursery stage can be used till the desirable size is reached for further use. This procedure is also particularly of interest when single spat or single oysters will be used for biodiversity projects, creating oyster populations or reefs on the seabed.

Photo's 28, 29. Single spat 2023 out of hatchery Zeeschelp as juveniles after nursery ready for bottom culture and on oyster collectors as spat. Both as a source for farming or establishing an oyster reef on the seabed.

Bonamia analysis of spat 2023

Spat produced in august 2022 was kept in the hatchery-nursery of ZS for several months upon sampling in January 2023. The spat was analysed as single spat and 150 analysis showed no infection with *Bonamia*. This period of five months shows the robustness and reliability of the hatchery-nursery system because it normally takes one or two months when the spat is placed out in open seawater. Once again, and now for the third time disease free spat was produced from out of a disease free broodstock from a *Bonamia* infected area.

Spat produced at Zeeschelp in June 2023 from broodstock of 2023 was raised till half July. WMR took samples and the Veterinary Institute analysed for presence of *Bonamia*. Result: all 150 spats were free of *Bonamia*. For the fourth year in row Zeeschelp produced *Bonamia* free spat from a broodstock originating from a *Bonamia* infected area (Oosterschelde-Grevelingen).

Outplacement of spat on shell in the Voordelta

The experimental plot stayed in place, despite winter storms of 2022/23. The rebar pins (12mm thick, 100 cm long, 25c m wide) showed to be an easy method for fixing the cages to the seabed by divers. Shells with spat spread over the bottom showed spat of 2022 still present. Also some scallop shells with spat from 2020-2021 were still present with flat oyster juveniles alive.

In March 2023 monitoring of the spat of 2022 showed a good survival in crates of about 50-70% and also growth. The experimental setup was still good anchored and biofouling was acceptable, so water exchange was still good.

A subsample of four crates (one from each batch and age) was retrieved in March 2023 to obtain intermediate results (table 6). The remaining samples were retrieved in June 2023. Of each sample the total amount of shell material and total live spat was recorded. Length and width of a subset of at least 50 individuals per cage were measured with digital calliper (mm, two decimals). Measured spat too small (< mean T-zero) was removed from data. This ratio was used to correct survival in the cage.

Table 6. Overview of experimental set-up; start date of spat culture in hatchery, the date of outplacement in the Voordelta, the date of retrieval, the number of cages per structure and subsample size.

	Batch start	Out-placement 2022	Time in hatchery (months)	Retrieval date	Crates	Oyster-baskets	Sub-sample March '23
Batch 1	End June 22	August 23rd	2	26-06-23	3	3	1 basket
	End June 22	September 13th	3	26-06-23	3	3	1 basket
Batch 2	End July 22	August 23rd	1	26-06-23	3	3	1 basket
	End July 22	September 13th	2	26-06-23	3	3	1 basket

To determine effect time in hatchery and method on size (length) stepwise model selection was used. Full model comprised of a mixed linear model with method and time in nursery as covariates. A unique cage number was included as random intercept in the model to correct for nested structure of the data. The significance of the covariables "method" and "time in hatchery" were assessed via model selection based with likelihood ratio testing. Model assumptions were inspected visually.
The effect of cage type on survival was assessed with a weighted mixed binomial model. Corresponding groups (batch-time in hatchery combination) were included as random factor in the model. This ensures that the crate versus basket is assessed within the same treatment groups. Other factors (month in hatchery and month of release) were assessed visually, as

data indicated strong interactions between these factors, but not all levels exist in the data to incorporate these in a model.
The effect of outplacing in August versus September on size and survival was analysed by mixed linear modelling. This could only be done in the 2-month hatchery samples. The remaining data could only be explored visually, as data is rank deficient for statistical analysis.

Growth
Intermediate results in March indicated that the one-month hatchery group (outplaced in September) was smaller than the other groups (figure 20, table 7). In June, however, oysters from all treatment groups fell within a narrow range (28.3-32.6mm, (table 6)) and no significant effect of either method (X^2 (1, N = 1204) = 0.00, p = 0.93), nor time in hatchery (X^2 (2, N = 1204) = 2.47, p = 0.29) on size was found (figure 6). Thus, considering the almost equal final size of the spat, an increased hatchery time does not provide a growth advantage after a year in the field.

Table 7. Size of spat at the tree moments of monitoring; outplacement (T-zero), intermediate (March) and final (June).

		Length (mm ± SD)		
		September 2022 (T-zero)	March 2023	June 2023
1-month hatchery	August	-	11.7 ± 6.1	28.3 ± 8.2
2-month hatchery	August	10.7 ± 3.8	21.7 ± 6.4	32.6 ± 7.9
2-month hatchery	September	10.7 ± 3.8	18.2 ± 7.1	28.7 ± 8.2
3-month hatchery	September	18.7 ± 7.5	20.2 ± 8.0	31.1 ± 8.6

