Deliverable No. 1.2

Project acronym:

AquaVitae

Project title:

New species, processes and products contributing to increased production and improved sustainability in emerging low trophic, and existing low and high trophic aquaculture value chains in the Atlantic

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[1] PU: Public, PP: Restricted to other programme participants (including the Commission Services), RE: Restricted to a group specified by the consortium (including the Commission Services), CO: Confidential, only for members of the consortium (including the Commission Services)
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Deliverable D1.2

Report on first development phase in case studies WP1

11/03/2021

Executive Summary

The objectives of AquaVitae's WP1 is to develop hatchery and seedling production of low trophic species (LTS) which includes hatchery/seedling innovation and exploitation activities in the specific CSs linked to WP1. This and previous deliverable (D1.1) aim to establish and address barriers to production in the AquaVitae project area, along with the development of new and novel hatchery protocols for LTS production across the five value chains incorporating 7 out of 13 case studies:

- CS1 Macroalgae, new species production
- CS3 Land-based IMTA
- CS7 Sea cucumber species, site selection and key hatchery steps
- CS8 Improving seed availability and grow-out of native and non-native oysters
- CS9 Offshore production of blue mussels
- CS10 Optimisation of freshwater fish production in Brazil
- CS11 Marine fish farming

Coordination of reporting of Case Study (CS) tasks are managed between the leaders of the three innovation WPs' (WP1, WP2 and WP3).

The CS reporting coordination has resulted in the Draft CS Reporting Template contained in the Appendix 1 and a tool to access these data in Appendix 2 of this deliverable (D1.2). Innovation potential and exploitation activities for WP1 specifically linked to the seven case studies listed above outlines the innovation potential and technology transfer for work carried out in linked case studies for D1.2. It also gives an overview of the status of each linked CS with a compiled number of 28 exploitable processes, prototypes and or products. The current status of the listed CSs' is generally in line with the submitted work plans for each CS with an average completion rate of 27% across the seven CSs'. Tables 2-9 presented in this deliverable outline the key output and deliverables for each CS including the type of exploitable output. D1.2 is in effect the completion of the first 12-months of research and innovation for WP1, the completion of this deliverable gives a clear overview of where each CS task at the beginning of the next 24-month prototyping loop and what the exploitable output will be (Figure 1, D1.1).

All detailed planning, scientific, technical and innovation information for each CS which advance the completion of WP1 tasks are presented in Annex 3 of Deliverable D1.1 (CS specific work plans) and in Annex 3 of D1.2 (Detailed Case Study Reports (M1-M12)). Annex 3 of D1.2 specifically contains an abstract/summary for each CS. The Case Study Reports (M1-M12) detail the methods used and results obtained. Where applicable the results are discussed. In a final section the progress, deviations, problems/solutions and planned future outlooks for next 12-months are provided.

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1. Introduction

i. Synopsis AquaVitae

AquaVitae is a research and innovation project funded by the EU's Horizon 2020 programme. The project consortium consists of 35 partners, from 16 different countries, spread across four continents. In addition to Europe, partners are situated in countries bordering the Atlantic Ocean, including Brazil, South Africa, Namibia, as well as in North America. Its broad objective is to introduce new low trophic species, products and processes in marine aquaculture value chains across the Atlantic.

ii. Scope and motivation of D1.2

The objectives of WP1 are to develop hatchery and seedling production of LTS (SO1 & SO2), specifically:

- Carry out hatchery/seedling innovation and exploitation activities in the specific CSs linked to WP1
- Coordinate WP1 CSs work with work on the same CSs in WP2 and WP3
- Identify industry barriers to the optimisation of production of seed for low trophic and extractive species
- Develop robust new and novel hatchery protocols and production processes for new and emerging species in each of the five AquaVitae value chains, formulate a "Good Practice" recommendation based on these protocols, and publish this recommendation as a low level, voluntary industry standard
- Optimise and test wild collection methods for native and non-native LTS species

The specific objectives of WP1 and the collaboration between the three innovation WPs aims to address T1.2 within this deliverable. Specific CSs' involved in WP1 are listed in Table 1. The role of WP1 is to develop hatchery and seedling aquaculture production of LTS through the five AquaVitae value chains (VC).

Table 1: Overview of the CSs' directly linked to WP1 and their lead partner organisation. These lead partner organisations are responsible for reporting to the WP1 on specific tasks and outputs. CS3, CS7 & CS9 are common CS to WP1, WP2 & WP3.

VC1 **Macroalgae**	**VC2** **IMTA**	**VC3** **Echinoderm**	**VC4** **Shellfish**	**VC5** **Finfish**
CS1: Macroalgae, new species production **CS Lead: CIIMAR**	**CS3:** Land-based IMTA **CS Lead: FCPCT**	**CS7:** Sea cucumber species, site selection and key hatchery steps **CS Lead: AWI**	**CS8:** Improving seed availability and grow-out of native and non-native oysters **CS Lead: IVL**	**CS10:** Optimisation of freshwater fish production in Brazil **CS Lead: EmBraPa**
			CS9: Offshore production of blue mussels **CS Lead: DTU**	**CS11:** Marine fish farming **CS Lead: FURG**

2. Report on first development phase for hatchery/seedling production in CSs, including requirement specification

i. Methodology

The approach and method adopted in WP1 is the same as that presented in D2.2 and D3.2:

Following the spiral model of innovation methodology (Figure 1, D1.1) CS leaders have completed their first innovation loop and reached a first prototype stage. Here, a prototype translates to any sort of output from a CS, may that be a new or improved product (including new species & technical hardware), process or a report.

WP1-3 leaders have developed a Draft CS Report Template (Appendix 1 in D1.2). The draft template has been reviewed by the Project Management Group and sent to each CS leader to be completed at 6 monthly intervals. This will allow both WP leaders as well as case study leaders to track the progress of each CS and to have the information needed to supply the relevant industry with the case study developments. The document will also be used to keep track on the stakeholders involved and detail their interest and input towards the potential applications resulting from each CS.

Included in this deliverable as part of appendix 3 are 13 detailed case study technical reports which provide all technical, scientific and innovation progress for all CS. All tasks outlined and their progress in tables 2-9 of D1.2 are described and discussed in detail in Appendix 3 of this deliverable. The detailed CS reports are directly aligned with the detailed CS workplans submitted in full as part of D1.1 (Appendix 3).

ii. Terminology

The development process (Figure 1, D1.1) utilised by the AquaVitae project identifies what outputs are derived from both WPs and CSs' based on two development loops. This process along with D1.2, D2.2 & D3.2 also supports the identification of innovation potential, exploitable outputs and products. Prototype's resulting from the project have been identified as one of the following:

- **Proof-of-Principle Prototype:** serves to verify some key functional aspects of the intended design, but usually does not have all the functionality of the final product
- **Working Prototype:** represents all or nearly all of the functionality of the final product
- **Visual Prototype:** represents the size and appearance, but not the functionality, of the intended design
- **User Experience Prototype:** represents enough of the appearance and function of the product that it can be used for user research
- **Functional Prototype:** captures both function and appearance of the intended design, though it may be created with different techniques and even different scale from final design
- **Paper Prototype:** is a printed or hand-drawn representation of the user interface

Outputs from CSs may not be developed into a protype or product until after the second loop at M36 however the innovation potential is determined after the first loop and is monitored through the CS reporting templates ever 6-months. A prototype can be a system design, functional pieces of equipment or an innovative process. Production processes are being developed mainly in WP1 and WP2 where the hatchery production (WP1) is interlinked with the on-growing activities carried out in WP2. The overlap between shared WP and CS tasks are mapped in Tables 2-9.

To gather the necessary information for this deliverable, two tools were used: firstly, the completed Case Study reports that used the "*CS Report Template"* (Appendix 1 in D1.2), completed by CS leaders at month six and month 12. These will be updated at 6-month intervals for the duration of the project; and secondly, a new Excel tool developed by the leaders of WP1-3 to make the entire dataset of the progress made by all 13 CS more manageable and available – the "*AquaVitae WP 1 - 3 database"* (Appendix 2 D1.2). In order to match the work and outputs of each case studies with the best fitting WP (WP1-3), the terminology used within AV and the database was refined as explained in this deliverable.

To generate the tables summarising the outputs of the first development phase, a number of filters were set in the database (Appendix 2). This allowed extraction of the information specific to WP1. The product specifications were requested by email from all partners and added to the tables. Alligned with the tables presented Appendix 3 contains the detailed CS technical reports on the first 12-months of AV.

ii. First development phase for new or improved products in CSs

There were 28 potential, exploitable outcomes identified by the CS in AV (Tables 2 - 9) that reported to WP1. Each output was assigned a specific identifier, type category, a detailed explanation describing the outcome, its requirement specifications, and the potential for becoming a future marketable product, the level of completeness with regard to what is expected by the end of the project, the current technology readiness level (TRL) and the WP task(s) that it reports to.

iii. A synopsis of the progress to date

A common structure was developed to facilitate efficient reporting of the progress of all CS for WP1, WP2 and WP3 (Appendix 1, D1.2). The CS reporting at month-10 for WP1 has resulted in all seven linked case studies reporting a total of 28 potential exploitable outputs from their activities related to hatchery and seedling production (Table 2 – 9). Out of this CS reporting period the breakdown of potential exploitable outputs is: 18 processes, 8 technical reports and 2 products (coming from CS8) coming from the first 12-month prototyping loop. Prototypes coming from the exploitable outputs can be a system design, functional pieces of equipment or an innovative process.

The overall reporting has shown an average of 27% completeness on proposed activities and outputs with some tasks nearing full completion at 90% respectively. All CS leaders have reported in detail any expected delays or deviations however the majority are currently not majorly impacted due to the current Covid-19 crisis. The project management realise that the COVID 19 crisis will be ongoing and risks and impact of COVID 19 will be continuously reported and a review conducted each 3-months by the project management group. Any major changes to the DoA will be immediately reported back to the European Commission.

Table 2: All outputs related to WP1 for CS1 (Macroalgae, new species production) and their requirement specifications (with: CS = corresponding Case Study Number; Ident. = specific identifier; Pot. Product (Y/N/tbc) = potential for becoming a future sellable product (Yes, No, to be confirmed); Complete = level of completeness with regard to what is expected by the end of the project; current technology readiness level (TRL); WP task = the WP task to which each output reports, according to description of action).

CS	Ident.	Output type	Detail	Requirement Specifications	Pot. Product (Y/N/tbc)	Com-plete	Current TRL	WP task
1	1.1.1	Process	A new reproduction method for seedling production of *C. tomentosum.*	A novel reproduction method for *C. tomentosum* that will allow improvement of genetic diversity of the biobank, and the development of efficient procedures for seeding on substrates.	tbc	10%	3	T1.2
	1.3.1	Process/ Technology Transfer	New protocol to cultivate *Ulva* Spp. in Southern Brazil	This will allow to stablish efficient vegetative and reproduction methods for local Ulva sp. in order to boost the cultivation of *Ulva* Spp. in Southern Brazil	tbc	0%	4	T1.2
	1.4.1	Process	A new method for cultivation of *Ulva* Spp. in substrates in earthen pounds	Cultivation of *Ulva rigida* in substrates will improve the deployment and harvest of biomass, and will allow upscale its production in underutilised, low cost, earthen pounds. This will be done in an organic certified land based IMTA.	tbc	0%	4	T1.2, T2.2

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Table 3: All outputs related to WP1 for CS3 (Land-based IMTA) and their requirement specifications (with: CS = corresponding Case Study Number; Ident. = specific identifier; Pot. Product (Y/N/tbc) = potential for becoming a future sellable product (Yes, No, to be confirmed); Complete = level of completeness with regard to what is expected by the end of the project; current technology readiness level (TRL); WP task = the WP task to which each output reports, according to description of action).

CS	Ident.	Output type	Detail	Requirement Specifications	Pot. Product (Y/N/tbc)	Com-plete	Current TRL	WP task
3	3.1.1	Process/ Technology Transfer	Use of *Ulvella lens* for settlement under organic certification standard.	The output of the task will provide protocols including data for integrated hatchery production to be implemented at an industry/commercial level under organic certification standard	N	90%	4-5	T1.1, T1.2, T1.3
	3.1.2	Process/ Technology Transfer	Land based abalone hatchery system.	The output of the task will provide protocols including data for abalone hatchery systems to standardise settlement induction protocols for tech-transfer throughout the project area.	tbc	90%	4-5	T1.1, T1.2, T1.3
	3.1.3	Process/ Technology Transfer	Method to ensure consistent settlement of South African abalone using species specific cues (abalone mucous) were developed for instances when the natural diatom cultures were inconsistent.	Standardise the commercial production method and provide relevant settlement protocols and data for abalone hatchery systems to standardise settlement induction protocols across the project area.	N	90%	4-5	T1.1, T1.2, T1.3
	3.2.1	Process/ Technology Transfer	Understanding the changes associated with global warming.	The output will investigate abalone nursery production under a global warming scenario and provide protocols to mitigate direct impact.	N	25%	5	T1.2
	3.3.1	Process/ Technology Transfer	Optimised nursery system for European abalone.	EU abalone production is in a state of flux and this output will compile available technologies including robust protocols to aid the advancement of the current industry standard	N	80%	3/4	T1.2

Table 4: All outputs related to WP1 for CS4 (Sea-based IMTA) and their requirement specifications (with: CS = corresponding Case Study Number; Ident. = specific identifier; Pot. Product (Y/N/tbc) = potential for becoming a future sellable product (Yes, No, to be confirmed); Complete = level of completeness with regard to what is expected by the end of the project; current technology readiness level (TRL); WP task = the WP task to which each output reports, according to description of action).

CS	Ident.	Output type	Detail	Requirement Specifications	Pot. Product (Y/N/tbc)	Com-plete	Current TRL	WP task
4	4.5.1	Report	Production site evaluation.	The first 3D hydrodynamic model for the region Vágar, Faroe Islands.	N	25%	3	T1.5, T2.2
	4.5.2	Process	Development of mussel seeding lines for wild settlement and optimal growth.	The mussel spat availability in Faroese waters will be clarified and settlement on two types of seeding lines investigated.	tbc	25%	3	T1.5, T2.2
	4.5.3	Report	Evaluating the IMTA potential with salmon/blue mussel coculture.	The potential waste assimilation by blue mussel around a commercial scale fish farm will be modelled taking into account the spatial constraints. This will add to the knowledge already established around the subject of fish mussel co-culture.	N	25%	3	T1.5, T2.2
	4.5.4	Report	Evaluation of the influence of salmon/blue mussel/seaweed coculture on fjord ecology	Evaluation of the influence of IMTA on the fjord ecology, when the lower trophic species are not feeding directly on the waste from the higher species.	N	25%	3	T1.5, T2.2

*Table 5: All outputs related to WP1 for CS7 (*Sea cucumber species, site selection and key hatchery steps*) and their requirement specifications (with: CS = corresponding Case Study Number; Ident. = specific identifier; Pot. Product (Y/N/tbc) = potential for becoming a future sellable product (Yes, No, to be confirmed); Complete = level of completeness with regard to what is expected by the end of the project; current technology readiness level (TRL); WP task = the WP task to which each output reports, according to description of action).*

CS	Ident.	Output type	Detail	Requirement Specifications	Pot. Product (Y/N/tbc)	Com-plete	Current TRL	WP task
7	7.3.1	Process	Hatchery protocols for sea cucumber larvae production in Brazil and South Africa.	These protocols will include details of the application of adapted methods broodstock collection, broodstock spawning, fertilisation, larval rearing and larval settlement to first artificial feeding of settled juveniles. These methods will be of significant value to the farmers interested in the integration of sea cucumbers in South Africa and Brazil in Abalone and Oyster farms respectively.	tbc	5%	4	T1.2, T1.4

Table 6: All outputs related to WP1 for CS8 (Improving seed availability and grow-out of native and non-native oysters) and their requirement specifications (with: CS = corresponding Case Study Number; Ident. = specific identifier; Pot. Product (Y/N/tbc) = potential for becoming a future sellable product (Yes, No, to be confirmed); Complete = level of completeness with regard to what is expected by the end of the project; current technology readiness level (TRL); WP task = the WP task to which each output reports, according to description of action).

CS	Ident.	Output type	Detail	Requirement Specifications	Pot. Product (Y/N/tbc)	Com-plete	Current TRL	WP task
8	8.1.1	Product	A new diet for *C. gasar* larvae in hatchery production	*Crassostrea gasar* is a new species in Brazilian aquaculture and hatchery protocols for the species are not well developed. The food requirements of *C. gasar* larvae is not well known and survival in hatchery production using traditional microalgae species is low. Native microalgae species may improve hatchery production of the species.	Y	30%	3	T1.2, T1.3, T1.4, T1.5
	8.1.2	Report	A new conditioning protocol for *C. gasar*	To enable control of the reproductive cycle in hatchery conditions, a condition protocol must be developed for *C. gasar.*	N	30%	3	T1.2, T1.3, T1.4, T1.5
	8.1.3	Report	A new protocol for water improvements in small-scale oyster hatcheries using estuarine water	To enhance survival of *C. gasar* larvae in hatchery production, the importance of water quality and means to improve water quality must be evaluated.	N	10%	3	T1.2, T1.3, T1.4, T1.5
	8.1.4	Report	A new protocol for enhanced survival of flat oyster seed using small-scale, low-tech nursery systems	The production of Ostrea edulis, the European flat oyster, is hampered by low seed availability. One of the major bottlenecks for small scale industries is transfer of seed from hatchery systems to sea based grow out systems as large scale industry nursery systems are often lacking. Alternative systems for small scale farmers must therefore be developed.	N	10%	4	T1.2, T1.3, T1.4, T1.5

8.1.5	Report	A new production protocol for flat oyster spat pond production	Hatchery production is only economically viable in larger industries and less cost intensive seed production techniques suitable for small scale industries must therefore be developed. Spatting ponds is a promising technique but there are no protocols enabling implementation of this technique in new areas.	N	30%	4	T1.2, T1.3, T1.4, T1.5
8.2.1	Report	A new protocol for sea based native oyster spat production including recommendations on new seed collector materials and new protocols adapted to local species	Seed production using sea-based collectors is a common strategy for extensive seed production. However, existing techniques are adapted to large scale industries and alternatives for small scale industries must be developed. Moreover, in areas where more than one oyster species exists a mixture of seed from different oyster species will be obtained on the collectors. Protocols to optimize capture of target species must therefore be developed.	tbc	25%	4	T1.2, T1.3, T1.5, (T3.2)
8.2.2	Product	A new software for automatic identification of oyster species	Seed production using sea-based collectors is a common strategy for extensive seed production. In areas where more than one oyster species exists a mixture of seed from different oyster species will be obtained on the collectors. Automated methods to separate oyster seed by species must be developed.	Y	50%	4	T1.2, T1.3, T1.5, T3.2

Table 7: All outputs related to WP1 for CS9 (Offshore production of blue mussels) and their requirement specifications (with: CS = corresponding Case Study Number; Ident. = specific identifier; Pot. Product (Y/N/tbc) = potential for becoming a future sellable product (Yes, No, to be confirmed); Complete = level of completeness with regard to what is expected by the end of the project; current technology readiness level (TRL); WP task = the WP task to which each output reports, according to description of action).

CS	Ident.	Output type	Detail	Requirement Specifications	Pot. Product (Y/N/tbc)	Com-plete	Current TRL	WP task
9	9.1.1	Process	Production protocol for hatchery produced blue mussel seed.	Development of a standard protocol for hatchery-based production of mussel seeds for industry scale production. Due to the feeding of the mussels with microalgae it might not be possible to get the final mussel product organic certified but could most likely be ASC certified.	tbc	25%	4	T1.4, T1.5
	9.2.1	Process	Method for grow-out of hatchery produced blue mussel spat.	Testing on-growing of hatchery produced mussel seed by different deployment methods and monitoring the biomass growth and biofouling. Due to the feeding of the mussels with microalgae it might not be possible to get the final mussel product organic certified but could most likely be ASC certified.	tbc	5%	4	T1.4, T1.5

Table 8: All outputs related to WP1 for CS10 (Optimisation of freshwater fish production in Brazil) and their requirement specifications (with: CS = corresponding Case Study Number; Ident. = specific identifier; Pot. Product (Y/N/tbc) = potential for becoming a future sellable product (Yes, No, to be confirmed); Complete = level of completeness with regard to what is expected by the end of the project; current technology readiness level (TRL); WP task = the WP task to which each output reports, according to description of action).

CS	Ident.	Output type	Detail	Requirement Specifications	Pot. Product (Y/N/tbc)	Com-plete	Current TRL	WP task
10	10.1.1	Process/ Technology Transfer	Optimized protocol for captive reproduction of pairs of *Arapaima gigas* in earth ponds.	Protocol designed to increase spawning rates of *A. gigas* using synthetic hormonal inducers for pairs in earth ponds.	tbc	10%	3	T1.2, T2.2
	10.1.2	Process/ Technology Transfer	Novel method for milt collection in *Arapaima gigas*.	Method to allow the collection of milt in *A. gigas*, needed to develop artificial fertilisation in the species and thus increase control of captive reproduction. Method should include a description of the collection process (fish handling, hormonal manipulation, sample collection).	tbc	10%	3	T1.2
	10.1.3	Process/ Technology Transfer	Novel method for egg collection in *Arapaima gigas*.	Method to allow the collection of eggs in *A. gigas*, needed to develop artificial fertilization in the species and thus increase control of captive reproduction. Method should include a description of the collection process (appropriate timing for fish handling, hormonal manipulations, sample collection).	tbc	10%	3	T1.2
	10.2.1	Process	Protocol of triploid induction in *tambaqui* Spp. with minimum of 70% induction success.	A protocol to induce triploidy in *tambaqui* Spp. using pressure shock onto fertilised eggs.	tbc	5%	3	T1.2

Table 9: All outputs related to WP1 for CS11 (Marine fish farming) and their requirement specifications (with: CS = corresponding Case Study Number; Ident. = specific identifier; Pot. Product (Y/N/tbc) = potential for becoming a future sellable product (Yes, No, to be confirmed); Complete = level of completeness with regard to what is expected by the end of the project; current technology readiness level (TRL); WP task = the WP task to which each output reports, according to description of action).

CS	Ident.	Output type	Detail	Requirement Specifications	Pot. Product (Y/N/tbc)	Com-plete	Current TRL	WP task
11	11.1.1	Process	Optimised process for larviculture in RAS	Flounder larviculture has been usually carried out in static (first few days after hatching) or semi-static systems (up to complete metamorphosis). We are looking for a complete larviculture cycle in a recirculating aquaculture system. We have done it this past breeding season, and juvenile output has been positive. We are looking forward to upscale it, in order to make it appropriate for the industry.	tbc	10%	4	T1.2, T1.3, T1.4
	11.1.2	Process	Optimised process for grow out in RAS	Despite successful spawning and juvenile production in captivity, production of flounder up to commercial size has not been reliable so far. A few attempts were carried out in the past, but we are looking forward to optimizing the process, including the application of results from several experiments carried out in the last few years, and a new RAS system, using raceways instead of circular tanks.	tbc	10%	4	T1.2, T1.3, T1.4

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3. Conclusion

The overview of the current CS activities linked to WP1 are on-time and on-track to achieve proposed tasks. This deliverable has outlined 28 potential outputs which are outlined in Tables 2-9.

Monitoring of these CS tasks for WP1, WP2 & WP3 is crucial for the management of activities, monitoring of expected outputs, identification of innovation potential and development of products.

The 10-month reporting on the first prototyping development process for WP1, WP2 & WP3 has resulted with on-time submission of outputs, innovation potential and products from the 13 CS studies from the 35 project partners. This level of relevant and varied information required a shared reporting format for the three innovation WPs'. WP1, WP2 & WP3 have developed a common reporting system that allows CS leaders to report on individual or shared tasks between the WPs on the same digital reporting form (Appendix 1).

To utilise the information in the CS reporting template a tool was developed (AquaVitae WP 1 - 3 database) to extract the relevant CS details for WP leaders for the purpose of reporting and monitoring outputs (Appendix 2). The data base used to extract information can be utilised for outputs from WP9 and it is envisaged that WP4-8 may also integrate into the CS reporting template, thus expanding the use of the template and data base for the advancement and monitoring of the overall AquaVitae project.