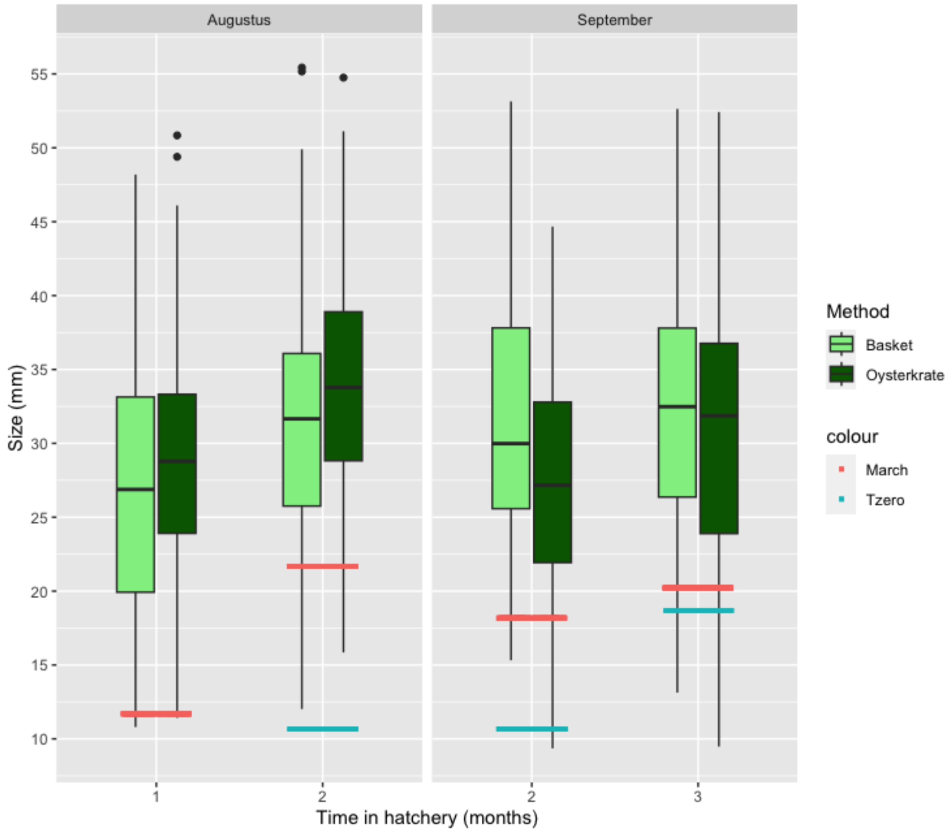


Figure 20. Size of spat at final measurement (June 2023) per hatchery time, method and month of outplacement. Blue lines indicate size at T-zero (moment of outplacement). Red lines indicate size at intermediate measurement in March.

We did find a small negative (estimator: -7.5mm, 95% C.I. -4.26 to -1.02), yet significant effect (X^2 (1, N = 613) = 6.61, p = 0.01) of outplacement in September compared to August on final size within two-month-old spat. Indicating that earlier outplacement in August provides better growing conditions. However, this is under the assumption that batch 1 and batch 2 are of identical quality, and the observed difference is therefore not caused by to variation in batch quality.
Above comparison was only possible within for two-month-old spat, as the other two age categories were only released in either August (1-month old) or September (3-month-old).

Survival
Survival of all treatment groups (crates, baskets and age) in March was high, with a minimum survival rate of 51% and maximum of 68% (table 3). Highest survival was recorded in the September outplaced batches, with no clear age distinction (2 versus 3-month-old spat) (table 7). The good survival of spat until March of the 1-month hatchery group indicates that later observed higher mortality cannot be attributed to a batch quality effect.

Table 8. Survival of spat in March (intermediate results) and in June 2023.

		Survival (%)		
		March 2023	June 2023	
		Crate	Crate	Oyster basket
1-month hatchery	August	51.2	31.8 ± 7.6	26.7 ± 9.6
2-month hatchery	August	53.5	62.5 ± 11.3	47.1 ± 8.7
2-month hatchery	September	66.3	33.7 ± 1.5	28.3 ± 6.7
3-month hatchery	September	68.0	37.5 ± 6.4	38.8 ± 6.2

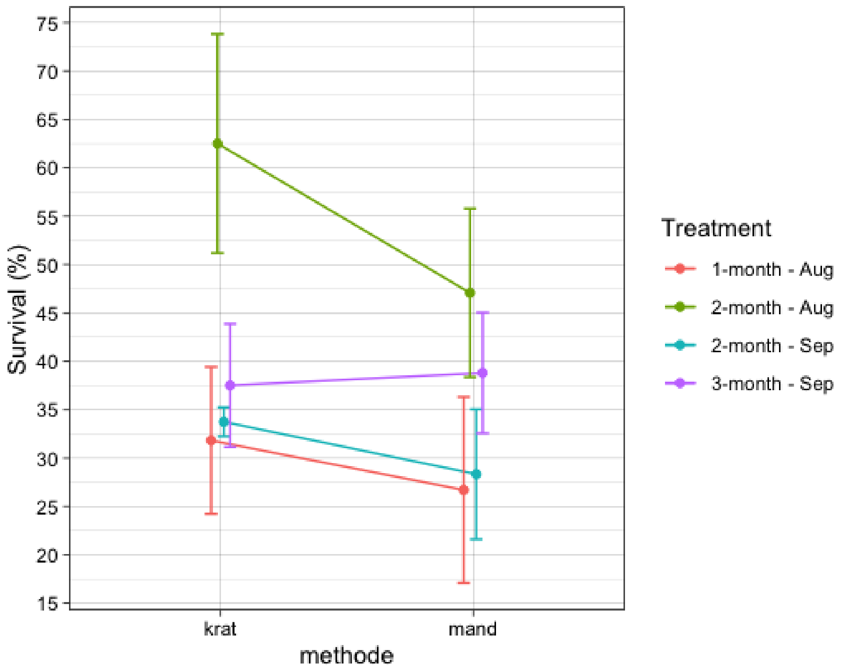


Figure 21. Survival of spat in June 2023 per time in hatchery and month of release.

Over the period March to June mortality in all groups occurred, except in the 2-month-old August outplaced oysters (table 8). This group has the overall highest survival (above 60%), and survival decreased when these oysters were kept a month longer in the hatchery and outplaced in September (figure 21). This would suggest either increased hatchery time above 2-months decreases survival in the field, or September was worse for outplacement. This effect of timing did not occur so strongly in the 1-month august versus the 2-month September group. However, our recorded survival rates result from the interplay of multiple simultaneous factors, therefore individual effects for each factor cannot be disentangled.

In general, the crates showed better survival then the oyster basket. We suspect due to slightly larger mesh size (crate 1,5 x 1,5cm square holes in the wall and 2 x 2 cm lid; oyster basket, 1,5 x 1,5 x1,5 cm hexagon). Overall, the survival of spat on shell in this experiment was good and on average 34% in oyster baskets and 43% in crates over the year.

We did find that oysters in crates performed slightly better in survival than in the oyster baskets (X^2 (1, N = 1204) = 15.61, $p < 0.01$), see figure 20 and table 7. This could also be associated with the coarser mesh size of the crates, which ensures better water flow and thereby abiotic conditions withing the cage.

The reef cube placed in 2021 with many spat on shell on it was checked for flat oysters. On the inside of cube one living flat oyster was found, besides a number of empty flat oyster shells.

Overall, these results indicate that outplacement in August of an intermediate spat age is optimal to ensure maximal survival, preferably with a mesh size of the housing as large as possible, compared to outplacement in September.

***Bonamia* protocol**

Followed as in 2021-2022. As in previous years, in 2023 still no progress in practice has been achieved for certification, since there is no policy active. However upscaling is in progress, dependence on demand. For deployment at sea, as a *Bonamia* unknown area, from out of a Dutch hatchery, there are serious limitations without any degree of certification, even a permit for short term does not seem feasible.

Conclusions and recommendations 2023

- Predictable production of *Bonamia* free oyster spat continued in 2023 (other projects), scaling up is possible. For this, the hatchery-nursery technique needs continuity over longer time, so availability and demand should meet or be prepared to each other.
- Monitoring of outplaced spat on shell shows reasonable growth and survival after one and two years. 2 months old spat at outplacement showed highest survival, outplacement early in season probably enhances growth (and survival in winter)
- Single spat cultured in nursery to juveniles is usable for farming, and also for creating an oysterbed on the sea floor for biodiversity goals.

There is one clear recommendation to be made for further progress and development of providing young flat oysters for projects at sea: get a procedure for permit or certification operational for deploying *Bonamia* free spat of flat oysters.

Overview results 2020-2023

Bonamia analysis and production of *Bonamia*-free seed

The non-destructive screening of broodstock oysters identified 8-32% of the tested oysters as *Bonamia* infected (Table 9). The oysters that tested *Bonamia* negative were used to produce larvae and seed. All larval and seed batches were *Bonamia* free.

Table 9. Overview of number of broodstock oysters screened at Wageningen Marine Research; number of oysters without *Bonamia* infection used in hatcheries (RvY = Roem van Yerseke and ZS = Zeeschelp); negative *Bonamia* results for larval batches released and seed produced by these pre-screened broodstock oysters. Number of pools for pooled samples is presented in parenthesis.

Screening date Broodstock	Total # oysters	# Neg	Sampling date larvae/spat	# Larvae batches	# Neg	# Seed	# Neg	Hatchery
			June - Aug 2018	9	9	(2)	(2)	RvY
30 April 2018	149	137	28 July 2019	12	12	(3)	(3)	RvY
7 October 2019	200	136	-	-	-	-	-	RvY
11 May 2020	145	105	June & July 2020	9	9	240 (56)	(56)	ZS
12 April 2021	182	163	13 July 2021	-	-	145 (27)	(27)	RvY
			August 2021	-	-	150 (30)	(30)	ZS
17 May 2021	180	149	31 January 2023	-	-	150	150	ZS
25 April and 2 May 2022	200	99	-	-	-	-	-	RvY
13 March 2023	227	162						ZS
1 May 2023	200	103						RvY
5 June 2023	205	125				150	150	ZS

At the hatchery of Roem van Yerseke, 20 broodstock oysters died in 2020. These were kept for later analysis. Eight were positive for *Bonamia*. By which cause there was a *Bonamia* presence in a screened oyster is not clear. It can be that the screening method is not 100% reliable or that infection took place during conditioning, and therefore the (UV) water treatment was not sufficient. *Bonamia* infection is going via the water phase, so water treatment can be a cause. This underlines the importance of continuous monitoring of both broodstock and seed. Dead broodstock of Zeeschelp was also collected for analysis, no *Bonamia* was found. Here an ultrafilter was used.

The results showed that the non-destructive screening method provides information on the status of the oyster with regard to *Bonamia* infection. In addition, *Bonamia* negative broodstock always produced *Bonamia* negative larvae and seed. De Melo et al (2021) produced disease free seed of Pacific oysters (*Crassostrea gigas*) through after spawn testing of broodstock. Unlike *C. gigas*, *O. edulis* cannot be strip spawned. Therefore, a non-destructive method was developed. This provides opportunities for *Bonamia*-free hatchery production with broodstock from a *Bonamia*-infected area. A prerequisite is that production takes place in a *Bonamia*-free zone of the hatchery. With the installation of (ultra)filtration this can be secured. In addition, regular monitoring needs to be carried out to confirm the disease-free status and to become certified as a *Bonamia* free production site.

Producing *Bonamia*-free oysters with broodstock originating from a *Bonamia*-infected area provides disease-free oysters that may also have developed tolerance to the disease

(Kamermans et al. 2023). This is very useful for restoration projects since transfer of diseases is not wanted, but protection against disease is desired, in case it does show up in a newly established bed.

Hatchery performance

New broodstock was taken in several times by both hatcheries. After *Bonamia* analysis some mortality occurred, probably due to gill sampling and stress. Mortality ranged from a few to 40-60 % in a batch in the first two weeks. The method of *Bonamia* analysis by sampling the gills can have a serious and acute mortality of broodstock in the beginning of conditioning. In general, only a few oysters per month may die, once acclimatised. During conditioning, it was experienced that lowering food availability also lowered mortality, although the food availability was in optimal range as suggested in literature. This might be due to quality of algae culture or just too high cell numbers, since pseudo faeces were also present. In practice of this project, to cover the loss of (occasional) mortality, it was necessary to update the broodstock with new numbers of oysters every year or every other year.

All broodstocks gave larvae, in enough numbers to start larvae cultures. Conditioning to 22-24 degrees will last in larvae releases up to at least 8-10 weeks. Quality of the larvae was sufficient in general, occasionally good. In 2020-2021 the larvae started to grow in the first days of culture, mortality started after a few days and growth declined as well. Some batches were lost completely, some batches produced small numbers of competent larvae and a small number of batches yielded in spat. Reason(s) of the poor performance were not clear. It could be something they suffer (*Vibrio*), but also could it be that a (unknown) component in nutrition was missing. These hypothesises are still not validated, but in time the larvae culture at Zeeschelp became more stable in 2022. The hatchery of Roem van Yerseke had still visible *Vibrio* effects in the larvae culture. Table 9 shows that the hatchery of Roem van Yerseke produced spat in 2019 and 2021, the hatchery of Zeeschelp produced spat in 2020-2021-20222-2023. All batches of spat were free of *Bonamia*. Also NIOZ produced larvae and spat in small amount from for research in 2021flat oysters out of the Waddenzee, however not analysed for Bonamia. The spat performance in the hatchery of Roem van Yerseke was complicated by *Vibrio*-infections, as seen by behavioural symptoms of the larvae. At Zeeschelp, no *Vibrio*-effects were seen on the larvae (however *Vibrio* was present) and the number of spat increased over the period 2020-2023 from 0,03-0,02-2-12 million respectively as combination of spat on shell, single spat and spat on substrate (in 2023).