D1.2 is the culmination of the first 12-months of research and innovation carried out in the AquaVitae Project across the Atlantic, considerable effort was seen from each CS team on the development of the detailed CS technical reports included in this deliverable which give a clear overview of the significant technical and scientific activities carried out for the first 12-months of AV project. The stated average completions rate of 27% across the seven CSs' connected to WP1 is a direct result of the coordination of the activities by each CS leader and the on-time reporting to each of the innovation WPs'. Twenty-eight exploitable outputs were identified using the CS common reporting system (18 processes, 8 technical reports and 2 products) after the first 12 month prototyping loop, these exploitable outputs and their further envisaged prototype (process, prototype or product) have also been mapped.

Contained in this deliverable (Appendix 3) are 13 CS detailed technical reports which align with the work plans submitted as part of D1.1 (Appendix 3 D1.1). The scientific and technical reports outline all tasks directly related to tables 2-9 of this deliverable. Each CS task is introduced along with the methods and results. The results for each task are discussed and each CS has presented a detailed progress which is aligned with tables 2-9 of D1.2. All technical, scientific and progress updates for the first 12-months of AV are contained here for each CS in Appendix 3.

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4. Appendix 1

Person: Björn Suckow, Clifford Jones, Collin Hannon
Date: 10/03/2020
Reason: Need of reporting for case studies to WP1-3 and info to be used in Deliverable D

Info: New template, updated already three times
designed for more intuitive use by the owner of case study task (CST)
can now be send by CS leaders to CST leaders directly
automated "summary spread sheet"

READ ME FIRST - Information for partners

Dear CS leaders,
for our work in WP1, 2 and 3 we need you to fill in this "Case Study Report Form" on SharePoint (and Sh

In order to edit the file, you have to choose "Open in Desktop App" in the middle of the Excel menu ribb
If you are asked whether or not to open the file, chose "Yes". You will be asked to login into your Micros
Do that by inserting your email address and password (potentially the one your created by yourself e.g
Depending on your connection it will take a while until your Desktop Excel App will open and allow yo
You can save your file anytime. Finally close and save the file. All changes will be uploaded to SharePo

It was designed to allow CS leader to ask their CST (case study task) leaders to describe the pr
There is one Excel-file per Case Study containing all of its CST (up to 12) in different tabs/shee
Each "CST (#) tab" can be used for one CS task (currently you cannot have more than 12 tasks
Each Tab is password protected only giving access to those fields that you have to fill in. This b
You can only adjust the size of each line/row (making it bigger/smaller) or hide those rows tha
You are actually encouraged to hide lines that you do not use! Currently there are way too mar
For each CS task you can add up to 10 prototypes that result directly from your work within tha
Once the CST task leaders have filled in their tab, they will inform their CS leader who will hav
When the last CST leader report was acknowledged by the CS leader, the CS leader has to fill i
After that the CS leader will inform the WP leaders, Colin Hannon (WP1), Cliff Jones (WP2) an

The information from the "progress log" will be used, as indicated in the document, for future

Therefore the form forces you to be concise and short in your progress description!
We know that this is trickier than explaining yourself on multiple rows per problem etc. but it

The report is a living document that you will add to on biannual basis.

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To be compile by the leader of each case study task (as outlined in the case study workplans):

Instructions:

(1) Before filling in this table make sure to read the info on the "INFO" tab first!!!
(2) Complete one form for each case study task (CST) that is listed in the case study workplan.
(3) Give each CST a clear number - and use that number for the rest of the project.
(4) Whenever you are asked to update your form, simply unhide the respective rows in the progress log.
(5) Use the exact same form for all future reports and update sections where needed.

Name of reporter:				Date of current report:	
Case study number:		CS task number:		CS task name:	
Work package/s to which this case study task reports:					
Tasks (per DoA) to which this case study task reports:					
Partners involved (lead partner first):					
Completeness (%):			Risk (type 0 = red, 1 = amber, 2 = green):		
Planned start month:		Actual start month:		Current TRL:	
Planned end month:		Actual end month:		TRL by M48:	

Shortened task description as in CS workplan (max 200 words; basic material/methods, where and how the work will be carried out):

Progress log - Main progress of the CS task (max 200 words - limit it to 10 bullet points per time period):

Please highlight any deviations/problems experienced (bullet points only):

5. Appendix 2

Appendix 2 (D1.2, D2.2 and D3.2)

"AquaVitae WP 1 – 3 database"

Purpose of the database-tool

The data collected from the AquaVitae (AV) case study (CS) reports have been banked in a database. The purpose of this database was initially for the Work Package (WP) 1, 2 and 3 leaders to check and evaluate the progress of the CS that will contribute to the various WP1, 2 and 3 tasks. It was essentially designed and converted into a "tool" using the filter-functions in Microsoft Excel to make this function possible. As such, it can now also serve as a check/balance that the CS and CS task leaders can use to establish the accuracy of their reporting and the progress of their work. Similarly, the AV project management team will be to use this tool to do the same; as will the evaluators of the project; and, possibly in time to come, the greater European public with an interest in this work, might be able to use this tool to access the data generated in this project.

What the tool is made up of

All the data that are captured from the CS bi-annual reports will be linked to this database. A single spreadsheet will include all data captured from all the main tasks of all case studies 1 to 13. It will include the following details of every CS task:

- The time that the data were captured (e.g. M0, M12, M18, etc);
- CS and CS task number;
- Unique identifier number for each output;
- Description of the entry type, and the entry type will range from it being:
    - an exploitable outcome;
    - detail on its progress at that time;
    - any problems;
    - solutions to overcome the problem; and
    - progress beyond state of the art.
- The entry will also include detail on its:
    - requirement specifications,
    - its exploitation potential,
    - percentage completeness,
    - its current technology readiness level,
    - its expected and actual start and end dates.
- It will also include participant details, such as:
    - the CS leader,
    - task leader
    - partners involved in the work,
    - contact details, etc.

Table 1 An example of how the database tool can be used. Here it has been set to display all exploitable outputs from all case studies that will contribution to WP2 task T4.2 (i.e. the inclusion of algae into low trophic species). At a glance, the WP leader can prepare a report on the CS that will contribute to this particular WP-task. Note that the page was too small to display all the columns here; but this selection was chosen to provide a feel for what the tool can provide.

Report Date	CS Number	CS task number	Work Package	Ident.	Entry Type	Output type	Detail	Requirement Specifications	Product potential (Y/N/t…	Com-plete	Current TRL	WP task	CS leader name
M12	2	2.4		2.4.2	Exploitable output	Product	Ocean cultivated kelp included in an abalone diet	Cost effective feed with reduced environmental footprint. Feed will adhere to industry standards with regard to risk management and ingredient traceability.	Y	20%	4	T2.4	Urd Grandorf Bak
M12	3	3.6	2	3.6.1	Exploitable output	Process	Abalone IMTA production/nutrition and systems.	Provide data to demonstrate the effects of IMTA production on nutritional and environmental aspects.	N	30%	3/4	T2.2, T2.4	Gercende Courtois de Viçose
M12	3	3.7	2	3.7.1	Exploitable output	Product	Pelletised abalone feed containing land-based IMTA grown seaweed.	Produce compound feed integrating IMTA produced macroalgae to demonstrate the effects of IMTA production on nutritional aspects and benefits for circular processes.	Y	10%	3	T2.2, T2.4	Gercende Courtois de Viçose
M12	3	3.7	2	3.7.2	Exploitable output	Report	Life cycle analysis of land based IMTA.	Provide data to demonstrate the effects of IMTA production on system efficiency and environmental aspects.	N	10%	3	T2.2, T2.4	Gercende Courtois de Viçose
M12	4	4.1	2	4.1.1	Exploitable output	Process	Method to make algae biosecure when introduced to an abalone feed.	First method to make IMTA (and other sources) of algae biosecure when fed to abalone, where the nutritional value of the feed is not compromised. Process will adhere to industry standards with regard to risk management and ingredient traceability.	Y	15%	4	T2.2, T2.4	Cliff Jones
M12	4	4.2	2	4.2.1	Exploitable output	Process	Coproduction of algae with mussels	More efficient use of existing infrastructure aimed at job creation and reduced environmental footprint. Adhere to environmental legislation/monitoring and black economic empowerment legislation in South Africa.	tbc	80%	3	T2.2, T2.4	Cliff Jones
M12	4	4.3	2, 3	4.3.1	Exploitable output	Report	Data supporting use of abalone diet with alternative LTS dietary ingredient, that originates from sea based IMTA.	Cost effective feed with reduced environmental footprint. Feed will adhere to industry standards with regard to risk management and ingredient traceability.	Y	8%	4	T2.2, T2.4	Cliff Jones
M12	4	4.6	2	4.6.2	Exploitable output	Product	Saccharina latissima obtained in abalone IMTA co-culture.	Process will contribute to reduce environmental footprint of aquaculture production methods and will make production more cost-effective; contribute to developing new industry standards.	Y	40%	5	T2.2, T2.4	Cliff Jones
M12	13	13.1	2, 3	13.1.1	Exploitable output	Product	Diet formulation for European abalone macroalgae-based.	The formulation that will include macroalgae needs to be nutritional balanced (protein, amino acids, lipid, fatty acids, vitamin and minerals) for European abalone juveniles to meet the known requirements for optimal growth	Y	21%	4	T2.4	Sofia Engrola
M12	13	13.1	2, 3	13.1.2	Exploitable output	Product	Diet formulation for European abalone macroalgae- and vegetable-based.	The formulation that will include macroalgae and vegetable needs to be nutritional balanced (protein, amino acids, lipid, fatty acids, vitamin and minerals) for European abalone juveniles to meet the known requirements for optimal growth	Y	21%	4	T2.4	Sofia Engrola
M12	13	13.1	2, 3	13.1.3	Exploitable output	Product	Diet formulation for African abalone harvested kelp-based.	The formulation that will include kelp needs to be nutritional balanced (protein, amino acids, lipid, fatty acids, vitamin and minerals) for African abalone juveniles to meet the known requirements for optimal growth	Y	21%	4	T2.4	Sofia Engrola
M12	13	13.1	2, 3	13.1.4	Exploitable output	Product	Diet formulation for African abalone IMTA macro-algae-based.	The formulation that will include macroalgae needs to be nutritional balanced (protein, amino acids, lipid, fatty acids, vitamin and minerals) for African abalone juveniles to meet the known requirements for optimal growth	Y	21%	4	T2.4	Sofia Engrola
M12	13	13.1	2, 3	13.1.5	Exploitable output	Product	Diet for European abalone macroalgae-based.	The diet will have a pellet quality (e.g. density, durability, hardness, water stability), size, and shape suitable for the species and stage.	Y	21%	4	T2.4	Sofia Engrola
M12	13	13.1	2, 3	13.1.6	Exploitable output	Product	Diet for European abalone macroalgae- and vegetable-based.	The diet will have a pellet quality (e.g. density, durability, hardness, water stability), size, and shape suitable for the species and stage.	Y	21%	4	T2.4	Sofia Engrola

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Table 2 A second example of how the database tool can be used. Here it has been set to display all the details that were captured for a single case study task 4.1 at months 12.The window displays all information relating to the progress of CS task 4.1, including date of the entry, task name, output type, its progress, problems encountered, solutions that were implemented, percent completeness, etc. Note again, that the page was too small to display all the columns here; but this selection was chosen to provide a feel for what the tool can provide.

Report Date	CS Number	CS task number	Work Package	Ident.	Entry Type	Output type	Detail	Requirement Specifications	Product potential (Y/N/t	Com-plete	Current TRL	WP task	CS leader name
M12	4	4.1	2	4.1.1	Exploitable output	Process	Method to make algae biosecure when introduced to an abalone feed.	First method to make IMTA (and other sources) of algae biosecure when fed to abalone, where the nutritional value of the feed is not compromised. Process will adhere to industry standards with regard to risk management and ingredient traceability.	Y	15%	4	T2.2, T2.4	Cliff Jones
M12	4	4.1	2		Progress		PhD student was engaged on this project. A literature review and experimental design was completed during the first six months. Various meetings between Rhodes University and Industry partners were held and different approaches on the methodology was discussed. Workshop was held between industry partners and the stakeholders. Established collaborative relation with researchers from DAFF. During M10, a training course on microbiological and molecular techniques was attended and contributed immensely to the techniques that will be used in this task. The training included microbial growth media preparation, bacteria culture, serial dilution and basic PCR techniques. Numerous discussion were held between the PhD student and supervisors over the course of the months. Established collaborative relation with researchers from Ghent University (Belgium) to address knowledge gaps that were identified earlier. Task 1.2 and 1.3 have not begun; but all is in place to start now.			15%	4	T2.2, T2.4	Cliff Jones
M12	4	4.1	2		Problems/deviations		It was established that the industry was not interested in pathogenic viruses (bacteria and fungus important too - but second to viruses) - so focus needed to shift. A major challenge was finding an appropriate laboratory to carry out the virology experiments. Attempts were made at different departments at Rhodes University, but none were successful and as a result, the experiments were delayed. A virus strain is required as part of the test pathogens, but sourcing this has been unsuccessful.			15%	4	T2.2, T2.4	Cliff Jones
M12	4	4.1	2		Solutions		The laboratory at the Marine Research Aquarium (Department of Agriculture, Forestry and Fisheries) in Cape Town has been made available for the experiments. Instead of using a virus strain, it was suggested that a bacteria-phage may be viable. A collaboration with Ghent University is underway, where the virology experiments could potentially be conducted. Instead of using a virus strain, it was suggested that a bacteria-phage shall be used as the demonstration model. A collaboration with Ghent University is underway, where the virology experiments could potentially be conducted.			15%	4	T2.2, T2.4	Cliff Jones
M12	4	4.1	2	4.1.1	Progress beyond SotA		Method to make algae biosecure when introduced to an abalone feed			15%	4	T2.2, T2.4	Cliff Jones

How the tool works and how it can be used

It will be possible for the user to extract any combination of data entries required, depending on what the person conducting the search requires the data for, and this can be adjusted using the filter functions in Microsoft Excel. For example, WP2 leader might want to list all the exploitable outcomes that will contribute to one of the WP2, for example tasks T2.4. The outcome of this filter selection is presented in Table 1. The search might then be altered to retrieve the same information, but for another WP or WP-task. Alternatively, the searcher might want to retrieve greater detail on the progress of one the exploitable outputs in the list, and might change the search accordingly. For example, they might what a more detailed account of the progress of CS-task 4.1; Table 2 displays all the details that were recorded at month-12 for that task: It includes date, task name, output type, its progress, problems encountered, solutions that were implemented, percent completeness, etc. In time to come, the person using the tool might want to track the progress of this particular task across different reporting periods, and the tool will allow for this. The tool allows for any combination of searches, depending on the required report.

6. Appendix 3

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Appendix 3 – Detailed Case Study Reports (M1-M12) (D1.2, D2.2 and D3.2)

This appendix lists the progress accomplished in all 13 CS per CST within the first twelve months.

Content

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Summary of progress report for Case Study 1

Date of report:	**01.04.2020**
Case Study name:	**CS1: Macroalgae, new species production**
of relevane for WPs	**WP1, WP2, WP3**

Abstract/Summary

The experimental work has only been initiated for CST 1.1. The evaluation of seasonal variations on the reproductive status of *Codium tomentosum* has been initiated and is currently underway. The essential basic procedures required to control the life cycle of the species have been established under controlled lab and culture conditions, namely the release of gametes, the formation of zygotes, and the development of filamentous germlings, which are precursors of the adult *Codium* (spongy thallus). The evidence here provided of differentiation and development of filamentous thallus in *C. tomentosum* from isolated utricles (micropropagation), and further development of spongy thallus at sea is another very important result. A 10% completeness is estimated for this task. Literature reviews and preparation of material and methods have been carried out for some other CSTs.

CST 1.1 Reproduction of *Codium tomentosum*

Isabel Sousa Pinto/Gonçalo S. Marinho, CIIMAR

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
1.1	Reproduction of Codium tomentosum	T1.2	CIIMAR	10%	✓	M7	M8	M29	0	3	6

Introduction

In order to develop a new hatchery protocol to produce seedlings of *Codium tomentosum* it is essential to understated the basic reproduction biology of the species. Thus, in the first months a detailed literature review performed, and was complemented with extensive microscopic observation of the different stages of the life cycle of *C. tomentosum*. The goal was to understand the reproductive cycle, get experience with the microscopic observation and identification of the different stages of development, and perform basic procedures essential for the control and manipulation of the life cycle of the species, such as evaluation of reproductive status, gametes release, zygote formation and germination.

The genus *Codium* has a diplontic life cycle with sexual reproduction by fusion of biflagellate anisogametes and gametic meiosis[1,2]. Male and female gametangia are formed laterally from utricles[3]. The peak of maturation of gametangia for the species *C. tomentosum* has been reported to be reached in winter[4], but no comprehensive analysis on the reproductive status of the species has been done so far. Sexual reproduction in *Codium* genus is performed through zygotes resulting from the gamete fusion. The resulting zygote germinates into a germling[1], a siphonous filament, which eventually develops into the diploid adult thallus[3]. The control of the life cycle under controlled artificial conditions is challenging and growing the adult thallus in the laboratory has not been achieved so far.

[1] Borden & Stein, 1969 https://doi.org/10.2216/i0031-8884-8-2-91.1
[2] Miravalles et al. 2012 https://doi.org/10.1111/j.1440-1835.2012.00640.x
[3] Chang et al. 2003 https://doi.org/10.1515/BOT.2003.043
[4] Trowbridge et al. 2001 https://doi.org/10.1017/S0025315401004854

The formation and growth of filamentous thalli from isolated utricles with medullary filaments has also been described for *C. fragile*[5,6] and *C. bernabei*[7]. This type of propagation involves changes in the utricles morphology and the capacity to form elongated filaments, able to attach to the substratum and later branch into new utricles[5,7]. Results from a previous study suggest that utricles of *C. fragile* detached by strong waves and grazers can grow into a full-grown adult via the development of filamentous thalli leading to the wide and rapid spread of the species[5]. The development of filamentous thallus of *C. tomentosum* from isolated utricles in the laboratory (i.e. under culture conditions) has not been reported until now.

Methods

The state-of-the-art concerning life-cycle, reproduction methods and cultivation of *Codium* sp. was evaluated through a detailed literature review.

Study area and sampling

Specimens of *Codium tomentosum* were collected from two natural populations previously mapped by CIIMAR: Aguçadoura shore, 41° 26’ N, 8° 47’ O, located in the municipality of Póvoa de Varzim in Porto district, and Viana- Norte, 41°41'49.3"N 8°51'03.4"W, Viana do Castelo. The biomass was collected bi-monthly for evaluation of seasonal variation in the reproductive status, and to be utilized in the experiments panned in this task.

The species identification, observation of reproductive structures/evaluation of the reproductive status, gamete release, zygote formation, and early stages of development were followed through microscopic observation.

Species identification and presence of gametangia

To assess the presence of reproductive structure three fragments (2-3 cm) were cut from the apical part of the thallus for each individual specimen. The fragments from each specimen were mechanically crushed and diluted as needed with a known amount of autoclave seawater. Five mL of this suspension were transferred to graduated Petri dishes and observed under the stereo microscope. The presence of gametangia was accessed and recorded. Each Petri dish was then transferred to the inverted microscope to confirm the species identification for all specimens based on the morphology of the apical part of the utricles (absence of mucron).

Gamete release, zygote formation, and early stages of development

For the gamete liberation and further development three fragments (2-3 cm) were cut from the apical part of the thallus for each individual specimen. The fragmented were dried with laboratory paper and transferred to a previously tared Petri dish, and the weight recorded. Five mL of autoclaved seawater were added to the Petri dishes, which were then seal with Parafilm®. The Petri dishes were transferred to a culture chamber at controlled temperature, photoperiod, and light intensity. After one hour and the next day the gamete release was monitored, the formation of zygotes, germination, and early stages of development were monitored in the following days and weeks using the inverted microscope.

Micropropagation

Studies on micropropagation were performed with biomass kept at CIIMAR´s culture facility for several months (acclimatised/cultured biomass). In order to test if filamentous thallus of *C. tomentosum* could

[5] Nanba et al. 2002 https://doi.org/10.1016/S0304-3770(02)00036-0
[6] Yang et al., 1997 https://doi.org/10.1023/A:1007996207924
[7] González et al., 2014 https://doi.org/10.2216/13-251.1

differentiate and develop from isolated utricles the biomass´ macroscopic structure was mechanically crushed according to the procedure described by Hwang[8]. One mL of this suspension was transferred to a graduated Petri dish to observe the condition/integrity of the isolated utricles and estimate their density. The Petri dishes were then transferred to the culture room with controlled temperature, photoperiod, and light intensity.

In order to test the ability of the isolated utricles to attach, differentiate and grow on culture lines (polyester string) the same procedure was followed to prepare the suspension of utricles[8]. The polyester string attached to a frame was submerged in the utricles´ suspension to allow the attachment[8]. Then the string was transferred to small tanks inside the culture room with controlled temperatures, photoperiod, and light intensity. PES was used as nutrient medium. Aeration was initiated after some days and progressively increased every week.

The development of the cultures was followed on a weekly basis to access their development using the stereo microscope, and the inverted microscope with a coupled camera for image record.

Results

The absence of mucron in the apical part of the utricles indicates that the biomass collected from natural populations corresponds to *C. tomentosum*, and not the co-existing and invasive *C. fragile* (Figure 1.1.a A,B,C). The presence of reproductive structure, gametangia (Figure 1.1.b A), was observed in the samples collected in January and March, while in the samples from May they were absent, which could be indicative of the reproductive status of the specimens (Table 1.1a). From March 2020 onwards, the reproductive status was further confirmed by the liberation of gametes, and subsequent fecundation, zygote formation and germination.

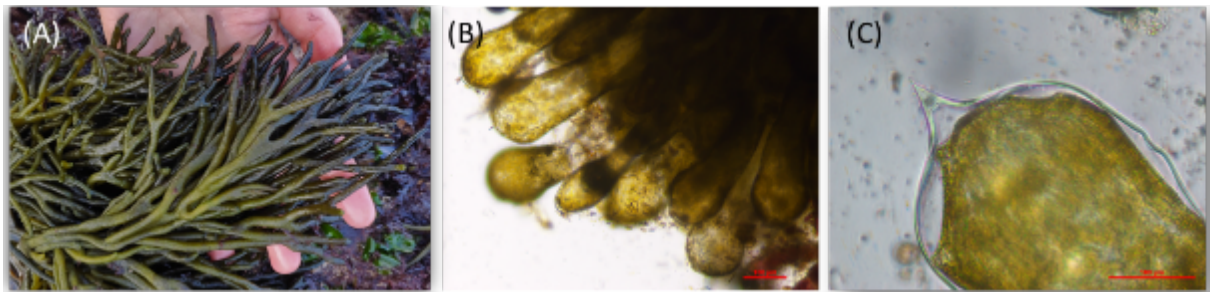


Figure 1.1.a: (A) General aspect of the C. tomentosum thallus in its natural habitat. (B) Detail of the apex of an utricle with the typical rounded appearance of the species C. tomentosum; (C) Detail of the apex of a typical mucronate utricle of the species C. fragile. (A): picture by Mª Francisca Sá. (B and C): pictures by Gonçalo S. Marinho

*Table 1.1.a: Presence of reproductive structures at different sampling months (January 2020 – May 2020). *Reproductive status confirmed by gamete release and fecundation (zygote formation).*

Sampling	Aguçadoura	Viana
January	Fertile	Fertile
March	Fertile*	-
May	Infertile	Infertile

Both male and female gametes were successfully release and identified (Figure 1.1b B), and after a few days in culture the development of a zygote could be observed. After approximately 3 to 7 days each zygote germinated into a filamentous germling (Figure 1.1.b C). The germlings developed (in length) for several weeks, and after some weeks some branching could be observed. However, even after

[8] Hwang et al. 2008 https://doi.org/10.1007/s10811-007-9265-5

some months in culture the formation of spongy thallus ("adult" *C. tomentosum*) could not be observed.

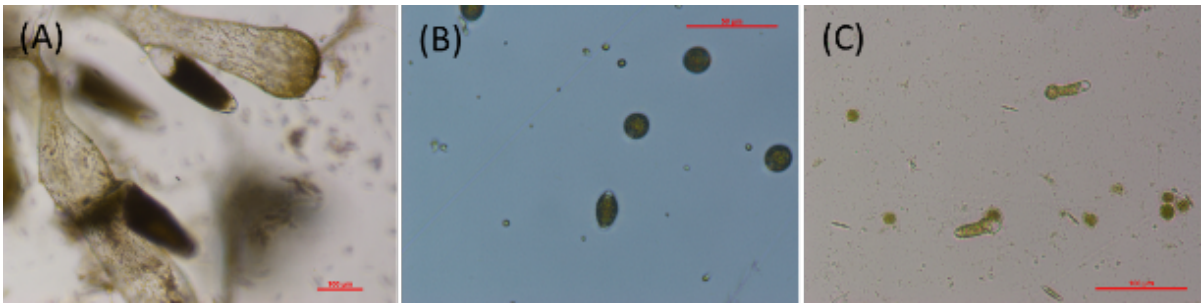


Figure 1.1.b: (A) Utricles with gametangia; Scale bar = 100 µm (B) Male and female gametes 24-hours after release; Scale bar = 50µm. (C) Zygotes started to germinate forming filamentous germlings (4 days in culture); Scale bar = 100µm. Pictures by Gonçalo S. Marinho

Micropropagation

For the study on Petri dishes, a few days after isolation the differentiation of filamentous thallus from the isolated utricles was observed, and they actively developed in the coming weeks to form long filamentous thallus (Figure 1.1.c).