Spatfall was used in various experiments and various substrates, resulting in various success of spatfall. Meaning that a lot of competent larvae were used in trials, to see how they behave or settle. In fact, given the choice, they prefer chalk or limed or stone like substrates but in tanks with only one substrate available, flat oyster larvae will settle on almost every substrate (plastic crates, collectors, PE, PP, silicone, glass). Only direct metal surface is avoided.

In years, the hatchery performance for flat oysters increased bit by bit and showed in the end a stable and also predictable production of *Bonamia* free spat, used in experiments in open seawater. In 2023 it was possible to order spat on shell or single spat which was also delivered conform planning. Nevertheless, the reproduction of flat oysters in a hatchery stays demanding, needs full focus every day of the week. Compared to Olympics, it’s like a marathon, combined with estafette and steeple chase. But doable nonetheless and in time it will become more routine.

Algae nutrition

The fatty acid analysis on algae show considerable variability in fatty acid content between species, but also in time over the day, between days, over seasons and light source. Where in 2021 the seacaps with daylight showed higher total fatty acid content compared to continuous TL-light, in 2023 the analysis showed the opposite while no structural changes were made to the algae systems. The cause of these fluctuations is still not clear. Fatty acid content is known to be related to cultivation temperature, nutrient concentration, actual photoperiod and light intensity, but these data are not monitored specifically.
The performance of the larvae culture does not seem to be related strongly to this variability of fatty acid content, because the algae system at Zeeschelp has not changed between 2021 and 2023, while the larvae started performing much better in 2023. Therefore indicating that the used algae species (mainly *Isochrysis, Pavlova, Chaetoceros*) are suitable for flat oysters and other factors are also important for larvae culture.

Once spat, there has been no major factor for problems, indicating that the supplied algae are nutritious enough to support growth. For maturation and larvae development, algae quality seems more critical. However with our experience and collected data, we are not able (yet) to explain the critical parameter for the right algae quality in terms of fatty acids or way of culture. This will be a recommendation for further research on the seacaps system.

Bacterial load

Bacterial samples from 2021 were analysed in 2023 and could therefore not be interpreted sooner or used for adjustment in the way of culture. Analyses of the bacteria community composition of larvae samples for both hatcheries revealed a high diverse species composition of the samples. The results presented in figure 22 and 23 show only the Vibrio species found (as most pathogenic species for oyster larvae have been described for this genus).
As shown in the figures, there are a high number of Vibrio-species present among all samples of larvae and in both hatcheries. Unlike different culture systems and -water, there are always a (high) number of *Vibrio*-species present.

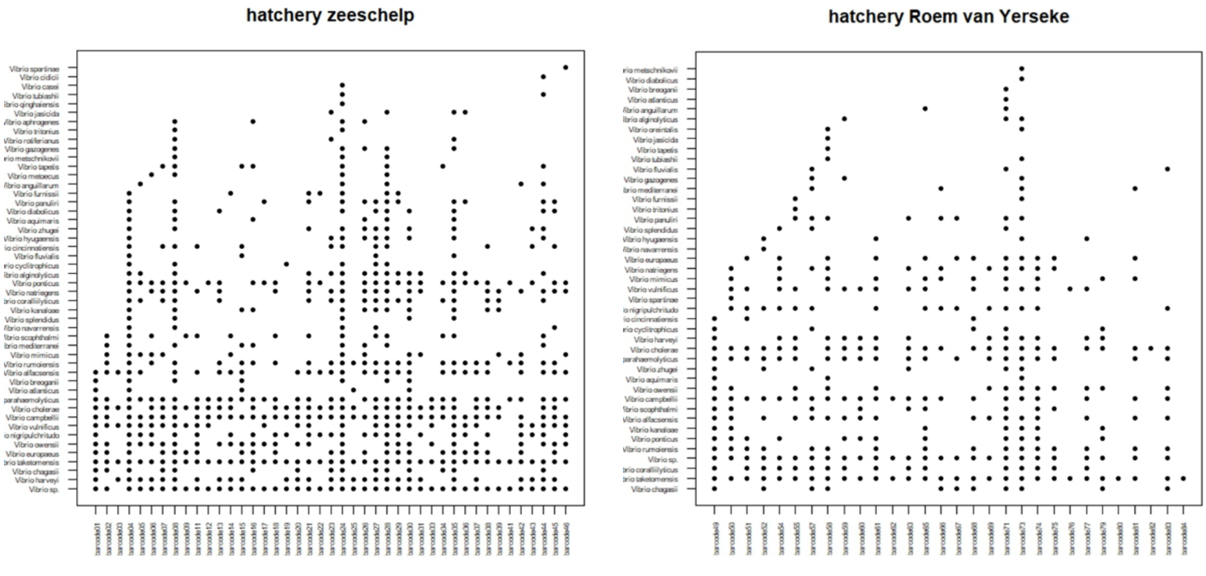


Fig. 22, 23. Bacteria analyses results (vertical) for a selected number of larvae batches from both hatcheries (horizontal). *Vibrio* 'all-over'.

Vibrio species which have been previously associated with disease of bivalve molluscs during early stages of development were found in samples for both hatcheries. The species found were: *V. alginolyticus, V. splendidus, V. kanaloae, V. coralliilyticus* and *V.europaeus*.
In addition, *V. tapetis* is a species associated with mortality in adult shellfish (pers. comm. Marc Engelsma). And *V. angularium*, *V. vulnificus* and *V. harvey* cause mortality in fish (pers. comm. Marc Engelsma).

Table 10. Larvae samples code presented on figure 22 (Zeeschelp). Batch date corresponds to the release date of the larvae and the result column shows whether larvae settled or died during the larval stages.