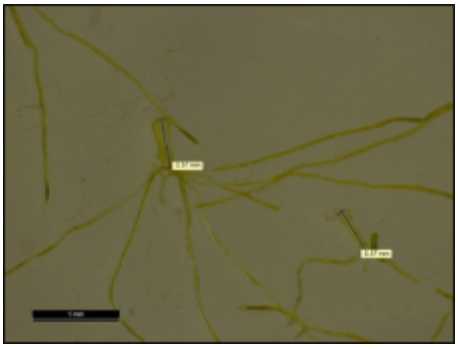


Figure 1.1.c: Long filamentous thallus of C. tomentosum differentiated and developed from isolated utricles (after 35-days in culture in Petri dishes following isolation); Scale bar = 1mm. Picture by Gonçalo S. Marinho

For the studies on polyester string, after three or four weeks, heavy development of filamentous thallus could be observed covering totally the culture line (Figure 1.1.d A). After two months at sea (and following one month in the hatchery) the development of spongy thallus ("adult" Codium) with approximately 2-3cm was observed in the culture lines (Figure 1.1.d B).

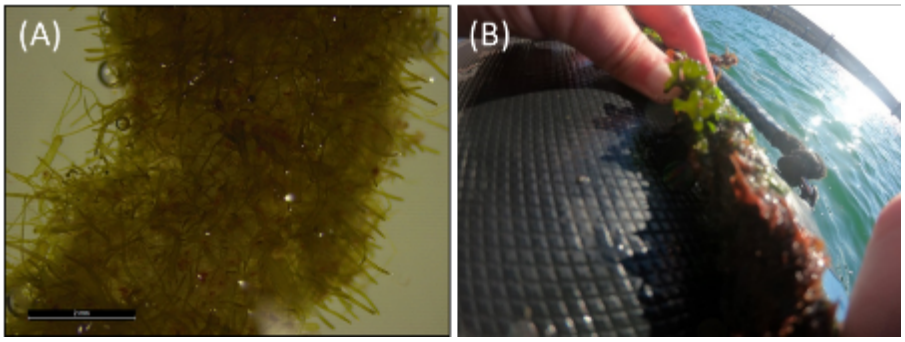


Figure 1.1.d: (A) Heavy development of filamentous thallus covering totally the culture line on March 2020 (after three weeks in culture in the hatchery following isolation); Scale bar = 1mm. (B) Development of spongy thallus ("adult" Codium) in the culture lines on May 2020 after two months at sea near by CIIMAR (following one month in the hatchery). (A): Picture by Gonçalo S. Marinho. (B): Picture by Bianca Reis

Discussion

The morphological analysis of the utricles from the collected biomass suggests that this is indeed *C. tomentosum*, and not the invasive co-existing species *C. fragile*. The observed release of gametes, the formation of zygotes, and the development of filamentous germlings, which are precursors of the adult *Codium* (spongy thallus), under controlled lab and culture conditions constitute a very important achievement as these are essential basic procedures required to control the life cycle of the species. The presence of reproductive structure in the samples collected in January (and March) may support the idea that the species is reproductive during winter months[9], however, the data set is still very limited to draw any final conclusions, and the sampling and evaluation planned for the coming months will be crucial for that. The evidence here provided for the differentiation and development of filamentous thallus from isolated utricles, both in Petri dishes and in culture line, and further development of spongy thallus (macroscopic *Codium*) at sea are important results that confirm the technical feasibility of this micropropagation method, which may constitute an alternative to current production methods based on fragmentation and tumble culture. Further investigation and optimization will be required.

Progress, deviations, problems & next 12M

Progress: Based on the work performed and results obtained so far, we estimate a 10% completeness for this task.

The observed release of gametes, the formation of zygotes, and the development of filamentous germlings, which are precursors of the adult *Codium* (spongy thallus), under controlled lab and culture conditions constitute a very important achievement as these are essential basic procedures required to control the life cycle of the species.

A preliminary protocol was developed for micropropagation of *C. tomentosum* in culture line through the development of filamentous thallus from isolated utricles. After two months at sea (followed by one month in the hatchery) the development of spongy thallus ("adult" upright thallus) of *C. tomentosum* was observed in the culture lines. This confirms the technical feasibility of this micropropagation method, which may constitute an alternative to current production methods based on fragmentation and tumble culture. Further investigation and optimization will be required.

Deviations & Problems: The laboratory work was planned originally to start in M7 but due to a delay in hiring the person that will conduct most of the research on this task it was postponed to M8. Due to covid-19 pandemic new experiments would not be allowed after the second week of March 2020

[9] Trowbridge et al. 2001 https://doi.org/10.1017/S0025315401004854

(M10) at our institution; however, the ongoing experiments were carried out as planned. In spite of this limitations, which resulted in some delay, a good progress was made towards an in-depth understanding of the basic reproduction biology of the species, and basic procedures essential for the control and manipulation of the life cycle were performed.

Outlook: In the next 12 months (M13-M24) the procedures developed until now will be applied to further evaluate the effect of season and origin of biomass on the reproductive status of *C. tomentosum*. Additionally, the effect of culture conditions (light quality and intensity, nutrient concentration, and water movement/aeration) on the production parameters will be investigated.

CST 1.2 Optimization of seedling methods for *C. tomentosum*

Helena Abreu/Andreia Rego, ALGAplus

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
1.2	Optimization of seedling methods for C. tomentosum	T2.2	AlgaPlus, CIIMAR	0%		M13	0	M30	0	3	6

Introduction

This task will be carried out at ALGAplus with the support of CIIMAR's research fellow from M13 to M30.

ALGAplus grows *Codium tomentosum*, in quantities below 1ton (fw), using a land-based tank system, where the biomass is kept in tumble culture. Internal and confidential procedures have been developed to prepare the biomass for seedling in artificial substrates.

The objective will be to grow-out the seeded material in the earthen ponds of ALGAplus, upscaling the production of this species.

Experiments will be done to test those procedures in two types of substrates, by different seeding methods. The influence of factors as: a) season b) seedling density c) substrate type on biomass yield will be tested.

The reproductive status, and production parameters will be evaluated through macroscopic and microscopic observation, image record and processing. Monitoring in the earthen-ponds will be done on temperature, irradiance, water turbidity, salinity and pH.

During the first 12 months of work will be performed three trials, testing two different types of seeding method, submersion and aspersion, in two types of substrates, biodegradable stripes and kuralon lines.

Methods

During the first months, a review of the literature was done in order to planning the trials. Literature about seeding cultivation methods and the reproductive status of *C. tomentosum* was studied and analysed.

The materials and methods were described and all the support tools were prepared, such PVC structures used for supporting the substrates, tanks, aeration and light systems. The methods will be described in the next report, together with results and discussion.

Results

The experimental work on this task will start on M13.

Discussion

The experimental work on this task will start on M13.

Progress, deviations, problems & next 12M

Progress: 0% completeness.

Deviations & Problems: The experimental work on this task will start on M13.

Outlook: In the next 12-months (M13-M14) experiments will be done to test seeding procedures in two types of substrates.

CST 1.3 Protocols of *Ulva* spp. cultivation in Brazil

Leila Hayashi, UFSC

CST #	Name	Reports to task(s)	Partners involved	complete d by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
1.3	Protocols of Ulva sp. cultivation in Brazil	T1.2	UFSC, AlgaPLUS	0%	✓	M13	0	M24	0	4	6

Introduction

ALGAplus has successfully been growing *Ulva* by vegetative propagation since 2013 in a land-based IMTA system. However, since all strains are clones, the production can decrease over the time, as happen with other commercial species, and there is no possibility to select strains with better performance. The scope of this case study task is developing a protocol to cultivate *Ulva* Spp. to improve the strains and the productivity of land-based cultivation. The goals of this 12-months of work were:

a) To identify one similar species of *Ulva rigida* cultivated in AlgaPlus, in Brazil, by phenotype and molecular biology;

b) Elaborate experimental design based on previous experiments with *Ulva* Spp. at UFSC;

c) Start preliminary experiments to verify if Brazilian species can easily produce spores.

Methods

A literature review is being carried out. The culture system in our lab facility has been prepared for the upcoming experimental work. The methods will be described in the next report, together with results and discussion.

Results

The work has not started yet

Discussion

The work has not started yet

Progress, deviations, problems & next 12M

Progress: 0% completeness. Meetings were held with the macroalgae group to prepare the laboratory for growing this new species. Potential algae surveys are being carried out that are similar to Portugal's *Ulva rigida*. A literature survey is also being carried out to familiarise ourselves with the cultivation conditions of *Ulva* species.

Deviations & Problems: The laboratory's infrastructure had to be adapted to receive and cultivate a new species of macroalgae. These problems have already been solved, and we believe that we will be able to start the experiments in the coming months.

Outlook: Select a species of *Ulva* Spp. from Florianopolis similar to *Ulva rigida* from Portugal, identify molecularly, and start the cultivation experiments.

CST 1.4 Testing the protocol for *Ulva* cultivation at commercial scales

Helena Abreu/Andreia Rego, ALGAplus

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
1.4	Testing the protocol for Ulva cultivation at commercial scales	T1.2, T2.2	AlgaPLUS, UFSC	0%	✓	M25	0	M42	0	4	6

Introduction

This task will be carried out at ALGAplus with the support of UFSC.

ALGAplus will test the Brazilian protocols for sexual reproduction developed in CST 1.3 with *Ulva rigida* cultivated in Portugal.

Experiments will be done to test the spore and gametic production. The seeded material will be placed for grow-out in the earthen ponds of ALGAplus.

Experiments will test the influence of factors as: a) season b) seedling density c) substrate type on biomass yield.

Monitoring in the earthen-ponds will be done on temperature, irradiance, water turbidity, salinity and pH.

Methods

During the first months, a review of the literature will be done in order to planning the trials. Literature about cultivation methods and the reproduction of *C. tomentosum* will be studied and analysed. The materials and methods will be described and all the support tools will be prepared.

Results

The experimental work on this task will start on M25

Discussion

The experimental work on this task will start on M25

Progress, deviations, problems & next 12M

Progress: 0% completeness.

Deviations & Problems: The experimental work on this task will start on M25.

Outlook: The experimental work on this task will start on M25.

CST 1.5 Biomass characterisation

Isabel Sousa Pinto/Gonçalo S. Marinho, CIIMAR

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
1.5	Biomass characterization	T3.2	CIIMAR, AlgaPlus, UFSC	0%	✓	M31	0	M48	0	4	6

Introduction

The macroalgae species investigated in CS1 are valuable biomass and food resources. Aquaculture has the potential to allow the sustainable production of this resources while ensuring the provision of biomass of reliable, traceable, and predictable quality. In this context, it is essential to evaluate the nutritional profile of the cultivated macroalgae, which may be significantly different and more stable compared to wild biomass. The analysis will include all the mandatory EU labelling nutrients for foods.

Methods

This task will start on M31. The analytical methods to be applied are yet to be discussed.

Results

This task will start on M31.

Discussion

This task will start on M31.

Progress, deviations, problems & next 12M

Progress: 0% completeness.

Deviations & Problems: This task will start on M31.

Outlook: This task will start on M31.

CS2

Summary of progress report for Case Study 2

Date of report:	**23.4.2020**
Case Study name:	**Offshore macroalgal cultivation**
of relevane for WPs	**WP1, 2, 3**

Abstract/Summary

Cultivated seaweed is one of the largest un-exploited global resources for the sustainable production of food, feed additives, industrial commodities, and petrochemical substitutes. Open sea-based cultivation of seaweed has huge potential. However, at present, there are challenges relating to expanding its cultivation. The algae beds and forests have important functions in the marine ecosystem; cultivated seaweed plots rapidly promote biodiversity, including many fish species. Unlike other seafood or livestock farming, seaweed does not rely on feed or fertiliser for growth – all the ingredients needed to sustain algae are in abundant free-supply in the ocean. To increase the Earth's NPP, we need to transfer the highly efficient NPP from algae beds to the large surface of the open ocean by open ocean seaweed production. Furthermore, brown macroalgae (kelp) is meant to be an important resource to improve and optimise feeding strategies for other low trophic species (e.g., abalone Spp.).

The main objective of case study 2 is to establish and prove large-scale offshore sustainable macroalgal production in the Faroe Islands and to test the feasibility of cross-Atlantic knowledge transfer for future upscaling. As well as developing the market and new seaweed products by implementing macroalgae in the feed for low tropic species (mainly abalone).

Case study 2 is focused on the analysis of the production of the macroalgae of the brown kelp species *Saccharina latissima* in the North Atlantic, with a specific study of the production in the Faroe Islands. The focus of this analysis is necessary as macroalgae cultivation in the Faroe Islands has successfully operated under offshore conditions for almost 10 years and has reached the commercialization stage. The cultivation methods used in the Faroe Islands have the potential to be replicated in other offshore areas of the North Atlantic, considering their specific conditions.

In this case study we will establish and prove large-scale offshore sustainable macroalgal cultivation in the Faroe Islands by using optimised logistics, re-use of aquaculture equipment, and site selection, to reduce the cost of production for the brown macroalgae *S. latissima*. We will test and develop alternative protein sources for the South African abalone by including *S. latissima* in the feed as an alternative dietary ingredient. Finally, through review and desk work we will select commercial interesting local macroalgal species for cultivation for a case study region and optimise the design of the cultivation rig based on the principles of the MacroAlgal Cultivation Rig and come up with a feasibility analysis of offshore macroalgal cultivation in that chosen area.

The key exploitable result of this case study found in the first year of the project was a technical report of suitable cultivation sites offshore in the Faroe Islands[10]. The work has involved several developing

[10] Report by Fiskaaling: Site selection for upscaled macroalgae production in open ocean environments in the Faroe Islands April 2020.

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement No 818173. This report reflects only the authors' view and that the Commission is not responsible for any use that may be made of the information it contains.

phases. A method for chosen target parameters was made and the GIS mapping of suitable sites was made in Python3. The final output can be used by seaweed farmers, governance, and other stakeholders.

The first year did also provide novel results for testing second-hand aquaculture equipment. The results showed that the components anchors, chains, and buoys are found to be most workable for re-use in macroalgal cultivation. Using second-hand equipment need to be less expensive than buying new equipment, and since the price of rope is relatively low, this is the case with second-hand lines and ropes. The use of second-hand equipment in the Faroes is most likely different from other countries where there is a less booming aquaculture industry. Finally, it was concluded that the more the seaweed industry will scale up, the more limited the reuse opportunities will be.

The key exploitable result for the work related to logistics and upscaling the testes seeding machine was still more time-consuming than manual seeding. Also, the mechanical harvesting machine still needs some modifications before it can be used commercially and compete with hand-cutting using a knife. It was suggested that underwater harvesting will be the most cost-efficient method, though it still requires a lot of innovation to be commercially used. The results showed that freshly harvested kelp biomass needs to be treated according to the end-use to reach a storage-stable stage in a short amount of time. The timing depends on the processing method. Furthermore, pre-processing is a critical process to preserve the quality of the biomass as deterioration is fast when the macroalgae are taken from the ocean. Finally, by processing the seaweed biomass through fermentation in bulk it is expected to add approximately 45% of the total potential value of the product.

CST 2.1 Find suitable sites for upscaled production in open ocean environments in the Faroe Islands with opportunities for higher yield, and organic certified cultivation.

Responsible CS Task Leader Gunnvør á Norði, Fiskaaling

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
2.1	Find suitable sites for upscaled production in open ocean environments in the Faroe Islands with opportunities for higher yield, and organic certified	T2.2	Fisk,ORF	100%	✓	M1	M3	M9	M12	3	7

Introduction

The industrial project partner Ocean Rainforest (ORF) has a pilot-scale operation with offshore macroalgal cultivation in the Faroe Islands. The cultivation has over the past years been restricted to licenses of local fish farmers and not been subject to qualitative site selection. The scope of this task is to identify relevant areas in the Faroese seas to be able to upscale the macroalgae production.

A GIS map will be compiled describing suitable sites for offshore macroalgae cultivation systems in open ocean environments in the Faroe Islands. The map will be based on existing current and wave models and available bathymetry. The sites will be selected based on established criteria for depth, current speed, wave height, and distance to pollution sources, marine traffic, and recreational areas.

The goal within the first 12 months of work in this Case Study Task was to:

- Establish criteria for site selection in the Faroe Islands.
- Conduct a GIS map with all relevant parameters using input data on current, wave height, etc. that is needed.
- Make a report describing selected parameters and map outcomes aiming at relevant stakeholders.

Methods

To select the most suitable sites, a set of parameters have been set up, that describe how the conditions should be to sustain farming. There are three main parameters: wave height, current speed, and bottom depth.

Firstly, the sites that meet these requirements will be identified, then using the remaining parameters; distance to fish-farming sites, cities, and harbours will eliminate any site that potentially might be in polluted areas.

To evaluate the **wave height** at the different sites, the results from the simulations run by Niclasena and Simonsen, 2012[11] using the SWAN model will be used. Because it is questionable how well the wave model handles reflections[12], only the runs that have no recollections are used. Thus, extra care should be taken when interpreting results from sites that might appear as sheltered in the simulations, where waves might hit a large surface, near deep waters and reflected waves from the surface might hit the site. An example of such a sight might be Funningsfjørðu, where waves coming from the northwest might hit the island of Kallsoy, and the reflections from the island might hit the site.

The requirements specify that the highest significant wave height should be less than 6m. The definition of wave height as the mean of the highest one-third of the waves in the wave record[13], significant wave height is denoted as H1=3. The significant wave is readily estimated from the wave spectrum as Hm0. Hm0, and is usually 5% to 10% higher than H1=3. As the difference in wave height is so minor, Hm0 will be used. This is also the output parameter of the simulation.

The forcing sea state was determined to be the average sea state on the model border which had the highest wave height and waves travelling into the model through that border. All forcing sea-states were binned into whole numbers in wave height and period and 15° intervals in wave direction. The model has a spatial resolution of 10 km[1], using 32 frequencies and 24 directions.

To find the highest wave height at each position, the files containing the results were read into Matlab©, and for each position, the wave height from the different wave directions and frequencies were compared, and the heights were found. This was done for each point. As the original bottom matrix was not found, the resulting grid was then transformed, stretched, and rotated to fit key locations on land. This process is not perfect, and some of the points are slightly off point.

The **current** simulations are based on data from a numerical simulation of the barotropic tides, which are validated towards available current and elevation measurements[14] (Figure 2.1.a). The constituents that are used are: M2, S2, K1, N2, O1, and M4. The simulation outputs a 200m x 200m grid, describing the Phase, Inclination, and Major and Minor components of each constituent. For each point of the simulation output, the contribution of each constituent c is calculated at the time t in the north and west directions.

[11] B. A. Niclasen and K. Simonsen, "High resolution wave climate for the Faroe Islands," tech. rep., Fróskaparsetur Føroya, 2012.

[12] B. A. Niclasen, "Technical information related to hindcast forced wave climate estimation for the Faroe Islands," tech. rep., Fróskaparsetur Føroya, 2012.

[13] L. H. Holthuijsen, Waves in Oceanic and Coastal Waters. Cambridge University Press,

[14] K. Simonsen and B. Niclasen, "On the energy potential in the tidal streams of the faroe islands," Tech. Rep. 1, University of the Faroe Islands, 2011.

$$u_c(t) = \text{Maj}_c \cdot \cos(\text{t} - \text{Pha}_c) \cdot \cos(\text{Inc}_c) - \text{Min}_c \sin(\text{t} - \text{Pha}_c) \sin(\text{Inc}_c)$$

$$v_c(t) = \text{Maj}_c \cdot \cos(\text{t} - \text{Pha}_c) \cdot \sin(\text{Inc}_c) + \text{Min}_c \sin(\text{t} - \text{Pha}_c) \cos(\text{Inc}_c)$$

Combining the contribution from each of the different constituents results in a description of the current for each position.

$$u(t) = u_{\text{M2}}(t) + u_{\text{S2}}(t) + u_{\text{K1}}(t) + u_{\text{N2}}(t) + u_{\text{O1}}(t) + u_{\text{M4}}(t)$$

$$v(t) = v_{\text{M2}}(t) + v_{\text{S2}}(t) + v_{\text{K1}}(t) + v_{\text{N2}}(t) + v_{\text{O1}}(t) + v_{\text{M4}}(t)$$

Since the current direction is not an issue in this case, but only the current speed, the description for each direction can be combined:

$$s(t) = \sqrt{u(t)^2 + v(t)^2}$$

Now that we have a description of the current in one position, we can insert the values for time to estimate the maximum current in the specified time frame:

$$S = \max\left(\{s(t) \,|\, t = 0, 0.25, \ldots, 24 \cdot 365\}\right)$$

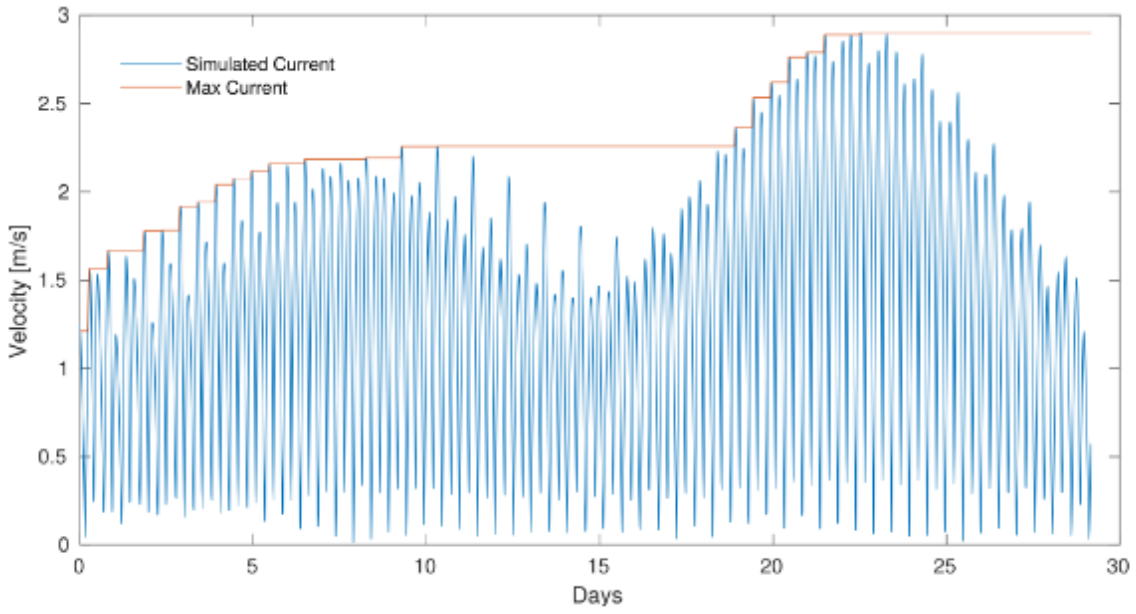


Figure 2.1.a: Current simulation for 30 days for one point near Kirkjubønes. Blue line is the velocity of the current s(t), red line is the highest current speed simulated up to each point max (fs(t) j t = 0; 0:25; : : : ; xg).

ROMS outputs one set of constituents for each point on the simulation. Thus, there is a description of $s_{x;y}(t)$ for each point *x; y*, and the maximum current speed can be calculated for each of these points.

$$\text{S}[x, y] = \max\left(\{s_{x,y}(t) \,|\, t = 0, 0.25, \ldots, 24 \cdot 365\}\right)$$

After defining the maximum current at each position, the next step was to find sites where the

current is high enough to bring in new nutrients and low enough to ensure that structures and seaweed do not get damaged. The values that were used to determine suitable currents were between 0.1 and 0.5 m/s. It is important to note that the currents in these simulations were only due to tidal forces, and do not consider wind-driven currents.