	Batch date	Result
Barcode01	29 june 2021	Spat outside tanks
Barcode02	29 june 2021	Spat outside tanks
Barcode03	29 june 2021	Spat outside tanks
Barcode04	2 july 2021	Mortality
Barcode05	2 july 2021	Mortality
Barcode06	2 july 2021	Mortality
Barcode07	2 july 2021	Mortality
Barcode08	2 july 2021	Mortality
Barcode09	18 july 2021	Spat inside tanks
Barcode11	18 july 2021	Spat inside tanks
Barcode12	18 july 2021	Spat inside tanks
Barcode13	18 july 2021	Spat inside tanks
Barcode 21	2 august 2021	Mortality
Barcode 23	2 august 2021	Mortality
Barcode 24	2 august 2021	Mortality
Barcode 33	12 august 2021	Mortality; larvae 300 um
Barcode 34	12 august 2021	Mortality; larvae 300 um
Barcode 35	12 august 2021	Mortality; larvae 300 um
Barcode 36	12 august 2021	Mortality; larvae 300 um
Barcode 41	14 august 2021	Spat
Barcode 42	14 august 2021	Spat
Barcode 43	14 august 2021	Spat
Barcode 44	14 august 2021	Spat

Fig. 24. Bacteria analyses results for a selected amount of larvae batches from the Hatchery Zeeschelp. Good batches that resulted in spat (blue) and bad batches that suffered mass mortality (red) are indicated as well as pathogenic *Vibrio* species for shellfish (pink) and fish (green).

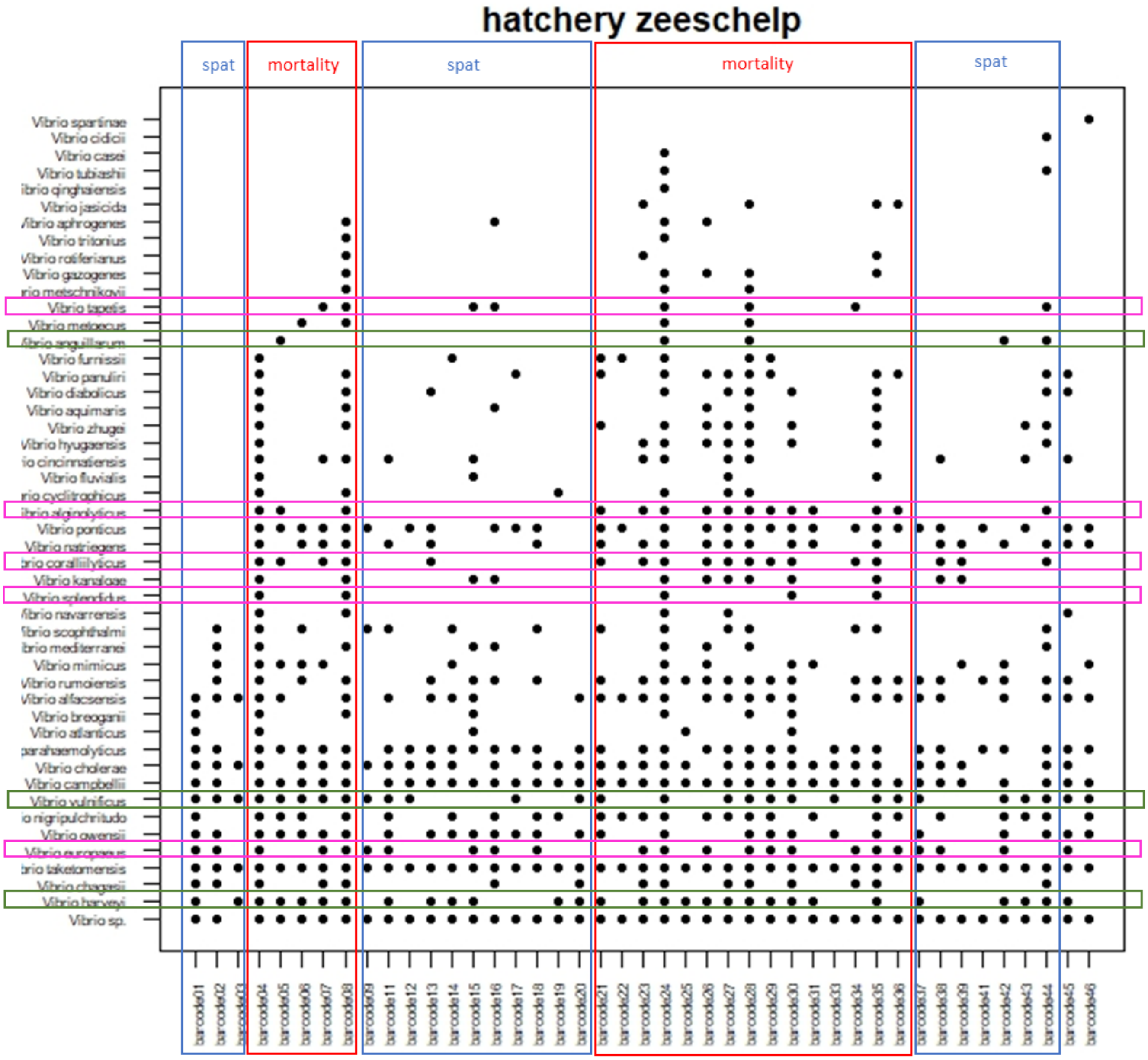
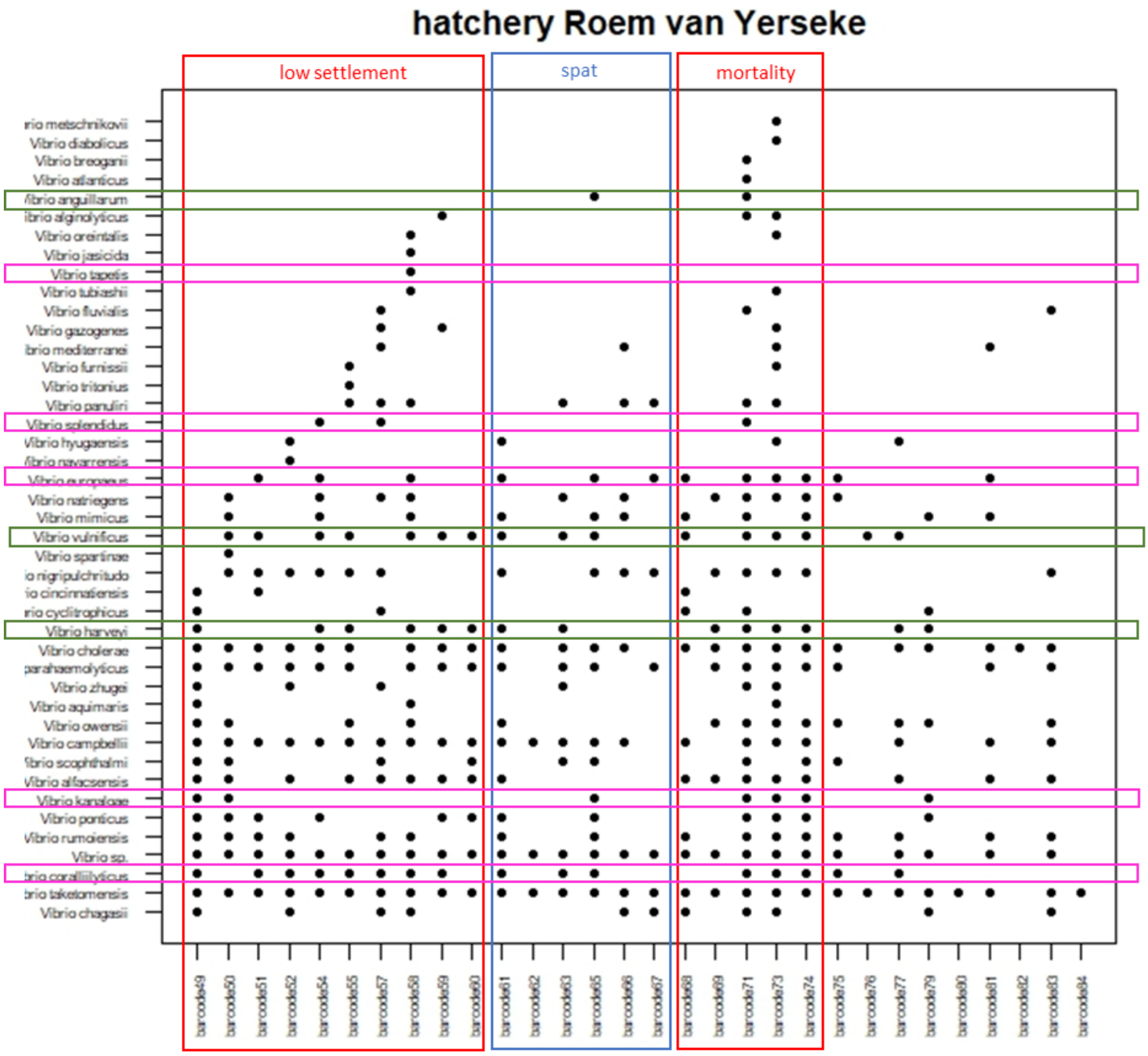


Table 11. Larvae samples code presented on Figure 23 (RvY). The result column shows whether larvae settled or died during the larval stages.

	Batch date	Result
Barcode49	Oe21BS6 (diet 3)	Low settlement_Long period larvae stage
Barcode50	Oe21BS6 (diet 3)	Low settlement_Long period larvae stage
Barcode51	Oe21BS6 (diet 3)	Low settlement_Long period larvae stage
Barcode52	Oe21BS6 (diet 3)	Low settlement_Long period larvae stage
Barcode54	Oe21BS6 (diet 3)	Low settlement_Long period larvae stage
Barcode55	Oe21BS6 (diet 3)	Low settlement_Long period larvae stage
Barcode57	Oe21BS6 (diet 3)	Low settlement_Long period larvae stage
Barcode58	Oe21BS6 (diet 3)	Low settlement_Long period larvae stage
Barcode59	Oe21BS6 (diet 3)	Low settlement_Long period larvae stage
Barcode60	Oe21BS6 (diet 3)	Low settlement_Long period larvae
Barcode61	Oe21BS10 (diet 1)	Spat
Barcode62	Oe21BS10 (diet 1)	Spat
Barcode 63	Oe21BS10 (diet 1)	Spat
Barcode 64	Oe21BS10 (diet 1)	Spat
Barcode 65	Oe21BS10 (diet 1)	Spat
Barcode 66	Oe21BS10 (diet 1)	Spat
Barcode 67	Oe21BS10 (diet 1)	Spat
Barcode 68	Oe26BS5B (diet 7)	Mortality
Barcode 69	Oe26BS5B (diet 7)	Mortality
Barcode 70	Oe26BS5B (diet 7)	Mortality
Barcode 71	Oe26BS5B (diet 7)	Mortality
Barcode 72	Oe26BS5B (diet 7)	Mortality
Barcode 73	Oe26BS5B (diet 7)	Mortality
Barcode 74	Oe26BS5B (diet 7)	Mortality

Fig. 25. Bacteria analyses results for a selected amount of larvae batches from the Hatchery Roem van Yerseke. Good batches that resulted in spat (blue) and bad batches that suffered mass mortality (red) are indicated as well as pathogenic *Vibrio* species for shellfish (pink) and fish (green).



Tables 10 and 11 present a description of part of the codes presented in the figures 24 and 25. Each barcode corresponds to one larvae sample. All species found are also present in seawater (pers. comm. Marc Engelsma). In general, more species (47) were identified in the samples from Zeeschelp than in the samples from Roem van Yerseke (42).
All species associated with disease were encountered in the samples. *V. alginolyticus V. coralliilyticus, V. kanaloae, V. europaeus* and also the fish disease species *V. angularium, V. vulnificus and V. harvey* were found in good and bad batches of larvae, making it difficult to establish whether pathogenic Vibrio's are the primary causative agent of mass mortalities of larvae batches or secondary opportunistic colonizers or commensals (Dubert et al., 2017).
The only species that was exclusively found in bad batches was *V. splendidus*. Monitoring for this species could be an early warning system for determination of the quality of the batch. However, the monitoring needs to be strain specific, as only certain strains of *V. spendidus* are pathogenic (pers. comm. Marc Engelsma).