Due to **the depth requirements**, it is necessary to find the depth at each location. Unfortunately, there exists no single dataset that covers all the depth data that is collected around the Faroe Islands. Instead, a dataset has been compiled with all the depth data that is available to Fiskaaling. This combined dataset consists of the following data.

- LV2000
- Magnus Heinason 1999 Track
- Magnus Heinason 2000 Track
- Magnus Heinason 2001 Track
- Magnus Heinason 2002 Track
- Magnus Heinason 2003 Track
- Magnus Heinason 2004 Track
- Magnus Heinason 2005 Track
- Magnus Heinason 2006 Track
- Magnus Heinason 2007 Track
- Magnus Heinason 2008 Track
- Magnus Heinason 2009 Track
- Tjaldri 2000 Track

In combination, these datasets included over 17 million data points. The LV2000 dataset was the

only dataset that was specifically produced to generate a complete picture of the seabed. This

dataset included data from inside most of the fjords at quite high resolution, but unfortunately, it did not cover every fjord and straight, and at many fjords, there was no data from near the entrance of the fjord. Therefore, the data from the other sources were included to increase the coverage area.

The following GIS work was written in Python3.

Results

In April 2019, the criteria for offshore macroalgae site selection in the Faroe Islands were established. The parameters that were used in the first round of locating potential sites are based on depth requirements and estimates on the strain the offshore farming equipment can manage[15].

- Significant wave height should be less than 6m.
- The current speed should be between 0.1 and 0.5 m/s.
- The bottom depth should be between 50 and 150m.

[15] U. G. Bak, A. Mols-Mortensen, and O. Gregersen, "Production method and cost of commercial-scale offshore cultivation of kelp in the Faroe Islans using multiple partial harvesting," Algal Research, vol. 33, pp. 36–47, 2018.

After finding sites that meet the above-mentioned requirements, the following distances should be considered to further narrow down what sites are suitable.

- Distance to fish farming sites.
- Distance to cities and harbours.
- Distance to important tourist sites.
- Distance to shipping lanes.
- Distance to protected areas.

Results of wave heights in Figure 2.1.b.

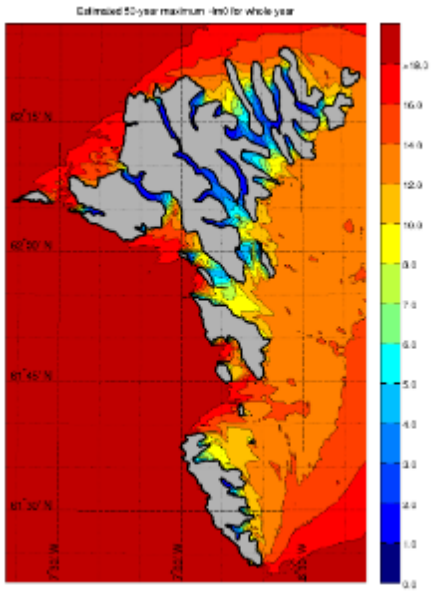


Figure 2.1.b: Example of a result from the SWAN simulations [16].

The "current" results are a matrix that shows the max current for each position in a one-year timeframe in Figure 2.1.c.

[16] B. A. Niclasen, "Technical information related to hindcast forced wave climate estimation for the Faroe Islands," tech. rep., Fróskaparsetur Føroya, 2012.

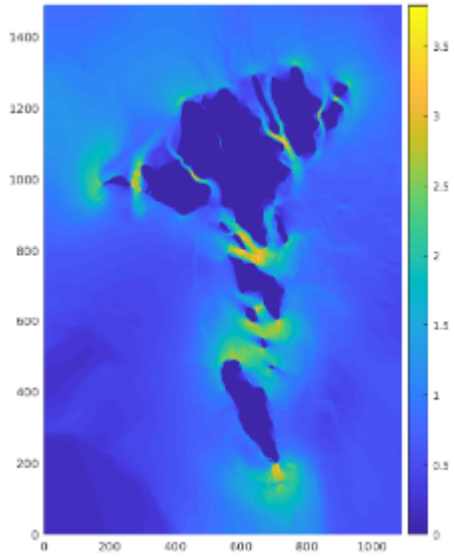


Figure 2.1.c: Max current in a one-year timeframe with 15 minutes increments at each of the simulation outputs.

The resulting depth dataset covers the most fjords, including the entrance, and out to sea in Figure 2.1.d.

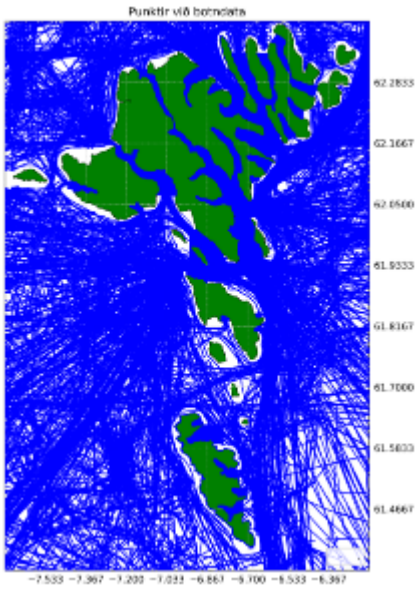


Figure 2.1.d: Datapoints containing depth information.

In November 2019, several GIS maps showing sites that meet the criteria for depth, current speed, and wave height were developed. One map encompasses the whole of the Faroe Islands. On this map, a few single points can be seen scattered around. These should mostly be ignored, as they are an artifact of the method that is used to highlight possible sites. Because the method is chosen to find possible sites that only needed a single simulation output to be inside each of the 200x200m grid points, a single simulation output can trigger a false positive. Some examples of sites that include these false positives are the points along with Kallsoy points North of Sandoy, and the points along Vestmanna straight. Other single points might be suitable, but in the following sections, only a larger collection of points will be further discussed.

Fish farmers are free to position their equipment within their farming sites. In this report, it is assumed that fish farmers position their equipment at the border of their areas. This is done to show potential areas for macroalgae production that will not be affected by fish farming, according to the 500 m distance requirements. A green line is drawn on the map 500m from the nearest fish farming area where it is applicable. Points that satisfy the environmental parameters but are within this line are marked with black to show that any macroalgal production in this area must be coordinated with fish-farmers.

Lobster fishing is done in many fjords and straights in the Faroe Islands. There is a risk that lobster traps will get tangled with potential production structures. It is recommended that lobster fishermen are contacted and asked if any planned production facility might affect their production, and how this can be minimised.

Every dot is the centre of a 200m x 200m box where depth, wave height, and current speed requirements for macroalgae cultivation are met in Figure 2.1.e. An example of a close-up map showing Funningsfjørdur in Figure 2.1.f.

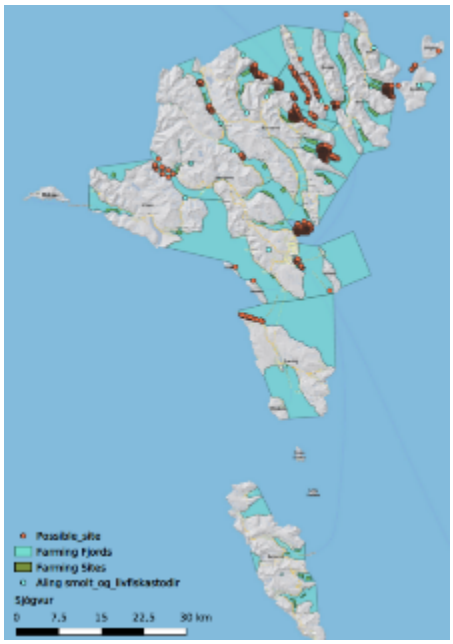


Figure 2.1.e: Potential sites for macroalgae cultivation.

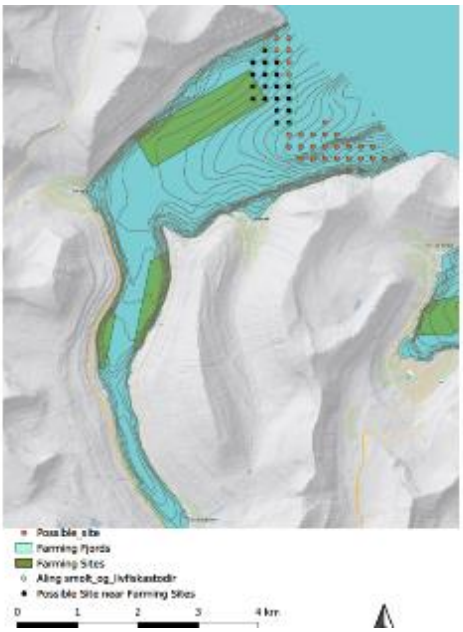


Figure 2.1.f: Result of site selection for Funningsfjørdur, the Faroe Islands.

By April 2020, a report describing parameters for site selection and map outcome was completed. The report is highly relevant for stakeholders as Government, seaweed farmers, the aquaculture sector in general, and investors. As it contains confidential information it is not publicly available but is available to project partners and shared with relevant stakeholders.

Frontpage of report deliverable in Figure 2.1.g.

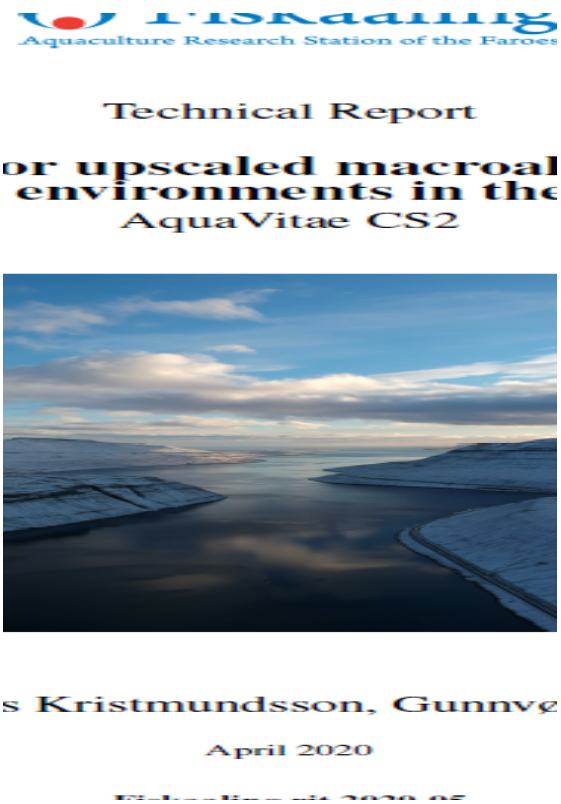


Figure 2.1.g: The technical report on site selection for upscaled macroalgae production in open ocean environments in the Faroe Islands made by Fiskaaling, April 2020.

The data management plan was made, and the expected data set name is: "Mapping the sea around the Faroe Islands to identify suitable cultivation site". The dataset will be owned by Fiskaaling, Ocean Rainforest, CS2, and WP2. Input sources are all from other available sources outside the project: Temperature data, Depth data, Wave data, Nutrients data, Current data, Marine use data. The datasets generated was:

1) Map of suitable sites.
2) Model using in Python3

Discussion

The Faroese legislation finally allows seaweed cultivation in Faroese waters. In the past, ORF used one of the (unused) licenses of the major salmon producer Bakkafrost to cultivate seaweed in a Faroese fjord. In July 2020, seaweed licenses were finally obtained. The licenses that were applied for by ORF were based on the outcome of this task result. ORF expects to obtain more licenses in the Faroe Islands in the (near) future and will base its application on sites suggested in task 2.1. For the industry, it is very important to have their own licenses to scale up the business and to attract investors.

The presented results are a major step for the seaweed producing company ORF to expand their operation. The maps and suitable cultivation sites identified are of course made from simplifications of the factors that influence seaweed farming in the real world. This means that some of the suggested sites may be insufficient for open ocean macroalgal production, but this will first be detected when *in situ* testing has been shown. Likewise, will there maybe be other sites that the model did not include that could be suitable cultivation sites, for example where the depth model has been lacking.

Progress, deviations, problems & next 12M

Progress: Task 2.1 is a 100 % completed. Still, the work will be disseminated and knowledge shared with relevant stakeholders. As well as implementing the gained results in the upscaling strategy of ORF.

All activities have been completed on time and the **key exploitable result** of this task is:

- Method for chosen target parameters.
- Codes used in Python3.
- Suitable sites can be used by seaweed farmers, governance, and other stakeholders.

Deviations & Problems: None

Outlook: Future activities will be further dissemination and exploitation of results found in CST 2.1 and know-how transfer to the work in CST 2.6.

Besides these activities, the task has been completed.

Figure 2.2.a: Aquaculture equipment to old or small for the fish farming industry, however useful in seaweed cultivation.

CST 2.2 Test and demonstrate large-scale re-use of fish equipment in macroalgal cultivation.

Responsible CS Task Leader Floor Marsman, Ocean Rainforest

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
2.2	Test and demonstrate large-scale re-use of fish equipment in macroalgal cultivation	T2.2	ORF, FISK	67%	!	M1	M1	M12	0	6	6

Introduction

The use of second-hand equipment from other marine industries has been suggested as one method to sustainably reduce installation costs (Figure 2.2.a). Instead of constant production of new goods which are used a limited number of times before being discarded, we are investigating how products can be reused so that they stay in use for a longer time. In this way, both the amount of waste and pollution is reduced as well as the investments in capital, which can be prohibitive for a pioneering industry. Understanding the opportunities and methods for the use of second-hand material is a fundamental part of building a circular economy that adds value to waste (Figure 2.2.b).

In the case of the Faroe Islands, salmon farming is a large industry and growing bigger every year. The upscaling of the salmon farming industry is an opportunity for smaller-scale ocean operators, like ORF, to re-use fish-farm equipment that has become too small.

Figure 2.2.b: Understanding the opportunities and methods for the use of second-hand material is a fundamental part of building a circular economy that adds value to waste.

The goal within the first 12 months of work in this Case Study Task was to:

- Buy/find used aquaculture equipment that could fit in the MACR design.
- Deploy 70,000 m growth line with 5 % re-used equipment.
- Evaluate the growth and economy of re-used material and the risks related to this.

The cultivation system is called the Macroalgal Cultivation Rig, or in short MACR (Figure 2.2.c). The design consists of a 500m long fixed line suspended horizontally 10 m below the sea surface. The mooring system consists of four anchor lines that are attached to the fixed-line and anchored to the seafloor with 700 to 1500kg steel anchors. Attached to the horizontal line vertical grow lines are provided uplift with 1-meter spacing and a buoy in the end.

The MACR relies on ropes, buoys, and anchors in its design – thereby offering simple and flexible structures that move with the ocean instead of resisting it.

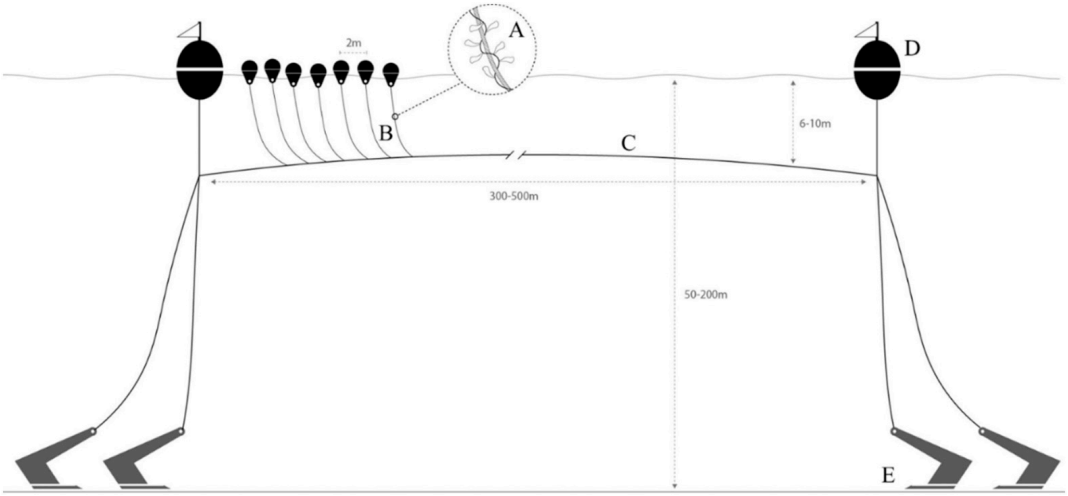


Figure 2.1.c: Schematic drawing of a Macroalgal Cultivation Rig (MACR) constructed by Ocean Rainforest Sp/f. The construction can be deployed for macroalgal cultivation at wave-exposed sites with a water depth of 50–200 m. Seed lines (A) are twined around growth lines (B) that are attached at 2-m intervals to the fix-line (C) by a loop and held in a vertical position by a buoy. Two main surface floats (D) and four steel anchors (E) ensure the right position of the rig.

Methods

In the first 3 months of the project ORF search for discarded aquaculture-equipment around the Faroe Islands.

One rig consisting of 8 main lines and 40,000 m seeded lines were deployed in October 2019 in which used aquaculture equipment is incorporated (Figure 2.2.d and 2.2.e).

ORF has assessed elements of the MACR whether it was suitable or not for the use of second-hand material. And a cost evaluation based on the optimal use of second-hand equipment.

In the period from February to May 2020, the growth performance and logistics related to re-used material were monitored and an economic evaluation of the cost-reduction vs. risks was conducted.

Figure 2.2.d: Handling of second-hand 1.5 tonnes steel anchors on the vessel Tongul in Funningsfjørdur.

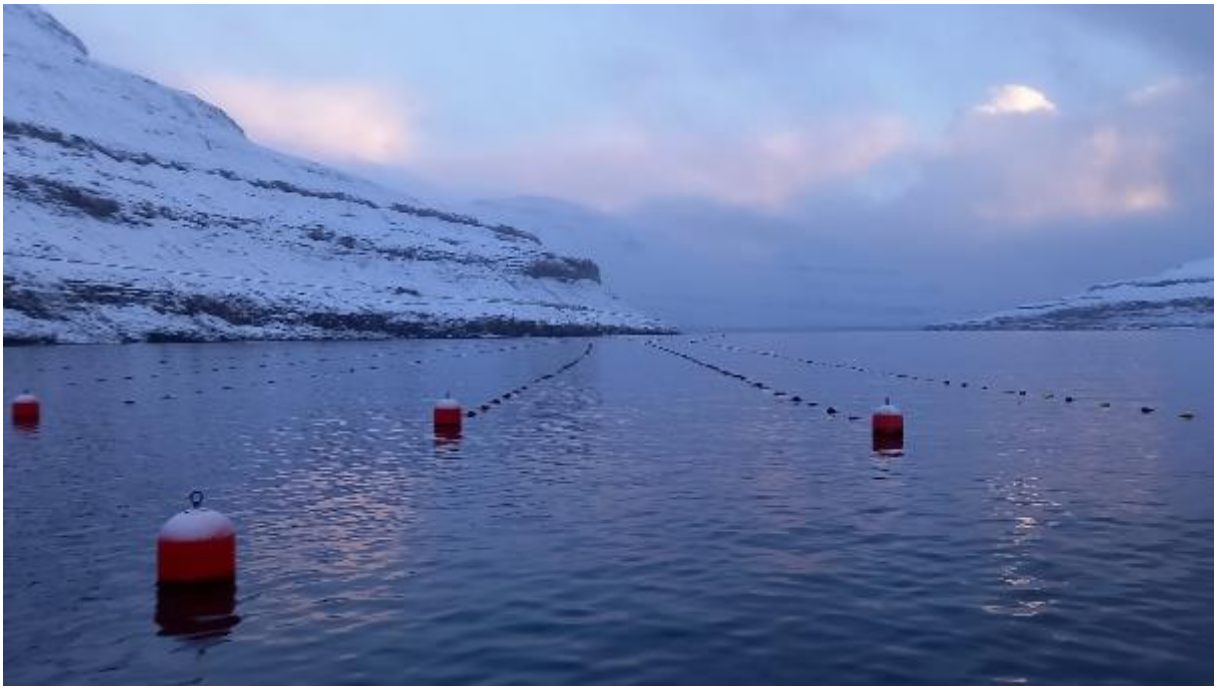


Figure 2.2.e: One rig consisting of 8 main lines and 40,000 m seeded lines was deployed in October 2019 in which used aquaculture equipment is incorporated.

Results

We were investigating how existing equipment can be re-used so it can stay in use for a longer time. In figure 2.2.f, all components of a MACR are shown and their percentage share of the whole costs of the MACR. The biggest cost items are anchors (17%), mainline floats (16%), seed material (16%), grow lines (15%) and anchor buoys (11%), and anchor chains (5%).

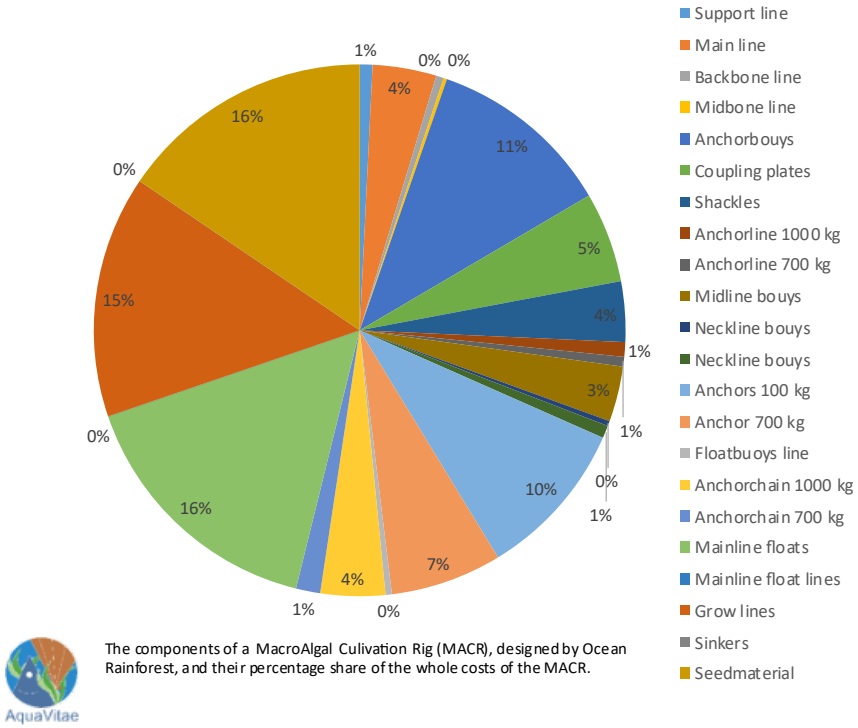


Figure 2.2.f: All components of a MACR is shown and their percentage share of the whole costs of the MACR.

From those components anchors, chains and buoys are found to be most workable for re-use in macroalgal cultivation. These elements often have a longer lifetime than used in the other marine industries. They are rejected because they have become too small for the other marine industry or because the required quality can not be guaranteed any longer for the ways it is used in the other industry, while the quality is good enough for the ORF purposes (Figure 2.1.g).

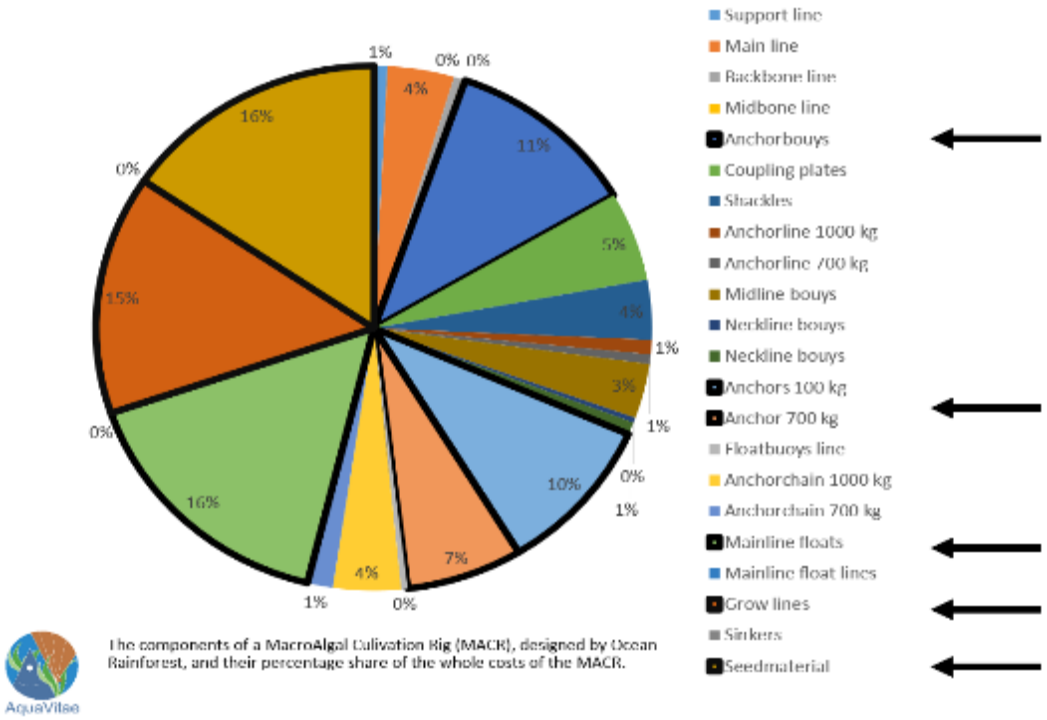


Figure 2.1.g: All components of a MACR is shown and their percentage share of the whole costs of the MACR. The highlighted components are suitable for re-use assessment per element and cost evaluation.