Outplacement of spat in open seawater
At the beginning of January 2021, spat on shell originating from august 2020 was placed in the Voordelta, outside the Brouwersdam. 3 months later the shells with spat had become scattered over the bottom, due to wave actions and currents. Scallop and oyster shells showed less dispersal due to weight and shape of these shells as substrate. The mussel shells began to disintegrate and were scattered with the spat in the environment. The reef cube was still in place, with oyster spats on it.

There was little loss of spat with the shells in the crates (15%), the spat showed clear growth margins. Larger oyster spat (approx. 20-25 mm) seemed to survive better than smaller oyster spat (approx. 5-10 mm).

In august 2022 remaining shells of scallop and oyster were still present on the bottom, with small flat oysters still attached. The juvenile oysters grew from average 2,6 cm wide in March 2021 to 5,5 cm wide in August 2022. These juveniles became scattered over the plot in time. Using larger shells (e.g. oyster of scallop) for spat on shell produces more stable substrate therefor limiting scattering. Using 12 mm rebar U shape pins (100 x 25 cm) proofed to be a simple and effective method for fixing the cages to the sandy seafloor.

Artificial substrate 'the Reefcube' was stable and stayed on top of the sediment. It gave housing to e.g. edible crab, shrimps and sponges. However it showed little survival of flat oysters, after 2 years only one flat oyster survived inside the structure.

In March 2023 a monitoring to the outplaced spat of 2022 showed a good survival in crates of averaged 57% and also growth. These results indicated also that the one-month hatchery group (outplaced in September) was smaller than the other groups, which were older or one month earlier outplaced.
In June 2023, however, oysters from all treatment groups fell within a narrow range (28-33 mm). Still there was a small effect of outplacement in September compared to August on final size within two-month-old spat. Indicating that earlier outplacement in August provides better growing conditions.
But, considering the almost equal final size of the spat, an increased hatchery time does not provide a growth advantage after a year in the field, this seems more related to food availability during the season. The average size of spat was 13 mm in September, 20 mm in March and 31 mm in June, which also supports the effect of seasonal food availability (and temperature).

Over the period March to June mortality in all groups occurred, where average survival in March was 57%, in June this was 43% in crates and 34% in baskets. Oysters in crates performed a bit better than the oyster in baskets. This could be associated with the coarser mesh size of the crates, which ensures better water flow and thereby better conditions for the spat.

The good survival of spat until March of the 1-month hatchery group indicates that later observed higher mortality cannot be attributed to a batch quality effect. From observations by diving, probable causes of mortality are sedimentation of lower layers of spat on shell on the bottom and predation by crabs and sea stars which occur in the cages in time.

Bonamia protocol

The concept *Bonamia* protocol (Kamermans 2021, Zu Ermgassen 2020) is followed since 2021 by both hatcheries in their daily practice, as described in the chapter over 2021.

The Breeding Line project showed that the procedures in the hatchery resulted in 3 years in row of *Bonamia* free flat oyster spat production. For other ongoing projects in 2023, already 4 years of *Bonamia* free production of spat is established.

For outplacement in near and offshore locations, a certified or permitted supply of *Bonamia* free oyster spat is urgently needed. As soon as the Dutch authorities activate their policy, the certification procedure can take place and oyster spat can be supplied in a formal way in projects for reef biodiversity in open sea.

Answering research questions

At start the research questions were:

-Which factors determine the success of conditioning the brood stock (period, temperature, origin of the brood stock, food quality/quantity, bacterial pressure)?

The answer is that broodstock of Dutch origin can be conditioned well to the moment that it releases larvae, the quality of the released larvae is quite stable. A gradual increase in temperature to 22-24 degrees at a diet of several diatoms and flagellates gives larvae production over about 10-12 weeks The bacterial pressure on broodstock and released larvae is present and can cause a bad start for the larvae culture. The algae diet can be a source of bacterial pressure, but seems nutritional sufficient. Focus on 'clean' algae cultures and hygiene in broodstock seems to lower the overall bacterial pressure in the culture systems.

-Which factors determine the growth and survival of the produced larvae (conditioning brood stock, origin stock, food quality/quantity, bacterial pressure)?

The answer is that broodstock originating from Grevelingen and Oosterschelde performs well, enough numbers of released larvae are produced. After harvesting adult flat oysters, anesthetising and sampling gill material for *Bonamia* analyses a small to sometimes serious number of mortalities occur in the broodstock in the weeks thereafter, which can affect the release and condition of larvae. The first week of larvae culture goes well in general, with growth and development. But the second half of the larvae culture is critical to bacterial pressure (*Vibrio*) and nutrition. In general one third of the larvae becomes competent to settle and one quart of this becomes spat, depending on condition of the larvae and substrates to settle on.

-Which factors determine the growth and survival of the brood (brood stock conditioning, origin, food quality/quantity, bacterial pressure)?

The answer is that the condition of the larvae seems to be the most critical issue for success in metamorphosis to spat, most related in this seems bacterial load and nutritional value of the diet for broodstock and larvae. Once spat and in good hygiene, the survival is good. In weeks after spatfall the size of several mm is achieved and in months it can grow to several cm in size.

General conclusions

The general conclusions are:

1. A predictable and reliable production method for *Bonamia*-free flat oyster larvae and young oysters ('spat') has been achieved.
   - Broodstock can be held for several years and releases enough quantities of larvae in suitable condition.
   - Better insight into good growth conditions for oyster larvae and spat have been achieved.
   - Bacterial pressure can still be a critical parameter throughout the culture process And should be maintained as low as possible.
   - The method towards the production of *Bonamia*-free oyster larvae and spat shows reliable results over 4 years of production.

2. A cost-efficient production of larvae and spat including a start for upscaling is possible to achieve in near future.
   - Lowering mortality during production has been the most important hurdle to get to a reliable production of spat. In 2023 the first scaling up shows more efficiency over the whole culture process.
   - In addition, important objectives stay: minimizing production costs, especially the important items of food, labour and space.
   - Continuity in the hatchery-nursery technique is essential to keep up with the upscaling process. It's a gradual process which has to grow in time. Therefore the real-time demand for oyster spat should be there as well.