Ropes, on the other hand, both grow lines and main- and backbone lines, are a lot more difficult to re-use because of two reasons: 1) The lines need to be clean, and 2) the line strength needs to be assured. Proper cleaning and rinsing of lines is very laborious, and therefore not scalable nor economically feasible (wages are high). Using second-hand equipment need to be less expensive than buying new equipment, and since the price of rope is relatively low, this is the case with second-hand lines. The strength of the second-hand equipment should comply with the required strength of equipment used in the ocean, for both safety and insurance matters. Second-hand ropes might not comply with these requirements so, in case of re-use of line, strength tests need to be done.

The price of second-hand anchors, chains, and coupling plates is calculated by their weight multiplied by the price of scrap steel (1.5 DKK per kg). The price of second-hand buoys and floats is calculated by a discount of 50%. We investigated a scenario in which there is optimal use of second-hand material, so 100% use of second-hand equipment in the above-mentioned categories.

*Figure 2.2.h: Cost evaluation of the components used in a MACR. All new scenario vs. optimal re-use scenario. Optimal re-use scenario: 100% re-use of anchors, chains, mooring plates, buoys, and floats. Steel elements: Weight (kg) * price of scrap steel. Second-hand buoys and floats: 50% discount on the new price.*

In an optimal scenario (with 100% re-used equipment) there is a cost reduction of 41% (Figure 2.2.h).

As already mentioned, the use of second-hand equipment is not only favorable for the costs of the rig, but it also reduces the production of new materials, and thereby it reduces the burden on the environment.

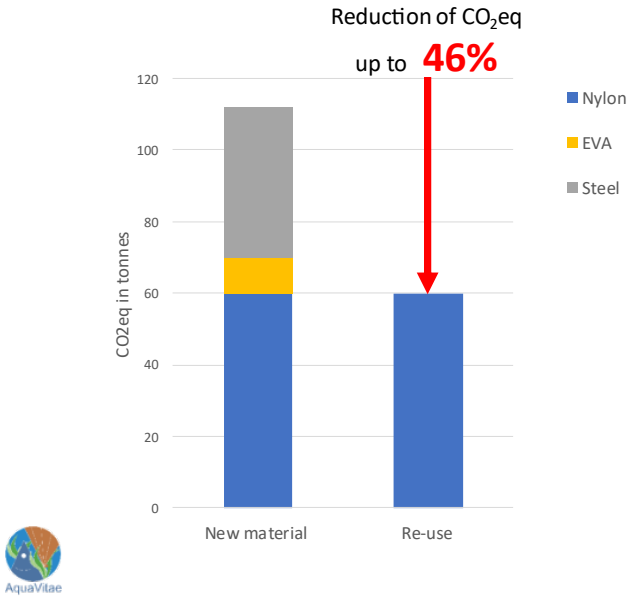


Figure 2.2.i: CO2eq reduction from the scenario with re-used steel (anchors and chains) and EVA plastic (buoys), but with new nylon ropes.

One way to quantify the impact on the environment is to calculate the CO_2 equivalent per element, by multiplying their weight by the emission factor of their material. The elements of the rig have been categorized in the categories steel, nylon, and EVA plastic.

With the assumption that all steel elements, buoys, and floats can be used second-hand, we can very generally state that there is a CO_2eq reduction up to 46% (Figure 2.2.i).

Discussion

The use of second-hand material can have large contributions to both price and environmental impact reductions. Steel elements like anchors, chains, and coupling plates, together with buoys and floats are found to be most suitable for reuse.

Unfortunately, scenarios are never optimal, therefore the numbers used here likely be a lot lower. Also, it has to be taken into account that how we experienced the use of second-hand equipment in the Faroes will most likely differentiate from different countries where there is a less booming aquaculture industry. There is no standard manual for the use of second-hand equipment in macroalgal cultivation. However, this knowledge can be used as inspiration for other seaweed cultivation companies, but in all separate cases, it needs to be assessed whether re-use is an option and in what way. Lastly, the more the seaweed industry will scale up, the more limited the reuse opportunities will be.

This work has gone beyond the state of the art by demonstrating the large-scale re-use of fish equipment in macroalgal cultivation, which has not been reported in the literature earlier.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained so far, we have completed 67 % of the assigned task as 40,000 m growth lines have been deployed on cultivation structures with re-used aquaculture equipment. However, we still need to test this on another 40,000 m growth line and disseminate these results.

The **key exploitable result** of this task was:

- From those components anchors, chains and buoys are found to be most workable for re-use in macroalgal cultivation.
- Using second-hand equipment need to be less expensive than buying new equipment, and since the price of rope is relatively low, this is the case with second-hand lines.
- The use of second-hand equipment in the Faroes will most likely differentiate from different countries where there is a less booming aquaculture industry
- The more the seaweed industry will scale up, the more limited the reuse opportunities will be.

Deviations & Problems: According to former experiences, ORF used less second-hand equipment in the deployment of the new rig than initially thought, for example, was ropes purchased from new. Also, ORF did not yet receive a license to deploy more rigs at different sites, so fewer rigs (and less seeded lines) have been deployed than initially thought.

Outlook: After licenses have been received (expected soon), it is expected to deploy 120,000-meter growth lines in 2020 at the Faroe Islands, which we expect to have re-used equipment in the rig structure. Other coming activities will be further dissemination and exploitation of results found in CST 2.2 and know-how transfer to the work in CST 2.7.

Besides these activities, the task has been completed within the planned time.

CST 2.3 Improve logistics to ensure low-cost handling and high-quality storage stable macroalgal biomass.

Responsible CS Task Leader Ólavur Gregersen, Ocean Rainforest

CST #	Name	Reports to task(s)	Partners involved	complete d by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
2.3	Improve logistics to ensure low-cost handling and high-quality storage stable macroalgal biomass	T2.2	ORF	75%	✓	M4	M4	M30	0	7	7

Introduction

CST 2.3 aims to improve logistics (seeding, harvest, landing) to ensure low-cost handling and high-quality storage of stable macroalgal biomass (value chain and hot spots in Figure 2.3.a). Also, we aim to provide relevant insights from the current value chain, processing, and market conditions in the macroalgae production in the North Atlantic, to identify pathways to improve competitiveness, by increasing efficiency and reducing costs.

The analysis will focus on the production method developed and applied by ORF in the Faroe Islands and their current value chain. This analysis will be the foundation for coming work in CST 2.7 focusing on profitability, socioeconomic impacts, and business plan development.

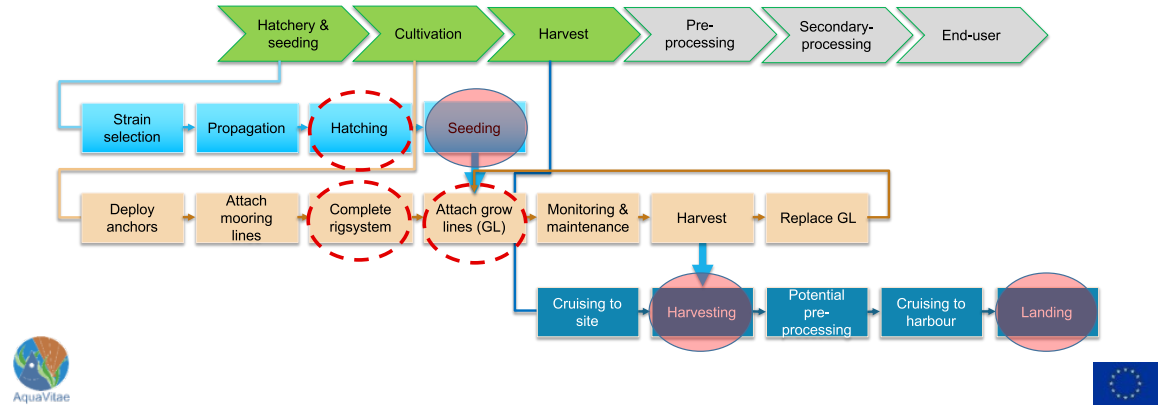


Figure 2.3.a: Logistical «hot spots» in the supply chain. Red circles indicating the main focus of this task: seeding, harvesting, and landing.

The successful pilot-scale operation of an innovative large-scale kelp cultivation system under nearshore exposed conditions has proven robust enough under highly energetic conditions by AV industry partner ORF in the Faroe Islands since 2010. The rig structure was designed to operate even further offshore[17] and it is ready to scale up.

With a scientific and innovative approach to macroalgal farming, ORF has tried out for several years a non-destructive cultivation method that allows them to harvest up to 6 times during the productive season without re-seeding, called multiple partial harvesting (Figure 2.3.b). This method has produced the highest known harvesting yield per meter of a seeded line in Europe so far, with the potential to reduce the production cost by 75%.

[17] U. G. Bak, A. Mols-Mortensen, and O. Gregersen, "Production method and cost of commercial-scale offshore cultivation of kelp in the Faroe Islans using multiple partial harvesting," Algal Research, vol. 33, pp. 36–47, 2018.

Figure 2.3.b: Manual harvesting of Saccharina latissima in Funningsfjørður, Faroe Islands, August 2016. Photo by Anja Mazuhn.

However, this promising industry still faces many challenges, which reflects on the novel state of the macroalgal industry in the West. Even more cost-efficient methods are needed (from seeding and deployment to harvest), further technological developments, and an adequate and updated regulatory framework are necessary to enable the profitable operation of macroalgal aquaculture in non-traditional producing countries to consolidate themselves alongside a well-established and mature industry in Asia.

This task was initiated in September 2019 and the goal of the first 12 months of work within the project was:

- To test 1 prototype of the seeding machine.
- To test 1 prototype of the harvesting machine.
- To initiate the work related to landing, transportation, and processing of macroalgal biomass.

However, innovation is moving fast in the company ORF, and also work planned for the second year of AV has been completed within the first 12 months.

Methods

Through a fruitful collaboration with the companies, IHC and Hortimare, who have developed a mechanical seeding machine and a mechanical harvesting machine optimised logistics were tested in Autumn 2019. The two machines were shipped to the Faroe Islands from the Netherlands. The test of the seeding machine took place in the factory of ORF in Kaldbak and the test of the mechanical harvesting machine was done at sea.

Planning of efficient landing, transportation, and processing system was initiated as scheduled and small internal workshops were made to discuss the innovation process.

Results

Improving seeding and deployment

In November 2019, a seeding device developed by IHC was tested at the factory of ORF (Figure 2.3.c). The seeding machine was not suitable for the currently used growth line system at ORF, mainly because of the use of pre-cut growth lines instead of one long line. Seeding with the seeding machine was still more time-consuming than manual seeding.

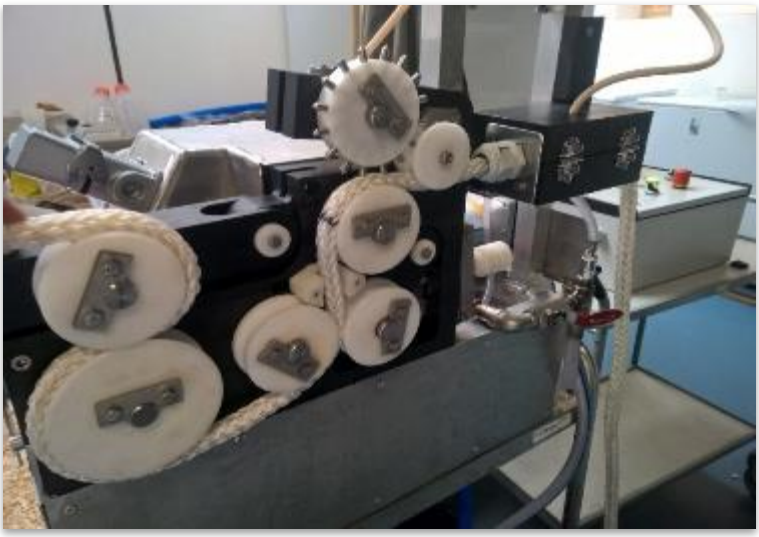


Figure 2.3.c: A mechanical seeding device developed by IHC and Hortimare.

The cultivation of the kelp *S. latissima* begins with the production of seeding material from spores. Seeding methods differ between Asia and what is done by ORF. ORF seed with free-floating sporophytes (grown in culture flasks) and do not use the thin seed lines which are twisted on coils (see old picture from ORF 2016 in Figure 2.3.d).

Figure 2.3.d: Sporophytes seeded on coils and nursed in the hatchery for 3 weeks (out-dated method).

The direct seeding method developed by project partners of a former EU funded called AT~Sea has been further developed by ORF. Instead of a thin seed line that is twisted around a growth line, we use glue to direct seed the sporophytes on ropes the day before deploying them at sea. This requires less capacity in the hatchery and has a lower risk of contamination (Figure 2.3.e).

Figure 2.3.e: Seeding material in the culture flask. Sporophytes under micoscope. Direct seeding using a glue and sporophyte mixture. Seeded lines deployed at sea.

The breeding process is done through a commercial arrangement between ORF and the company Hortimare in the Netherlands. Spores are released under controlled conditions. The gametophytes are

nursed in vitro for 3-4 months in the company's laboratory facilities until sufficient biomass is reached (Figure 2.3.f). This process needs to be done once every year.

Figure 2.3.f: Hatchery container in Kaldbak, the Faroe Islands. Picture of Elsa Berg, hatchery manager, and Urd G. Bak, Research manager.

The gametophytes develop into juvenile sporophytes within 2 to 4 weeks under white light. Juvenile sporophytes are then seeded on 12-mm lines using a binder mixture.

A milestone was achieved in October 2019 where ORF produced seeding material for a production of 150-300 tonnes wet weight (to be harvested in 2020). This was mainly possible due to trained staff and easy access to fertile parent algae.

Deployment of lines

After being directly seeded the growth lines are transported to the farm site. Seeded lines are transported by land from the hatchery to the port, where they are transferred to the vessel, which transports them by sea to the farm location. A skipper and two operators are needed for this process. At the farm site, the growth lines are attached along the fix-line.

Improving harvesting

Open water farms commonly use longlines where seed strings are deployed, and the kelp thali will grow[18]. In the Faroe Islands, the benefit of clear water and high light penetration allows vertical growth lines of 10 meters and thus expands the suitable cultivation area from 2D to 3D. The methods used in Asia do not fit the Faroese methods, as 1) the lines are kept in the sea for several years and partial cutting is crucial for the algae to survive on the lines. But also, the growth lines are 10 meters and not a continuously long line. The design of a harvesting machine, therefore, has to be special designed for ORF.

[18] Kim, J.K., Yarish, C., Hwang, E.K., Park, M. and Kim, Y. 2017. Seaweed aquaculture: cultivation technologies, challenges, and its ecosystem services.

Figure 2.3.g: The mechanical harvesting machine by Royal IHC was tested at the cultivation site of ORF in November 2019.

The mechanical harvesting machine was tested at the cultivation site of ORF in November 2019 (Figure 2.3.g). This prototype still needs some modifications before it can be used commercially and compete with hand-cutting using a knife.

Table 2.3.a: Cost comparison between four harvest scenarios at ORF; [1] summer of 2019, [2] current situation (the expected situation for harvest season 2020 including harvest machine), [3] new situation (improved harvest machine), and [4] underwater harvester (implementation of a harvester that can harvest lines without taking them up from the sea, launch within a few years from now).

Harvesting scenario	Summer 2019	Current situation	New situation		Underwater harvester
harvest speed (lines/hour)	17	30	60		240
length lines (m)	7	10	10		10
yield (kg ww/m)	6	6	6		6
harvest hours per day	21	21	21		21
yield (kg/vessel/day)	14994	37800	75600	> x19	302400
crew (persons/vessel)	3	2	2		3
harvest period (days)	120	120	120		120
salary (€/hour/person)	30	30	30		30
crew costs (€/day/vessel)	2160	1440	1440		2160
vessel costs (€/day)	1500	1500	1500		3000
total costs (€/day)	3660	2940	2940		5160
costs (€/kg)	0.24	0.08	0.04		0.02
biorefinery requirements (kg/day)	240000	240000	240000		240000
required vessels	16	6	3		1
total costs (€/day)	**58583.43**	**18666.67**	**9333.33**	- 86 %	**4095.24**

ORF uses the multiple partial harvesting method, currently, this is a manual process where the seaweed is cut with a knife. This is a non-destructive harvesting method that aims to ensure regrowth of the thali[19]. The goal is to mechanize this process, by keeping the non-destructive practice, as it will increase the harvesting capacity over tenfold. This will require investment in vessels with tailor-made equipment. Mechanisation is also relevant to control biofouling, which can constitute an issue when cultivating macroalgae (Picture of the seaweed underwater in Figure 2.3.h). Biofouling can be controlled through adequate site selection, the timing of harvesting periods, and effective harvesting technics[20]

[19] Bak, U.G., Mols-Mortensen, A. and Gregersen, Ó. 2018. Production method and cost of commercial-scale offshore cultivation of kelp in the Faroe Islands using multiple partial harvesting. *Algal Res.* 33:36–47.

[20] Visch, W., Nylund, G. and Pavia, H. 2020. Growth and biofouling in kelp aquaculture (Saccharina latissima): the effect of location and wave exposure. *J. Appl. Phycol.*

The harvested biomass is contained in plastic containers and transported by sea to the port and then by land to the processing facilities (Figure 2.3.i). Ideally, land transportation should be avoided, when processing capacity is improved, the biomass can reach these facilities directly by the sea. As the quality of macroalgae biomass decreases quickly when extracted from the ocean, transportation to storage facilities should be swift and to ensure better quality the biomass should achieve a storage-stable stage promptly. The mechanization of the harvesting processes and improving transportation methods, avoiding land transport to processing facilities, will contribute to this end.

Figure 2.3.h: The growth on the seeded lines seen underwater.

Figure 2.3.i: Harvested seaweed ready for transporting to processing factility.

Improving Biomass Treatment

Kelp species can be processed in multiple ways (for example dry, freeze, fermented, pellets, powder, etc.), where the key point is to achieve a storage-stable state, that preserves the qualities of the macroalgae according to the needs of the end-user. Macroalgae can be used for biofuels or bioenergy, where the biomass may require very little transformation and be sold in bulk. Further processing will be required for the food, feed, pharmaceutical, and cosmetic industries, where additional requirements and quality controls might be needed. The biomass treatment is a critical point in the production process, as the harvested macroalgae require to be treated and processed quickly, due to the risk of deterioration. Freshly harvested kelp biomass needs to be treated according to the end-use

to reach a storage-stable stage in a short amount of time. The timing depends on the processing method.

In the case of ORF processes, the harvested biomass is among others sold as an additive for pig feed. The pig farming industry in North Europe is highly interested in seaweed additives since they have proven to have several benefits in the pig's digestion, reducing the need for antibiotic treatment, reducing piglet mortality rates, and increasing weight at the time of slaughter, factors that have a major positive impact in the economic performance of pig farms. This type of processing for animal feed additive can be expanded to the market of farmed cattle and other rearing herbivores; additional trials are undergoing. For this type of end-use, the macroalgae biomass is fermented and sold in bulk, to be incorporated into the pig feed. A small portion of ORF production is dried, however, that type of processing is only secondary.

Figure 2.3.j: Equipment installed for better logistics of the processing of seaweed.

Improving the pre-processing

Washing and screening for debris. Remove rocks, snails, and other entangled objects. First, the seaweed is ground into a 1mm pieces soup, then it is mixed with the bacteria and sugar solution, and then it is stored in airtight IBC containers of 1-tonne capacity. Pre-processing is a critical process to preserve the quality of the biomass as deterioration is fast when the macroalgae are taken from the ocean. The biomass must reach a storage-stable state in a short period.

Secondary-processing

Lactic acid bacteria and sugar solution are then added to the ground wet biomass to bring pH levels down from 7 to 4 and make the product storage stable. This fermentation process requires about 1-2 weeks and does not require additional energy consumption from the processing facilities.

The seaweed is ensiled and ready to transport to the buyer, in this case, a pig feed processing company located in Denmark (Figure 2.3.k).

Figure 2.3.k: Fermented seaweed ready for shipping to customer.

Transportation and Distribution

The ensiled product is transported by a hired transportation service to the port where the end product is shipped via sea to the pig feed producer located in Denmark, which is a natural market for the Faroe Islands.

Currently, the product is delivered with transport and duty paid (DDP incoterms) to the buyer's factory.

The company ORF participates in the seaweed value chain until this point. The product is sold in bulk to be further processed by the buyer. During the process little to no waste is expected, as the biomass is fully utilized in the fermentation process and no by-products remain. This type of processing does not require intensive energy utilization as drying or freezing the biomass, making the fermentation a more cost-efficient process. By processing the seaweed biomass through fermentation in bulk, ORF is expected to add approximately 45% of the total potential value of the product.

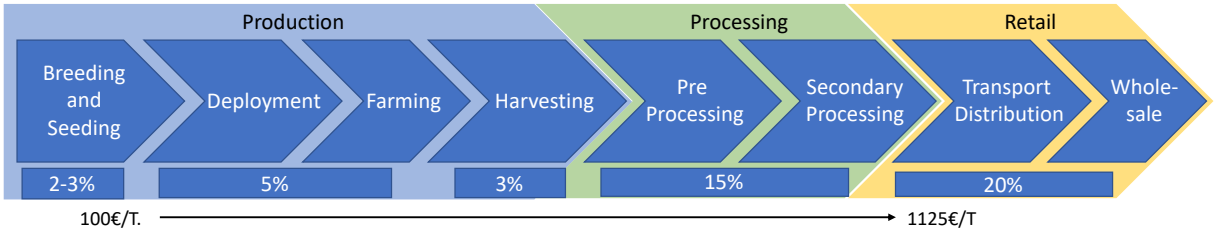


Figure 2.3.l: General diagram of the processing stages and value-added along the value chain for the seaweed bulk industry.

Figure 2.3.l shows the different stages for processing seaweed in bulk, including also the value-added to the seaweed along the different stages. The biomass at this stage requires additional processing to be able to reach the end-consumer. For the specific case of the fermented seaweed in bulk sold by ORF, the product will be added to pig feed at a 2% ratio, the pig feed will be then sold to the pig farming industry. At this level of transformation, the market of the fermented seaweed in bulk can expand into the cattle industry, where tests are undergoing to ensure animal safety and nutritional benefits of a seaweed component.

Discussion

This section presented a complete evaluation of the value chain of the macroalgae cultivation in the North Atlantic, with particular focus on the experience from AV partner ORF. The stages of the macroalgae production were described, specifically for the processing macroalgae into an additive for animal feed for pig farming. Several issues have been raised and elaborated throughout this analysis,

which will be summarised in this concluding section, to highlight the critical aspects that this industry is currently facing. Some of these issues will be taken to further analysis in future deliverables from the AV project.

The first critical aspect is the importance of selective breeding for producing resilient and highly productive seaweed seeds. Breeding is critical to achieving maximum yield in seaweed farms which were identified as the most important aspect of this operation. Furthermore, adequate breeding ensures higher quality from seaweeds produced which can contribute to the marketability and price of the product. There are not many diseases affecting seaweed farming in the North Atlantic in particular with the conditions of the Faroe Islands. They don't either face major fouling or animal grazers, due to the location of the farm, operating under nearshore exposed conditions with the possibility to move further offshore.

The next critical aspect is site selection for ORF is to receive licenses to operate more sites in the Faroe Islands which allows the growth of the operation. Yet ORF faces regulatory challenges as regulations are still biased towards animal aquaculture, which is fundamentally different from seaweed farming. Adding to it, there is a lack of recognising of the ecosystem services that seaweed farming provides to animal farms by uptaking nutrients in some of the sites, which can be translated into regulatory incentives to ORF operation and further financial instruments that could contribute to upscale their operation. Two critical issues arise from this one, the need to upscale and the need for an adequate regulatory framework.

The need to scale up is another critical factor that entails the development of new technologies to reduce operating costs and take advantage of economies of scale. To develop the necessary equipment and vessels, capital investment is needed, yet the industry is currently competing for investors instead of customers, as the market is still immature. For upscaling, an adequate regulatory framework is fundamental. For this, it is fundamental to acknowledge the particularities of seaweed farming instead of translating laws from fish or animal farming into it. Recognizing that seaweed provides a fundamental ecosystem balancing service, could facilitate the task of the regulators when considering the use of marine space with an emission-balancing approach at a regional level. IMTA as theoretically described so far appears to bring more risks to the novel seaweed industry due to the co-use of marine space and the proximity of the operations. The latter highlights the importance to promote a participatory approach to developing the regulatory framework for the novel macroalgae industry in Western countries, where stakeholders are involved, and knowledge gaps are acknowledged. Developing a business-friendly, yet sustainable and adequate governance for macroalgae farming is a critical aspect to ensure the development of this emerging and yet greatly promising industry.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained so far, we are ahead of the planned activities. The expectation at this stage would be 30% completed, however, we see the CST has obtained a 75% completeness.

The **key exploitable result** of this task is:

- Seeding with the seeding machine was still more time-consuming than manual seeding.
- The mechanical harvesting machine still need some modifications before it can be used commercially and compete with hand-cutting using a knife.
- Freshly harvested kelp biomass needs to be treated according to the end-use to reach a storage stable stage in a short amount of time. The timing depends on the processing method.
- Pre-processing is critical process to preserve the quality of the biomass as deterioration is fast when the macroalgae is taken from the ocean.

- By processing the seaweed biomass through fermentation in bulk, ORF is expected to add approximately 45% of the total potential value of the product.

Deviations & Problems: None

Outlook: The work planned in this CST will continue in the next 12M according to the workplan. However, much of the planning and testing work has already been completed.

CST 2.4 Utilise macroalgae to improve and optimise feeding strategies for low trophic species.

Responsible CS Task Leader Cliff Jones, Rhodes University

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
2.4	Utilise macroalgae to improve and optimise feeding strategies for low trophic species	T2.2, T3.2	ORF	0%	✓	M26	0	M37	0	0	0

Introduction

This task is led by Rhodes University who will be responsible for developing alternative protein sources for the South African abalone by including kelp as an alternative dietary ingredient. This work will be done in close collaboration with the industry partner MariFeed. The kelp biomass will be supplied by ORF, but also by others (overlapping with other case studies). Note that algae produced in sea-based IMTA (CS4) and land-based IMTA (CS3) and sea harvested kelp (here in CS2) will be included in the abalone diets, but the formulation of these diets and their manufacture and development will be reported in CS13 (and not here or in CS3 or CS4). The work is related to Work Package 3 (New or improved products). The task will start in July 2021 and finalise in June 2022.

This task will be commenced in July 2021.

Methods

The first sub-task, will be the biomass supply of biosecure *S. latissima* from ORF to MFeed.

In the second sub-task, the growth and feed conversion of South African abalone fed the following diets will be compared: 1) Basal diet (Control) (note that this treatment is shared with other tasks in CS3 and CS4 to save costs), 2) Basal diet supplemented with kelp powder supplied from ORF (CS2) included in the pellet. The basal diets will be isonitrogenous and the total lipids and energy of the diets will remain within a range know not to influence abalone growth.

In the third sub-task, growth will be monitored from September 2021 to April 2022. The two treatments will be assigned to baskets in the tanks on an abalone farm using a randomised-block design (each treatment will be represented at least once in each tank) to ensure standardised conditions, and each treatment will be allocated to at least three baskets of abalone (the abalone basket will be considered the unit of replication). The formulated feed for each treatment will be stored in buckets and the mass of feed that is fed to each replicate will be recorded, and feed conversion ratio (FCR) calculated: FCR = dry feed (kg)/wet weight gain (kg). Feeding will be carried out using standard farm feeding procedures.

The tanks will also be subject to standard farm production/husbandry procedures, and this will be standardised among the treatments (Note: this excludes feeding methods). The flow rate of water into each tank will be set regularly to ensure that this is standardized among treatments. Water quality measurements such as temperature (^{o}C), oxygen concentration (mg/L), pH, ammonia (mg/L), nitrite (mg/L) and nitrate (mg/L) will be taken at regular intervals throughout the trial.

At the usual split interval of roughly 4 to 6 months, the density of abalone in the baskets will be reduced, without grading, and the animals will be put back into their respective treatments at the required stocking density.

When a basket (replicate) comes into the split station its total biomass as well as the average weight of a sample of animals will be recorded. This will be used to determine the new basket biomass according to the farm stocking-density tables (standard farm practice). The required biomass will be placed back into the replicate basket and the basket re-tagged. The new basket will be returned to its replicate and feed treatment. That is, basket biomass before the split will be recorded (i.e. biomass at the end of the growth period); and it will be recorded again after the split (i.e. the starting biomass of the subsequent growth period).

The difference in biomass per basket and average weight per basket will be used to calculate metrics of growth-rate and biomass production. The amount of feed fed per treatment will be divided by the total biomass gain per treatment to determine the average FCR per treatment.

If a diet is replaced mid-experiment, all diets will be re-manufactured using the new dietary ingredients.

In the final sub-task, from May to June 2022, we will make a data assessment. The abalone biomass (kg basket^{-1} and g abalone^{-1}) for each treatment will be compared, to ensure that there are no significant differences between treatments at the start of the trial ($p<0.05$). Each basket will then be tagged indicating that it is part of an experiment, which replicate it belongs to, and the feeding strategy to which it will be subjected to.

Results

This task is planned to start in summer 2021.

Mfeed and ORF have had a phone talk and agreed to send kelp powder (100 kg DW) to Mfeed for feed testing, however, this was paused due to the discussion on the kick-off meeting regarding PEF treatment.

The data management plan was made, and the expected data set name is: “Testing abalone performance after a seaweed-based feed” with results on the growth rates vs seaweed included in the diet.

Discussion

Not applicable

Progress, deviations, problems & next 12M

Progress: Since the work has not yet started the results obtained so far correlates to 0% completeness.

Deviations & Problems: None

Outlook: The coming 12 months will most likely have no or little activities in this task as it is planned to initiate in July 2021.

CST 2.5 Select commercial interesting local macroalgal species for cultivation for each region and optimise design of cultivation rig based on the principles of the MacroAlgal Cultivation Rig.

Responsible CS Task Leader Urd Grandorf Bak, Ocean Rainforest

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
2.5	Select commercial interesting local macroalgal species for cultivation for each region and optimise design of cultivation rig based on the principles of	T2.2	ORF, UNE	0%	✓	M13	0	M22	0	1	0

Introduction

Initially, Brazil was the location to be subject to the identification of suitable "hot spots" for large-scale offshore macroalgal cultivation globally by conducting a feasibility study of potential species and sites. However, partner UFSC could inform that Brazil has only one kelp, which is very slow-growing and only origin is in a cold-water upwelling area. The kelps are the target species for offshore farming, and because of the lack of kelps and cold water in the Brazilian seas, we changed the scope of the area to North America. Hence, the Brazilian partner is no longer an part of the CS2 due to the lack of kelps in Brazil. Instead, the feasibility study will focus on North America and hopefully include the collaboration with UNE and Bigelow.

The task will start in June 2020 and be finalised in March 2022. We will through a review of scientific papers select commercial interesting local macroalgal species for cultivation for each target region and optimise the design of the cultivation rig based on the principles of the MacroAlgal Cultivation Rig (MACR). We aim for industrial stakeholder partners in the chosen target area.

This task will be commenced in June 2020.

Methods

Through a review, select commercial interesting local macroalgal species for cultivation for each region and optimise the design of cultivation rig based on the principles of the MacroAlgal Cultivation Rig (MACR).

Results

This task will initiate in M13 (June 2020). The task will use the method generated in CST 2.1.

Discussion

This task will initiate in M13 (June 2020).

Progress, deviations, problems & next 12M

Progress: Since the work has not yet started the results obtained so far correlates to 0% completeness.

CSTP2.5.1 will go beyond the state of the art by optimizing the cultivation system, harvesting, and landing logistics based on the principles of the MacroAlgal Cultivation Rig (MACR).

Deviations & Problems: The Brazilian case was changed to North America due to the lack of kelp species along the Brazilian coastline.

Outlook: In the coming 12 months we will make a review to determine native kelp species in North America (East coast) and investigate market possibilities for this species. We will develop a relevant cultivation system and report as input to site selection (CST 2.6).

CST 2.6 Find suitable sites for large-scale production (>500 ha) in open ocean environments in the Atlantic Ocean.

Responsible CS Task Leader Gunnvør á Norði, Fiskaaling

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
2.6	Find suitable sites for large scale production (>500 ha) in open ocean environments in the Atlantic Ocean	2,2	FISK, ORF	50%	✓	M23	M7	M34	0	3	7

Introduction

This task was led by Fiskaaling, who were responsible for the GIS mapping and site selection. Ocean Rainforest had the role of giving input about cultivation structure and environmental requirements. A new contact occurred after few months of the project. A Master student Drew Resnick took contact and a collaboration between the University of Edinburgh and the EU funded project iAtlantic (https://www.iatlantic.eu/) was initiated as a strong Atlantic collaboration! Drew Resnick was student at the GISciences department at the University of Edinburgh with a background in Marine Biology and Environmental Studies (Collaboration outline in the results section).

M6-M12 was about determine the work to be done in detail.

Methods

The cultivation structures we use

We cultivate large brown seaweed using two types of MacroAlgal Cultivation Rigs (MACR). The structures do only consist of steel anchors, steel chains, connection discs, nylon ropes, buoys and floats and can easily be deployed and removed without leaving damage to the seabed. The structures are handled from a vessel by crane (divers are never used for any of the handling operations related to the sea-based cultivation).

One MACR-structure is optimal for cultivation at exposed sites (wave heights up to 6 meters significant and current of <2,5 knots). This structure is called MACR-E and has been tested in Funningsfjørdur (site A71) since 2010 and is proven to survive hard weather and hurricanes. The structure is suitable for sites with a water depth of 50–200 m.

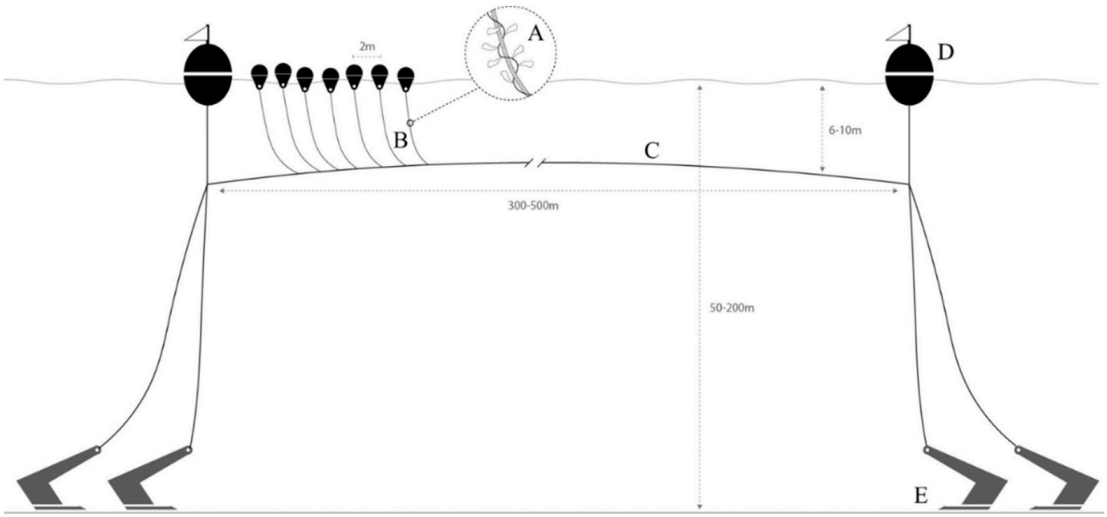


Figure 2.6.a: MACR-E constructed by Ocean Rainforest Sp/f.Seeding material (Figure 1, A) is added to a braided nylon ropes of 10 mm using a binder glue (alginate-product) and these seeded ropes (Figure 1, B) are attached at 1-m intervals to the fix line (Figure 1, C) by a loop and held in a vertical position by a buoy. The fix line is hold horizontally at 10 meters below sea surface level by two main surface floats (Figure 1, D) and four steel anchors (Figure 1, E) ensure the right position of the rig.

Figure 2.6.b: MACR-E seen from above sea surface.

In sites with more shallow water depth (15-50 m) another structure is used. These sites have typically a maximum significant wave height of <3 meters and there are no or little current. This structure is called MACR-S and has been tested in Funningsfjørdur since 2016.:

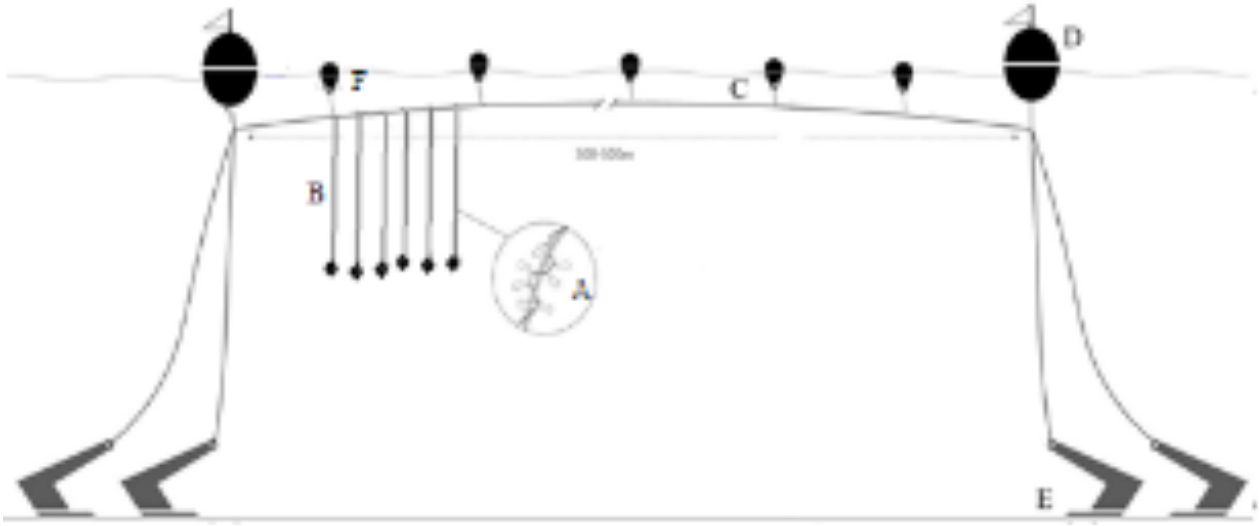


Figure 1 MACR-S constructed by Ocean Rainforest Sp/f. Seeding material (Figure 3, A) is added to a braided nylon ropes of 10 mm using a binder glue (alginate-product) and these seeded ropes (Figure 3, B) are then attached at 1-m intervals to the fix line (Figure 3, C) by a loop and held in a vertical position by a sink. The fix line is hold in place at 1 meter below sea surface by two main surface floats (Figure 3, D) and four steel anchors (Figure 3, E).

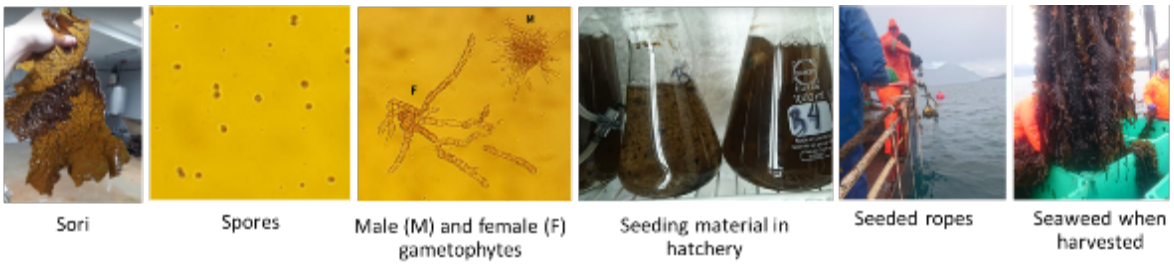


Figure 2.6.c: The different steps in producing seaweed seeding material from sugar kelp.

Figure 2.6.d: Multiple partial cutting method.

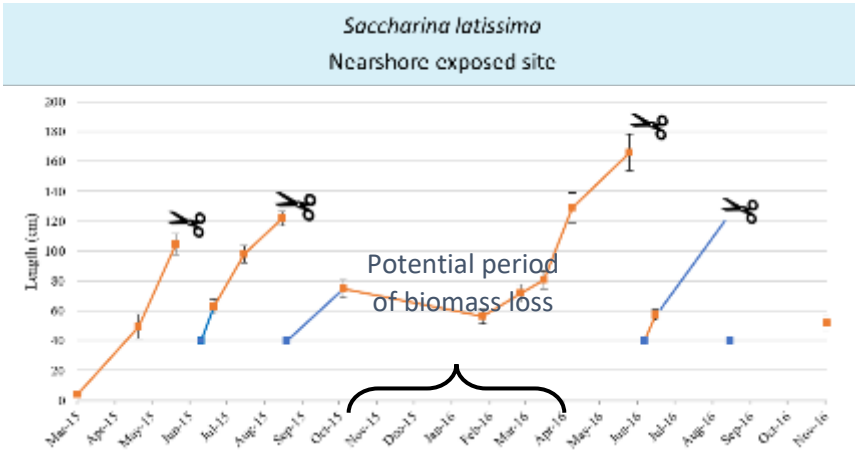


Figure 2.6.e: Mean length of Saccharina latissima grown at the nearshore exposed cultivation site in Funningsfjørdur (A71). The scissors represent harvesting. Blue lines are estimated from a cutting length of 40 cm from rope to blade tip. Orange lines are accurate measurements (ORF results).

Results

A data management plan was made, and the expected data set name is: "Mapping the Atlantic Ocean to identify suitable cultivation site".

M7-12: Data availability in the Gulf of Maine investigated and methods on site selection established → The task is ahead of schedule.

A highly motivated MSc student approached the AquaVitae consortium and the competence fitted CST 2.6 perfectly. Thus, a MSc project was established in collaboration between FISK, ORF and University of Edinburgh. The two approaches for site selection based on available data (task 2.2 and 2.6) together cover two highly relevant locations for macroalgal cultivation and describe a possible approach for site selection in other areas in the Atlantic Ocean.

Another unplanned result from CS2 which revolves around clustering and dissemination activities, several meetings and communication about the collaboration AquaVitae/iAtlantic has taken place. Minutes of the correspondence is shown below:

- Question by Drew Resnick: Lea-Anne and I were discussing plasticity in the context of whether or not the species you grow have been found to adapt in other ranges of, for example, temperature / salinity than have been measured around the Faroes, which we suspect would especially be

important if the seed potentially being used in the sites of Maine would be transplanted from the Faroes vs if they were to be seed from stock natively from the regions around Maine.

- Answer by Urd G Bak, ORF: Yes – *Saccharina latissima* is also growing in lower saline seas and at locations of higher temperature and colder sites. This is to some extend explained in my master thesis from 2012. **Attached.** Hope it helps a bit or you find relevant literature in the reference list.
  For your research it would be great to find local registrations in Maine of "where to find natural populations of *Saccharina latissima*" – where you find it naturally, it will most likely be "easy" to farm as well.
  You are also very welcome to screen for other relevant kelp species. When I say relevant, I mean kelps (large brown algae) that has a fast growth rate (not *L. hyperborea* or *L. digitata*) and which is already used in food, feed or extracts or has the potential to be implemented as a new species to use.
  It will not be relevant to deploy seedlings from not local material! This would be wrong as it could disturb local genetics.
  I suggest that the intervals that you use for "site selection" is:

  Temperature: Not above 15 degrees Celsius in more than one week/or maybe 15 days. Never above 20 degrees Celsius.

  Salinity: Not below 25‰ in more than one week/or maybe 15 days. Never below 20‰.

  Nutrients: Nutrients, especially nitrate is crucial for macroalgal growth, and concentration levels of 3 µM are the minimum requirements for growth in the brown algal species *Saccharina latissima* (Chapman et al. 1978).

- Question by Drew Resnick:This could be explored more qualitatively in the context of "could the named species successfully grow in a wider range of environmental conditions than is seen as its optimal growing conditions?" as grounds for theoretically expanding the suitable sites for the farm- or, also could be added quantitatively given proper data.
- Answer by Urd G Bak, ORF: The answer is yes, they can grow outside their optimal growing condition – but more harvests from the same crop is important for the profitability (like we harvest our lines 6 times without re-seeding in the Faroe Islands). And for example, temperature can be a "show stopper" for more harvests of the same line. Also, more sheltered sites than "the optimal sites of 50-150 m seawater column" could be used for seaweed farming. However, these nearshore shallow sites also have natural seaweed beds and therefore the shading from farming would be a problem. And nearshore sites are more extensively used for tourism, fishing, sailing, etc.

- Question by Drew Resnick: I will of course research into this on my own if it is not something you have literature / data to send me, we thought it was an interesting component nonetheless.
- Answer by Urd G Bak, ORF: Yes – please make your own research, we can only learn from it and you will need it in your thesis. And please read my PhD thesis to understand better the methods we are using.

Discussion

Not applicable.

Progress, deviations, problems & next 12M

Progress: The work has initiated. The work is now formed by the MSc student, Fiskaaling, Ocean Rainforest and iAtlantic. Results obtained so far correlates to 50% completeness.

Deviations & Problems: None (ahead of schedule)

Outlook: We aim to have a detailed site selection of the Gulf of Maine in the coming 12 months.

CST 2.7 Make a feasibility study and a knowledge transfer plan for at least one industrial partner outside Europe, optimal North America.

Responsible CS Task Leader Urd Grandorf Bak, Ocean Rainforest

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
2.7	Make a feasibility study and a knowledge transfer plan for at least one industrial partner outside Europe, optimal North America	T2.2	ORF, SYN	0%		M35	0	M46	0	1	0

Introduction

This task is led by Ocean Rainforest who will be responsible for the overall report and plan for knowledge transfer. Syntesa is a partner with the technical responsibility of the feasibility study. This task will get input from tasks 2.5 and 2.6. The work is related mainly to Work Package 2 (Post hatchery/seedling to harvest processes). The duration of this task is from April 2022 until March 2023. We will make a feasibility study and a knowledge transfer plan for at least one industrial partner outside Europe, optimal North America. A relevant industry partner in North America could be Bigelow (https://www.bigelow.org/). Planning will be detailed in a year-2 work plan. This task will be commenced in April 2022.

Methods

See work plan for CS2 in annex 3 of Deliverable D1.1.

Results

Not applicable

Discussion

Not applicable

Progress, deviations, problems & next 12M

Progress: Since the work has not yet started the results obtained so far correlates to 0% completeness.

Deviations & Problems: None

Outlook: No activities in the coming 12M

CS3

Summary of progress report for Case Study **3**

Date of report:	**29.03.2020**
Case Study name:	**Land Based Integrated Multi-Trophic Aquaculture (IMTA)**
of relevane for WPs	**1, 2, 3, 5, 6, 7, 8, 9**

Abstract/Summary

Considering the variety of species and production models that can be considered in Integrated Multi Trophic Aquaculture (IMTA) systems (Figure 3)[21], CS3 aims at identifying suitable combinations of low trophic species (LTS) in different environments and geographical locations while focusing on species from the same class or genus to facilitate information and technological and knowledge transfer between regions and academic and industry sectors. Further research for the development of IMTA production is required as IMTA is a central/overarching theme on which many variations can be developed and adapted to different scenarios and species. Figure 3 represents a conceptual diagram of an IMTA operation, however, multiple models including a variety of species are to be improved or developed as IMTA can be applied to open-water and land-based systems, marine and freshwater environments, and temperate and tropical climates. One definition of IMTA established within the Integrate project is: "Enhanced production of aquatic organisms (with or without terrestrial organisms) of two or more functional groups, that are trophically connected by demonstrated nutrients flows and whose biomass is fully or partially removed by harvesting to facilitate ecological balance". Taking this into account the diversity of systems and the participating regions; tasks within CS3 have been designed with the overall aim to develop and improve systems and processes in order to increase and improve the production of low trophic species, in Land Based IMTA systems, at different steps of the production cycle across the whole vale chain from hatchery production to end products. During the first 12-months of the project the experiments performed provided information on:

- The improvement of *Haliotis tuberculata* and *Haliotis midae* settlement processes for their future production in Land Based IMTA systems
- The effect of temperature on growth and survival of *Haliotis tuberculata* juveniles during the nursery stage
- The feasibility to integrate *Holothuria sanctori* and *Neostichopus Grammaticus* to existing Land Based IMTA systems used to produce *Haliotis tuberculata coccinea* and *Haliotis midae* by testing different systems prototypes and performing preliminary trials
- The suitability of recirculated and flow through Land Based IMTA systems to produce *Haliotis tuberculata coccinea*
- The effects of different feeding strategies on the performance of the Land Based IMTA production systems in terms of sediments and nutrient fluxes.