3. Providing suitable material for (large-scale) introduction for nature restoration is possible.
   - For a suitable male-female combination, the broodstock can be a dozen to a 'dozen-dozen' in number, depending on the amount of larvae needed.
   - Competent larvae in good condition will settle on almost all substrates in the hatchery tanks: empty shells of various species, baked clay, lime, side of tanks, plankton mesh and silicon tubes. They favour chalky or limed substance, but if not available they will use anything in the water phase to settle on.
   - Once spat, it's mostly a matter of enough food (algae) to grow them to a size for outplacement. Outplacement is technically possible at a size of a few mm, ecologically it looks like a size of 10 to 25 mm is preferred for better survival.
   - The start and implementation of a certification procedure of disease-free material with the authorities and further operationalisation of the necessary protocol is highly recommended. At the moment there is spat available conform protocol to be put out in the North Sea, free of *Bonamia*, but not available as certified material.
   - First trials with single spat and oyster collectors with the purpose of oyster farming are promising. A cooperation with oyster farmers has resulted in a first good survival and growth of spat from 2022 and 2023.

4. Co-operative approach is valuable.
   The expectation of open co-operation and knowledge sharing between a small group of project members has turned out to be efficient and valuable. Periodic

meetings with all project partners involved, discussing ongoing matter helps to understand the role and needs of each other. Ongoing insight and adaptation to this made important steps possible to achieve a quite stable culture of flat oyster spat in the end. This has been achieved due to constructive cooperation between NGOs, companies, foundations and research institutes.

Lessons learned

The expectation of open cooperation and knowledge sharing between a small group of project members has turned out to be efficient and valuable.

From the beginning we have started to work from an interactive working group perspective, with for everyone a moment for ongoing matter with good and bad things happened. In response, comments by others could be given, trying to get improvement in culture or make decisions with best info possible. These meetings have been periodically, every 6 to 8 weeks, to keep up with everyone's progress and to focus on the things to do in the next months. Also it was important to keep track of costs, when work packages change. After each year, results were evaluated and a new plan with ongoing insights for the next year was made.

Important issue was that the shared information stayed confidential within the group, otherwise scientists were not able to share their experiences and results in concept before publication for example.

In this way, all partners thought that best results could be found, by using everyone's knowledge and experience for the common goal of the project: predictable flat oyster spat.

Periodic meetings with all project partners involved, discussing ongoing matter helps to understand the role and needs of each other. Ongoing insight and adaptation to this made important steps possible to achieve a quite stable culture of flat oyster spat in the end.

Some items in this project showed serious delay in analysis or progress, like the bacterial and algae analyses, as the policy and process for certification as a *Bonamia* free production location. The reasons for delay are various and not always within the capacity of the project. A stricter planning and tuning will help to get these results available more early in the project so the results of analyses could therefore not be used at the moment of cultivation. The issue of certification was a remaining uncertainty during the four years of the project, as it still is. It was a time-consuming issue in the discussions.

In the end, the initial role of each partner evolved in sometimes a completely different way, but in need for the ongoing results in the project. This sometimes a bit chaotic and puzzling way has been most efficient for result and budget spreading in the end.

Recommendations/obligations for near future

Recommendation 1: Connect supply and demand of flat oyster spat for good planning

The hatchery-nursery with the culture of algae, quarantined broodstock and larvae-spat culture needs continuous attention and labour and therefore serious (background) costs over the year. This is only feasible when there is a serious demand for outplacement of flat oyster spat. Also for planning of delivery of spat it is needed that demand is clear because it can take up to one year on forehand for preparation to meet production and demand.

Recommendation 2: The use of *Bonamia* tolerant spat will secure survival once a clean area becomes infected compared to spat out of a *Bonamia* free area, where there will be low(er) tolerance for this parasite.

At the moment there are no hatcheries-nurseries that can provide high numbers of *Bonamia*-free spat. The Dutch hatcheries are able to scale up with delivery of *Bonamia* free spat, originating from broodstock out of a *Bonamia* infected area. It is expected that this type of spat has tolerance for this parasite and will be able to face infection without a lot of loss.

Recommendation 3: Scaling up flat oyster spat production needs development and attention and will take time before it will be fully operational.

Efficiency in scaling up will be achieved by growing demand and therefore cost efficiency will come in time. Scaling up techniques need to get operational as spat on substrate requires a much bigger volume than larvae or single spat. This will increase the water treatment and -volume for ongoing *Bonamia* free production. These facilities need to be built or organised. Also algae culture should be able to supply big volumes of seawater. When tanks outside buildings are used, special attention to prevent *Bonamia* is also needed.

Recommendation 4: Settle substrate for flat oyster spat for offshore use should be tested in practice.

Spat on shell is a good way in sheltered areas, where it will remain in the same area and can be traced for monitoring. However, in future there might be a shortage in the availability of empty shells. More exposed locations in open sea (currents, wave actions) will probably need more robust substrates such as artificial reef structures, stones or other more rigid structures, which will remain in place A method should be found for spatfall on these substrates, or connect single spat to them later. Single spat can be produced more efficiently than spat on shell, which requires much more tank volume. Therefore implementation of substrates for off-shore use from hatchery on is recommended.

Recommendation 5: Implement a certified hatchery-nursery procedure for offshore outplacement of flat oyster spat

Regulations are under way for the supply of certified *Bonamia*-free flat oyster spat, for outplacement in open sea where there is no *Bonamia* present (yet) or the status is unknown. In the Netherlands these regulations are not active yet, and therefore certification is not possible. For ongoing improvement of hatchery-nursery-outplacement this procedure should be put in practice as soon as possible. There is already a continued production of *Bonamia* free oyster spat for 4 years and our suggestion is to combine the implementation of certification with ongoing production and outplacement at sea. Meaning that following the protocols ensures *Bonamia* free spat, which can be used directly in project, with a yearly permit to do so till certification is achieved. Otherwise a great gap of 3 years will appear before any of the earlier recommendations can be put in practice.

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