The different experiments enabled species interaction, data and initial results to be further analysed and will allow implementation of the other planned experiments and ultimately the possibility of technological transfer between regions as well as between research and industry partners. The information obtained in the different experiments was integrated to their corresponding WPs to contribute to the general overview of the entire value chain of production. The final results are

[21] (Chopin, 2017)

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement No 818173. This report reflects only the authors' view and that the Commission is not responsible for any use that may be made of the information it contains.

expected to address bottlenecks reported at biological and technological level to advance the production and product diversity and increase quality. The planned and ongoing experiments are also aiming at promoting environmental, economic and social sustainability in order to highlight environmental and economic benefits of such production systems at the different stages of the production cycle.

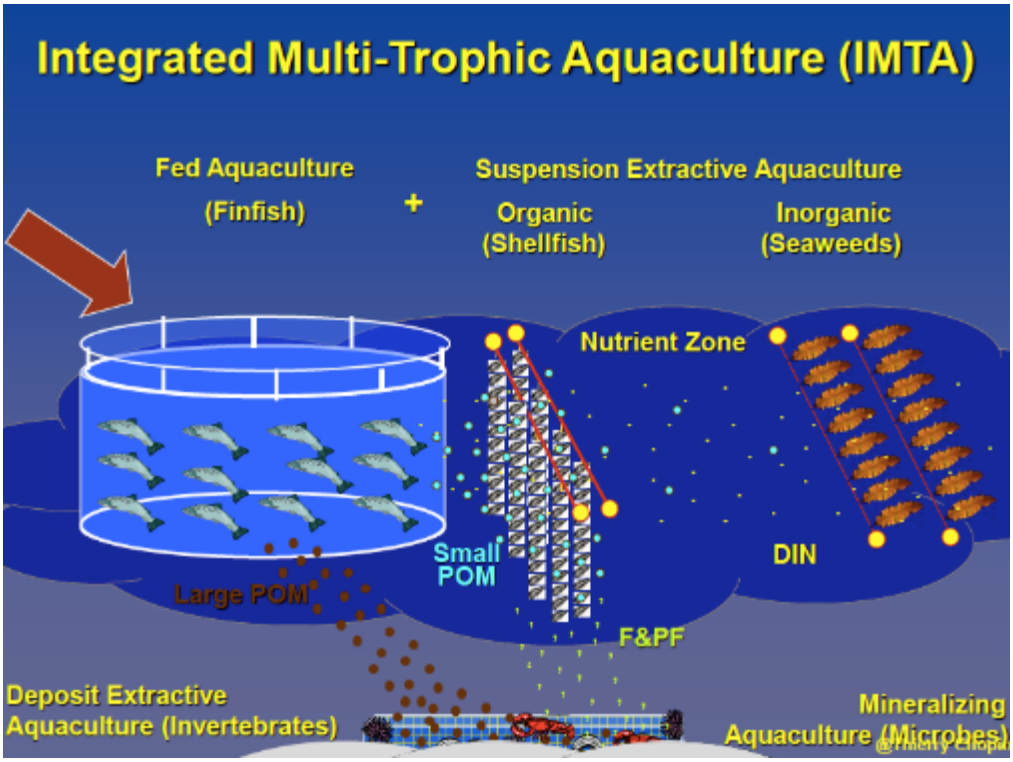


Figure 3: Conceptual diagram of an integrated multi-trophic aquaculture (IMTA) operation (Chopin, 2017).

Independently from each of the CS tasks a series of general organisation actions took place between the CS partners at the beginning of the project to organise the activities and contribute to the information required by the WPs. In order to define the methodologies to be implemented to perform the experiments and the activities required for the deliverables the partners have organised different exchanges.

- A first meeting was organised during the AquaVitae kick off meeting in Tromsø, June 2019 to clearly define the activities to be performed their possible methodology and start to draft a first workplan draft for CS3.
- A case study kick-off meeting was held in South Africa in conjunction with CS4 and CS7 between the 4th and 8th of November 2019 allowing to further describe the activities and methodologies to be included in the work plan and facilitate researchers/industries interactions.
- Various virtual meetings were held between partners to exchange about the tasks taking place in the different regions and to determine how the results will be transferred to the WPs requiring the CS information (WP5, WP6, WP8, WP9).

This resulted in

- A first workplan draft for CS3 was developed in September 2019
- CS3 workplan was detailed, including the methodologies for each experiment to be performed and defining how the transfer of information will be managed between the experiments and how it will be integrated in the key deliverables and task. This workplan was revised in December 2019.
- Questionnaires were sent to stakeholders regarding CS3 to provide information required for D1.1, D2.1 and D3.1.
- Data sheets describing the data sets expected from the various experiments to take place within CS3 were prepared and sent to contribute to the development of the data management plan.
- Exchange on product processing methods adapted to the requirements of WP5
- Contribution to the first prototype of challenge-structuring framework matrix WP6
- Contribution to WP8
- Dissemination articles were produced for WP9

CST 3.1: Abalone Hatchery settlement processes

Responsible CS Task Leaders: Sylvain Huchette, FrHa; Gercende Courtois de Viçose, ULPGC; Clifford Jones, RhU

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
3.1	Abalone hatchery settlement	T1.1, T1.2, T1.3	FrHa, ULPGC, HIK, RhU, Mfeed	0-90%	!	M3	M2	M12	M24	4-5	7-9

Activity 3.1.1: Haliotis tuberculata settlement processes

Introduction

The objectives of this activity were to improve the quality of abalone juvenile production using *Ulvella lens* produced under organic certification, by testing different treatments to increase the survival and growth of young abalone and improve their future performances while produced in Land Based IMTA systems. One experiment was set up; to study the concentration and frequency effect of addition of nutrients with Geogreen Plus ®, an organic nutrient obtained from vegetable molasses, on the:

- Growth of *Ulvella lens* (IMTA of macroalgae and abalone)
- The effect of preparation of *Ulvella lens* cultures on larval settlement,
- Post settlement growth and survival.

One of the main problems with organic nutrients is that they also promote the growth of other biofilms, of both bacterial and diatoms. These biofilms may hinder the settlement and subsequent IMTA production of larvae on the prepared settlement plates in the system. The second problem are their toxicity on the young animals because ammonia is introduced when the culture nutrients are dissolved in seawater. To ensure the feeding of post-larvae, it is essential that the *Ulvella lens* is

perfectly established to resist grazing long enough to allow for the post-larvae to grow to a minimum size of 4mm when their food can be change to other macroalgae sources.

Methods[22]

Biological material

- *Ulvella lens* cultured at France Haliotis' nursery (Figure 3.1.a) throughout the year.
- Abalone, juveniles from 2019 summer production cycle.

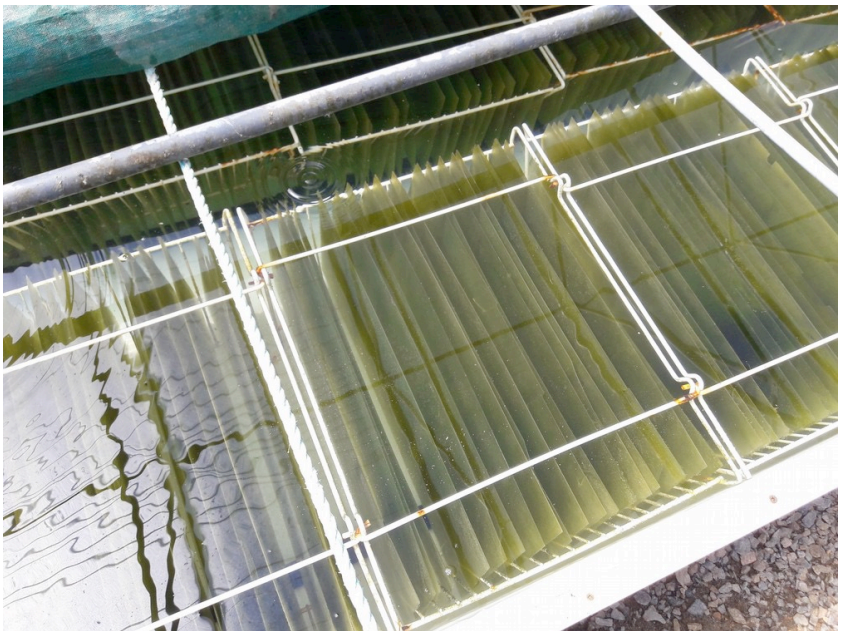


Figure 3.1.a: Baskets containing plates of *Ulvella lens.*

Structures

Ulvella lens is a green encrusting macroalgae which grows two dimensionally over surfaces (Nielsne, 1977; Hannon; et al., 2014). The use of this macroalgae was first identified in 1980s' in Japan for initial uses as an induction and settlement substrate. However, this is a known induction cue, its uptake in the abalone industry outside of Ireland, France, Japan and Australia is low. The use of this prime settlement cue and its preparation methods under organic production methods is been validated commercially in France offering possibilities of technology transfer to the large-scale abalone industry in South Africa and two commercial partner enterprises (HIK Abalone and Wild Coast Abalone) that are participating in the AquaVitae project.

[22] (Bansemer, M. S.,et al. 2016; Basuyaux, O., 1997 ; Berthelot, A., 2018 ; Dangeard, P., 1931; Daume, S., 2007 ; Courtois de Viçose, G., et al., 2012; Flassch, J. P., 1984; Hannon, C., 2015; Hannon, C., et al., 2013; Hannon, C., et al., 2014; Huchette, S., et al., 2003; Jones, A., et al., 2001;Lachambre, S., 2017**)**

Sporulated germlings of *U. lens* are grown on plastic plates 60cm long and 30cm high. There are 20 plates per basket and six baskets in each experimental tank of the nursery, i.e. 120 plates per tank. The experimental tanks have a capacity of 1000L of water and the incoming water flow was set at 3L/minute (which corresponds to 3 exchanges of water per day). The experiment was carried out in the 12 experimental tanks under a greenhouse.

Preparation of tanks and plates for *Ulvella lens* culture

Ten days before the inoculation of the experimental tanks, the *Ulvella lens* adult sporophytes were placed in 24Hr darkness; the culture tank is covered to exclude all light. Covering creates a stressful environment that prompts the adult sporophytes to produce and store spores. Setting up the culture medium: For each tank, seawater is filtered through 50µm pocket filters maintained at 10°C for 10-14 days. Then, six baskets of 20 clean plates are placed in the tanks.

Sporulation of *Ulvella lens*

After conditioning the adult sporophytes, the plates are scraped with a stainless-steel spatula to remove diatoms assemblages and other unwanted biofilms. *U. lens* resist to scraping due to its encrusting nature. Plates of adult *U. lens* sporophytes are then placed in the tanks all around the settlement baskets (Hannon et al. 2014). For each tank about 15 plates are needed. The sporulation of *U. lens* last over two or three days. Sporulation is triggered due to stress via an increase in temperature (culture tanks are maintained at 20°C) and increase in light (24Hr light 0Hr Dark) (Nielsen, 1977)[23].

Nutrient addition

The addition of nutrients for *U. lens* macroalgae is carried out with Geogreen Plus ® (Angibaud Derome & Specialties). The latter is a liquid organic nutrient/culture media of plant origin (vinasses and beet molasses). It is made up of 45g/L of total nitrogen (N), 25g/L of anhydride phosphoric acid (P2O5) and 50g/L1 of potassium oxide (K2O).

When adding nutrients to *U. lens* cultures, the incoming water in the tanks is turned off for four days. This prevents all the nutrients from being diluted during this process.

Temperature monitoring

Throughout the experiments, the temperature of the experimental tanks was recorded twice a day; in the morning and early afternoon, using a Voltcraft probe thermometer

Experiments set up

1 - Effect of concentration and frequency of nutrient addition with Geogreen Plus® on the growth of *U. lens*

Three treatments were tested on the 12 experimental systems with four replicates per treatment. The first treatment corresponds to the standard concentration used at France Haliotis (Berthelot A. 2018). The second treatment corresponds to an equivalent concentration, however in this case the

[23] (Nielsen, R., 1977)

concentration was twice the standard concentration. The last treatment corresponds to a double concentration, delivered over four days. The three treatments were carried out under the same conditions. For each treatment the experimental tanks were chosen at random (Figure 3.1.b).

Geogreen Plus® treatments:

- F1= 100 mL delivered twice every other day.
- F2= 90 mL every day for four days.
- F3= 50 mL every day for four days.

The seeding of the tanks was done on April 25th, 2019 (D), the second addition of nutrients on D + 12 and the last on D + 21.

The growth of *U. lens* is monitored by estimating the percentage cover of *U. lens* present on the plates. For this, three plates of algae per tank are photographed under uniform conditions, making a total of 12 plates per treatment. The measurement is performed using the ImageJ software, more precisely the "colour threshold" function. Two analyses of the plate coverage are performed; the first on seven days after seeding, then 14 days after the third addition of nutrients.

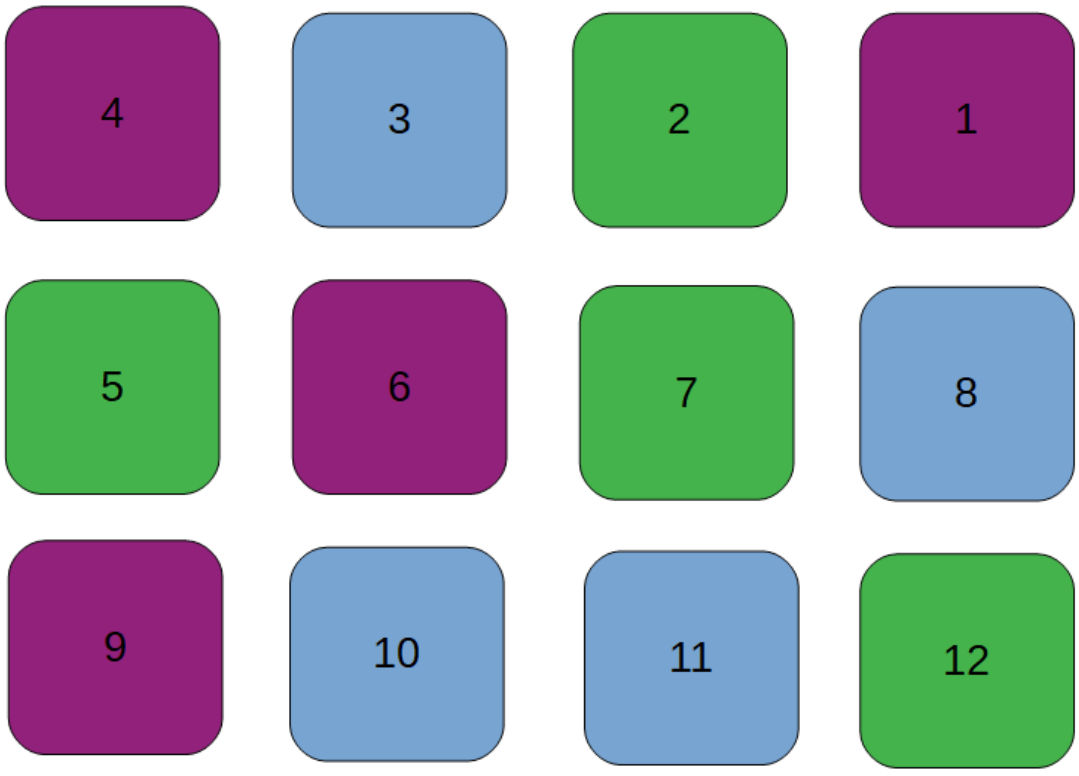


Figure 3.1.b: Diagram of the distribution of treatments in experimental tanks; green corresponds to treatment of 100mL, blue to 90mL and purple to that of 50mL.

2 - Effect of the preparation of *U. lens* cultures on the fixation of abalone

Using organic culture nutrients, it's frequent to observe overgrowth and clumping of diatoms and other biofilms on the plates and this may reduce the overall settlement rate. Although plates cleaned of diatoms and other bacterial biofilms will attract abalone larvae for settlement, removing this biofilm mays also deprive the post-larvae from their initial required food source and induction cue. With this experiment, we intended to evaluate the best technique to prepare plates for abalone settlement and integration into IMTA systems and further tech-transfer to industry partners.

At France Haliotis two techniques are used to prepare *U. lens* plates for settlement of competent abalone larvae; manual scraping using a scraper (G) or washing the plates with seawater using a hose connected to a submersible pump (L). This eliminates unwanted assemblages of diatoms and biofilms on the plates and leave a clean layer of *U. lens* for the larvae to settle on (diatoms, enteromorphs and other algae).

24-hours before the settlement, three baskets from each tank are scraped so that only *U. lens* coverage remains on the plates. The other plates are washed with a pump in order to reduce the coverage of diatoms present. Subsequently two baskets, made of scraped plates, of each treatment are divided into six tanks chosen at random (Figure 3.1.c). Baskets are identified (scraped and washed plates) (Figure 3.1.d).

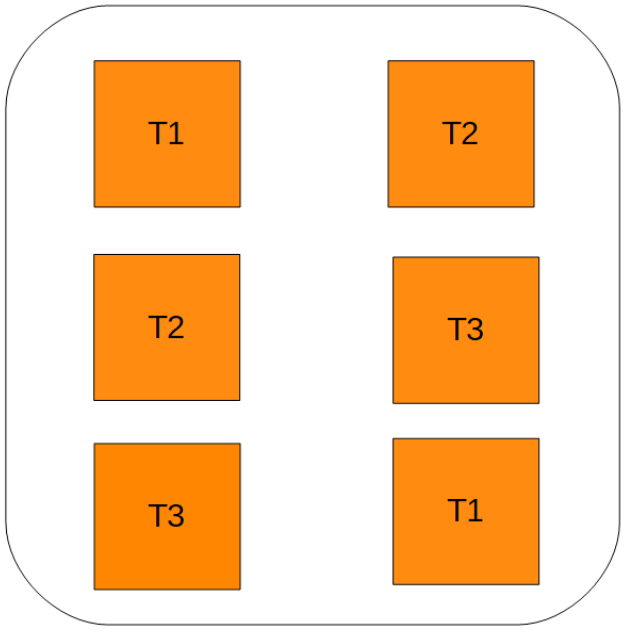


Figure 3.1.c: Arrangement of the different treatments in a tank; T1 corresponds to treatment of F1=100mL, T2 to that of F2=90mL, and T3 of F3=50mL.

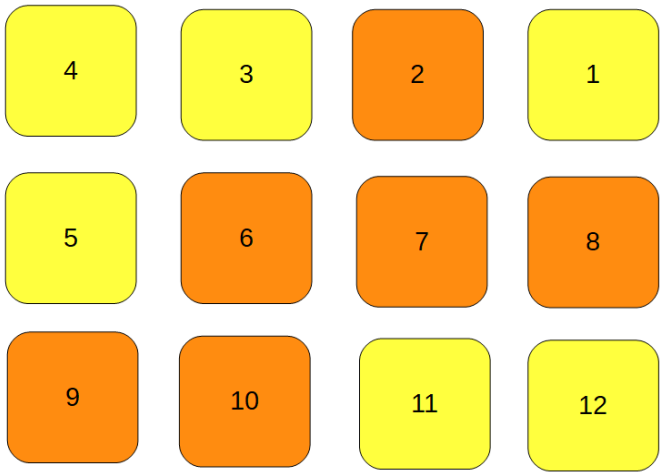


Figure 3.1.d: Arrangement of the tanks with plates scraped (G, in yellow) and washed ones (L, in orange).

The abalone post larval settlement on the *U. lens* plates was carried out on June 2nd 2019. The counting of settled post larvae occurred ten days post induction. For each experimental tank, the counting is carried out on one plate of each basket, *i.e.;* six plates per tank. Once the counts are completed, an estimation of the density per settlement plate and settlement rate was calculated.

3 - Effect of preparation of *U. lens* cultures on survival and growth

To estimate the overall survival rate, three counts are carried out at regular intervals (every 12 days). At each count, the plate selected is different, due to a certain life stage of growth, the juvenile abalone drop from their plates, due to a behavioural change.

Shell length was measured under the microscope. To perform the measurement, six experimental tanks are used: three tanks with scraped plates and three tanks with washed plates. The tanks were chosen based on the results obtained during the second counting. The three tanks with scraped plates with the results closest to the average of the six tanks. For each tank, three plates are used.

The first step is to estimate the density by visual counting of the animals on the plates. Then, using a Pasteur pipette, 15 abalone were removed and placed in a Petri dish. The animals are then fixed with alcohol. Once the samples have been taken, the abalone from each sample is placed in a well of a 24-well dish. Measurements were made with an Olympus inverted microscope using a measuring scale and x4 magnification. 39 graticules corresponded to 1mm.

Results

Temperature monitoring of experimental tanks

Throughout monitoring the temperature of the water in the tanks, a noticeable variation in temperature was observed, minimum measured temperature was 14.5 ° C and maximum 22.4 ° C (Figure 3.1.e).

Despite their identical flow, the experimental tanks did not have exactly the same temperature at a given time. These differences are due to the positioning of the tanks under the greenhouse. Indeed, by their position, some tanks had a longer exposure to the sun.

During settlement and the following days temperatures were optimal for the growth of juveniles. The temperature range for optimum growth is known to be between 15-18 ° C (Basuyaux 1997).

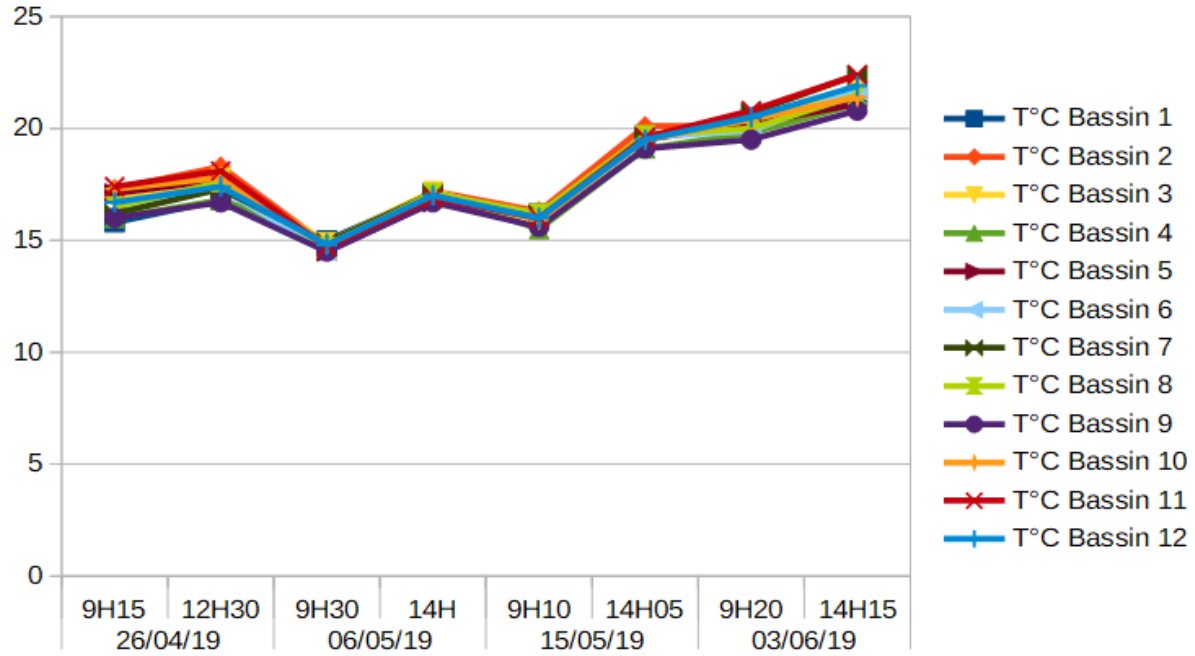


Figure 3.1.e: Temperature monitoring during the three fertilizations and the fixation.

1 - Dose effect and frequency of fertilization with Geogreen Plus®

To begin with, we observe with the naked eye a difference between the plates photographed after the various additions of nutrients with Geogreen Plus® (Appendix 1). Indeed, we see that the rate of coverage, reflected in the presence of green on the plate, increased between addition of culture media.

This observation is valid whatever the treatment carried out. According to the figure 3.1.f, there is no significant difference between the three treatments. Treatment recovery rates increase throughout follow-up.

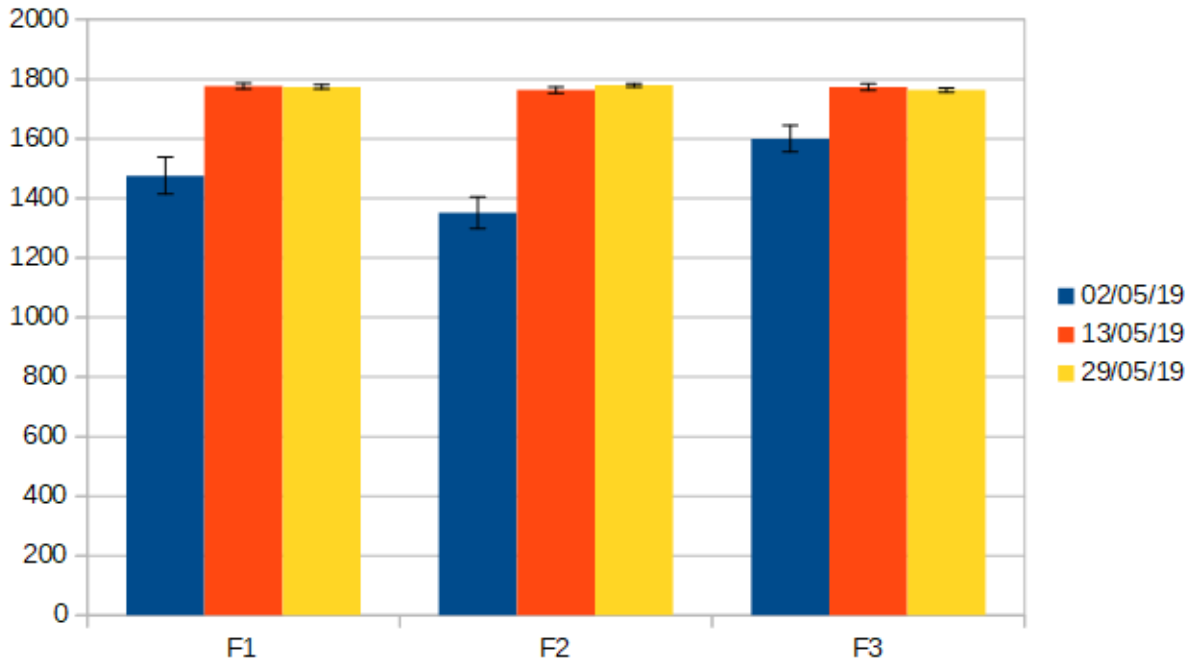


Figure 3.1.f: Average plate coverage in cm² by U. lens vs nutrient addition treatment F1 corresponds to the treatment of 100ml delivered twice every other day, F2 to that of 90ml delivered four times and F3 to 50ml delivered four times. 100% coverage is equal to 1800 cm².

2 - Effect of the preparation of *U. lens* cultures on the settlement of abalone

Table 3.1.a: Average of the first post-larval count according to the preparation (G for scraped and L for washed) and treatments.

Date	Préparation	Fertilisation	Moyenne – Comptage
13/06/19	G	F1	257
		F2	302
		F3	226
	L	F1	119
		F2	271
		F3	93
Total Résultat			211

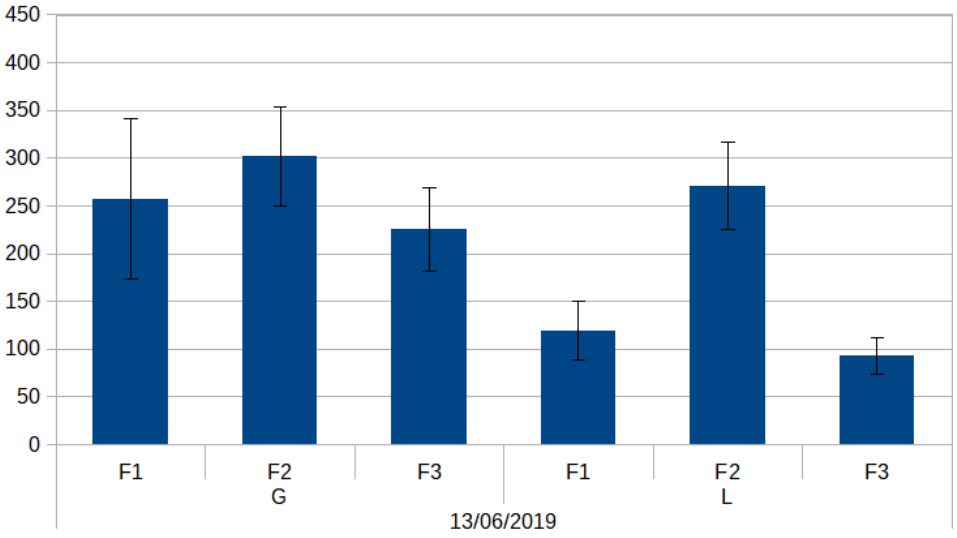


Figure 3.1.g: Average number of animals settled according to preparation and nutrient treatments, standard error is displayed with the error bars.

Based on the results obtained during the first count and Figure 3.1.g above, it is possible to say that there is no significant difference between the scraped plates of the three treatments and the washed plates of the second treatment. But the latter are significantly different from the plates washed from the first and third treatment.

3 - Effect of preparation of *U. lens* cultures on survival and growth

Effect on survival: According to the settlement counts carried out, regardless of the preparation or the treatment of settlement plates there is mortality observed in juveniles in all tanks. The level of this mortality varies depending on the settlement plate preparation, as well as the treatment.

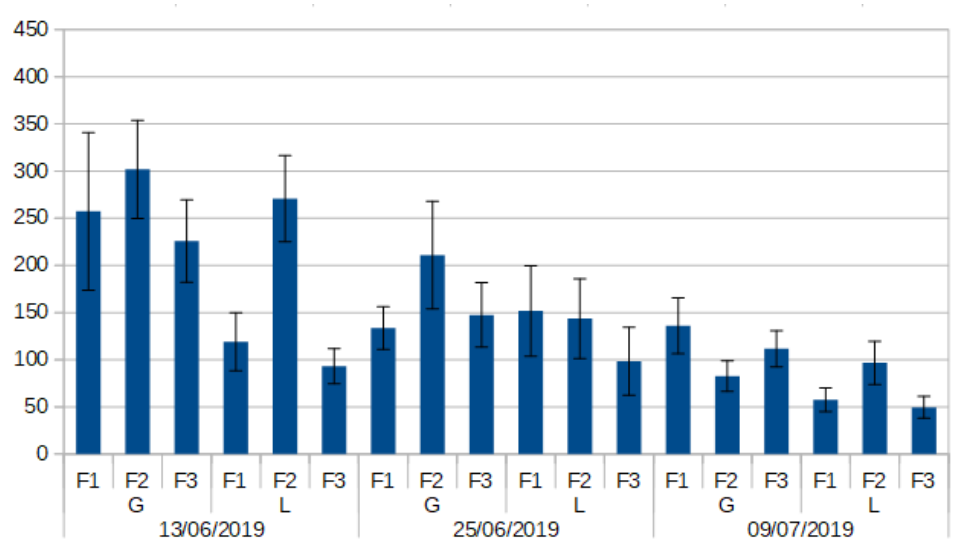


Figure 3.1.h: Number of surviving post-larvae over time according to treatment and plate preparation.

From the Figures 3.1.h, we see that there are significant differences between certain treatments. For example, the scraped plates from the third treatment of 07/09/19 are significantly different from the washed plates of the third treatment of the same date.

There is a significant difference between the washed plates of the second treatment and the washed plates. the other two.

As of 25/06/19 there are no significant differences between treatments.

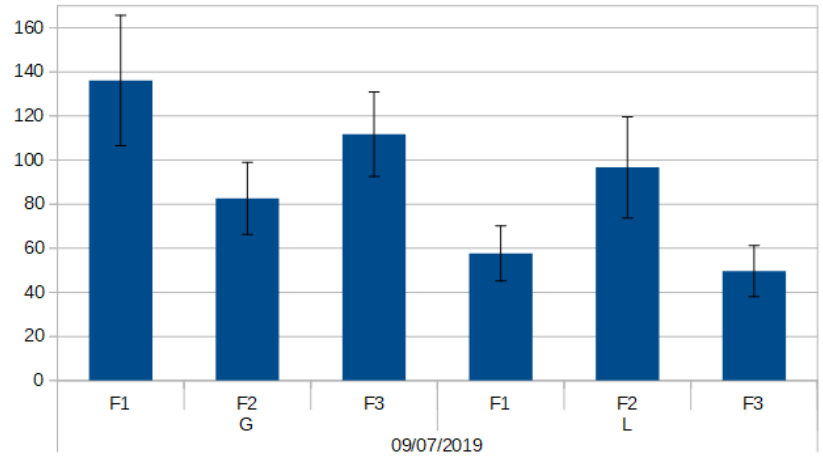


Figure 3.1.i: Final number of abalone in the tanks according to the processing and preparation of plates.

From the Figure 3.1.i, there was a significant difference between the scraped plates from the first and third treatment and the washed plates from the first and third treatment. There is no significant difference between the scraped plates from the second and third treatment and the washed plates of the second treatment.

Effect on initial growth: The size measurements and the Figure 3.1.j shows that there is no significant difference between treatments and preparations on the growth of abalone post larvae.

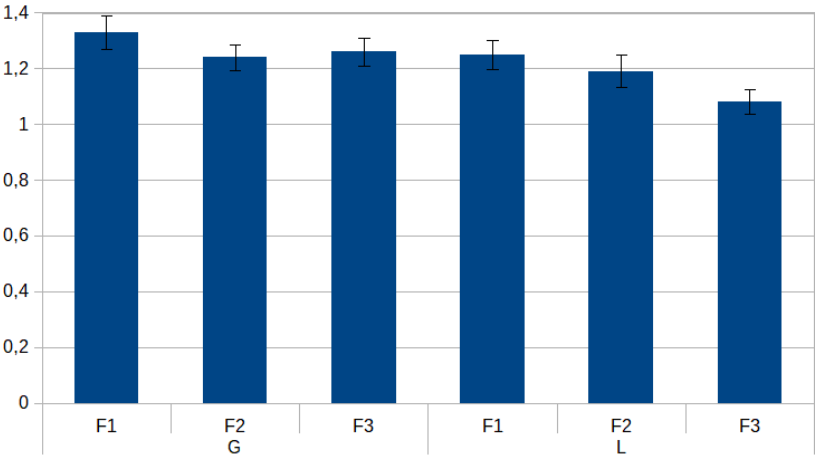


Figure 3.1.j: Average size of the juvenile according to the treatment and of plate preparation.

Discussion[24]

From the experiments carried out and the results obtained, CS3 partners observe that the treatment or preparation of the settlement plates of *U. lens* algae has an important role, in addition to water temperature, concentration of nutrients present in the medium and light, with regard to the rate of recovery.

The first experiment shows that the plates of the first treatment have the best *U. lens* coverage rate. The second treatment may have caused a lack of light and a lower nutrient level for *U. lens*. A low level of nutrients causes a lower germination of the settled tetraspores on the plates. The results obtained during the second experiment show that the preparation of the *U. lens* plates plays a role in settlement of larvae. Indeed, at equivalent concentrations the scraped settlement plates produced a greater settlement rate than washed settlement plates. Treatment also had an impact on the attachment of larvae. Indeed, the settlement of the larvae increased on the plates of the second treatment. This can be explained by the fact that in this treatment, *U. lens* received a higher concentration of nutrients, resulting in a higher protein concentration which aligns with previous initial experiments by Courtois de Viçose, et al. (2012). It is therefore possible to confirm that the more protein there is in the macroalgae the greater the settlement. At this point, the washed plates of the second treatment are most effective for settling larvae. The scraped plates of the second treatment were also effective in terms of settlement, but preparation takes time and labour. In fact, it takes around one and a half hours for two people to scrape the plates from an entire tank, i.e. about three hours for one person, while the action of washing the plates only requires only 30 minutes for one person.

The juvenile survival experienced during this trial, it is observed that juvenile abalone have a better survival rate on scraped plates. The action of scraping the plates before settlement would allow to remove the biofilm, made up of diatoms and enteromorphs, present on the plate, which allows for greater light exposure for *U. lens*. In addition, biofilms inhibit the attachment of the larvae of certain organisms (Lau et al. 2005).

The results also show that the plates from the first treatment gave the best rate of juvenile survival. In addition, the final number of juveniles present in the tanks is greater for scraped plates from the first treatment.

In regards to the overall size of the settled juvenile abalone, it is expected the increase in shell length to be higher on lower density plates (Flasch and Aveline 1984). However, in view of the results, we note that the treatment and preparation of the plates had no significant difference on the growth of juveniles. The hypothesis would be that there is no concentration effect of nutrient addition after a certain time.

Conclusion: The aim was to improve the quality and consistency of juvenile abalone production using *U. lens* by testing different treatments to increase survival and growth of young abalone produced in this and other IMTA system. The results of the various experiments showed that the treatment which initially gave the best settlement rate of abalone larvae is not the most suitable for growth and juvenile survival. The first treatment, which has the best percentage coverage rate of *U. lens*, is best suited. In

[24] (Courtois de Viçose, et al. 2012; Flasch and Aveline 1984; Lau et al. 2005)

fact, combined with the action of scraping the plates, it is the most effective treatment for larval attachment, juvenile survival and growth. The first treatment was selected because there is no significant difference between the second and the third treatment, due to the reduced production.

In general, the average increase in shell length of juveniles in just one month is 1.23mm. The increase in shell length experienced is above average under commercial conditions of France Haliotis and considering the existing growth observed up to date as generally a growth ranging from 1-2mm/month is observed in the nursery from 4mm onwards.

This growth rate experienced in this task, if continued would produce market sized abalone within 4 years, validating that the experiments performed are of commercial interest to establish future hatchery processes following organic methodological procedures being the overall aim of this task. It is expected that these processes will contribute to improve the growth and survival of abalone throughout their full production cycle; in order to improve the production processes; by increasing production performance of juvenile abalone along with integration into IMTA systems through management of integrated conditions (i.e. IMTA of algae and abalone) early in their life cycle.

The development and validation of production process utilising *U. lens* as part of integration of juvenile abalone production in IMTA systems. Processes developed and identified will be transferred and modified to suit local growing conditions for producers through the Atlantic region as part of CS3.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained so far, the activity has been completed at 90% and the results will be exchanged with the partners collaborating in this task to implement abalone hatchery production processes that would increase settlement consistency and survival in juvenile abalone production, while complying with organic certification standards and improving the performances in IMTA systems.

Deviations & Problems: None

Outlook: Post validation of trial results, knowledge exchange with the other project partners and stakeholders collaborating in this task to outline protocols that can be implemented with different produced abalone species and at different geographical locations (Spain, South Africa) and establish technology and knowledge transfer between partners, research and industry sectors.

Activity 3.1.2: Haliotis tuberculate coccinea settlement processes

Introduction

The objectives of this activity are to establish and validate robust repeatable hatchery protocols for abalone larvae to increase reliability, survival and growth of the early life stages, by testing different treatments, and improve their performances during grow out to take place in Land Based IMTA systems. There is a need to reduce the inconsistency between batches of abalone produced in the hatchery and ensure a reliable production of juveniles to be further on-grown in integrated Land Based IMTA systems. The experiments are to be performed using different species of micro and macroalgae in the different locations to further establish and validate consistent hatchery production processes that could be applied to the different commercially produced abalone species at the different

geographical locations and develop production processes to aid abalone juvenile production integration into IMTA systems through exposure to integrated conditions (i.e. improved IMTA production methods) early in their life cycle.

Methods

Review of the literature [25] to define the experimental protocols and treatments to be tested and set up of the experimental settlement infrastructure (Figure 3.1.k)

Figure 3.1.k: Set up of experimental settlement validation infrastructure.

Results

Results are not yet obtained as the experiments are to start during months 11-12 of the project.

Discussion

Results to be obtained will contribute to the development of abalone hatchery settlement processes.

Progress, deviations, problems & next 12M

Progress: Based on the current status of the experiment, it is estimated that the task is 0% completed

Deviations & Problems: COVID-19 resulted in the obligation to postpone the experiments. As they were about to start the restrictions lead to the closure of the experimental facilities.

Outlook: The experiments are planned to be performed during the next spawning season and will contribute to the establishment of settlement protocols for IMTA integration.

[25] (Courtois de Viçose et al., 2007; Courtois de Viçose et al., 2012; Daume, 2000; Daume, 2006; García-Lavandeira et al., 2005; Hannon et al., 2014; Khang et al., 2004; Li et al., 2006; Morse, 1992; Roberts, 2001; Sawatpeera, 2001; Wang et al., 2010; Yu et al., 2010)

Activity 3.1.3: Haliotis midae settlement processes

Introduction

The experiment was performed to develop a method to ensure consistent settlement of South African abalone using species specific cues (abalone mucous) when the natural diatom cultures were inconsistent in order to improve the integrated culture of abalone larvae with a diversity of diatoms and improve the hatchery production of this IMTA method of producing abalone in the hatchery, by increasing its economic stability, this later being one of the key considered in the development of IMTA systems.

South African abalone farm hatcheries rely on co-culture (i.e. IMTA) of diatoms (i.e. naturally occurring microalgae – one trophic level) as a feed for newly settled abalone spat (another trophic level). The farms culture these diatoms on settlement plates suspended in tanks of seawater ahead of abalone settlement, so that when free swimming abalone larvae are introduced to the tank, they have a ready source of algae to feed on when they settle. After the abalone settle onto the algal plates, the abalone and diatoms are co-cultured under conditions of high light intensity to encourage further algal growth (i.e. land based IMTA), and the system works until the abalone growth and feeding rate exceeds the algal growth rate or until the abalone become photophobic. The system works well but its success is inconsistent. The farms have little control over the composition of diatom cultures and, depending on this composition which varies seasonally, the cue for abalone to settle on the diatom cultures appears to result in inconsistent abalone settlement success. Anecdotal evidence suggested that the addition of conspecific mucous to the diatom culture would increase the rate of settlement and subsequent production rates in abalone hatcheries. The intention of this work is to establish if the addition of abalone mucous to microalgal culture ahead of abalone settlement would increase success and to develop methods that can to increase the consistency of settlement success in the IMTA system of production.

It should be noted that this work was carried out ahead of the AquaVitae project and funded by industry stakeholder HIK Abalone Pty Ltd. As declared in the proposed work plan, these data have not been presented elsewhere to the European Union or any other funder since the costs were carried by industry partner. They have made these data available since they contribute to the goals of AV, and ahead of the AquaVitae project our initial intention was to carry this work on as part of AV.

Parts of this report are extracted from sections of Mr. Jefferson van Staden's draft MSc thesis; and these sections may appear in that thesis also, and all data and data analysis should be considered preliminary and subject to further change.

This task was established to test the settlement success of South African abalone using various cues that included conspecific mucous and various algal diatoms in co-culture with abalone on a laboratory-scale only. The objectives included (a) the development of a laboratory-scale to develop and test the method, (b) to compare the settlement and post-settlement survival of abalone in the presence of conspecific mucous added to the diatom culture to a control without this mucous, (c) to see if the beneficial effect of the mucous can be maintained after making the mucous biosecure and (d) assuming positive results of all the previous objectives to test and scale the technology to a commercial-scale production.

Methods

These experiments were carried out on HIK Abalone Farm (Pty) Ltd, a commercial, land-based abalone farm located on the South West coast of South Africa (34°26'04.35"S; 19°13'12.51"E). An experimental-scale diatom/abalone settlement was designed and built to simulate commercial practice (see progress for detailed design).

Broodstock were induced to spawn with the addition of dilute hydrogen peroxide. This is a common method used in abalone farming for inducing gamete release in ripe *Haliotis* spp. Fertilisation and free-swimming, non-feeding larvae production followed normal farm practice.

The experimental-scale settlement system included small containers that were filled with 200ml of sea water from the larval rearing system. These containers were inoculated with free swimming large to a final stocking density of 1 larvae/ml.

Twenty-six settlement plates covered with a pre-cultured diatom film were placed into each settlement container. Thirteen of the diatom biofilms were either sprayed with conspecific mucous (DM). This was done by setting a household spray bottle to a fine mist of a solution containing abalone mucous and spraying each side of a settlement plate twice from a distance of 10cm. The plate was then gently shaken, allowing excess mucous to drip off before being placed back into its respective cup. The remaining 13 plates were left as a control with no conspecific mucous (D).

The lights were switched off and the larvae were left to settle overnight.

In the morning after larvae had been added to settlement containers, the pre-primed flow regulators were set to 100ml/h. Sampling began two hours later, after a full exchange of the water in the containers. This flushing period was employed to flush out unsettled larvae, which may stick to settlement plates as a result of surface tension when they are removed for observation (Jefferson van Stadens pers. obs.).

In the initial trail, sampling of settled larvae was carried out the morning after larvae were inoculated into cups (day-1) and on the last day of the trial (day-6). In the subsequent trial, the same procedures were used only samples were taken daily for the duration of the 6-day trail.

When sampled, a plate was removed from its container, held for five seconds to allow excess water to drip off, and then observed under a dissecting microscope (Motic SMZ-171B, Motic Group Co., Ltd, Kowloon, Hong Kong). The number of successfully settled larvae and the number of metamorphosed larvae were recorded for each plate observed. Metamorphosis was indicated by the development of peristomal shell (Figure 3.1.l).

Figure 3.1.l: A metamorphosed post-larval H. midae. Metamorphosis is identified by the growth of peristomal shell (PS), as indicated by the red outline.

Once the total number of larvae and the number of metamorphosed larvae had been recorded for each side of a plate, the plate was then discarded.

System design and construction details

The system was designed to facilitate the growth of an ambient diatom biofilm on the plate surfaces, similar to farm practice, but on an experimental-scale. It was designed to condition plates oriented vertically; using filtered ambient seawater and natural sunlight, i.e. industry standard practice. Prior to construction the system was modelled in Google SketchUp Pro 2016, to pre-emptively identify any potential design flaws (Figure 3.1.m).

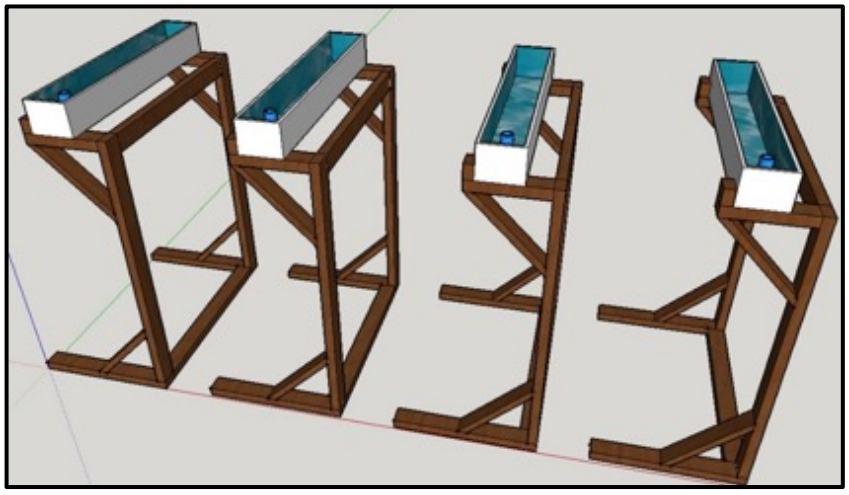


Figure 3.1.m: Design of system used to condition experimental settlement plates with ambient diatom biofilms prior to settlement induction.

Square lead-free polyvinyl chloride (PVC) guttering (Marley SGH510) was cut to a length of 120cm, and sealed at each end using the appropriate stop-ends (Marley SE501). Marine silicone was used to fill the joins between the gutter and stop-ends to prevent leaking. The gutters were white in colour; as are hatchery settlement tanks, which were intentionally constructed using the reflective properties of white materials to promote even light distribution within the tank for diatom growth. At one end of the gutter a hole was drilled, and a 32mm fitting that was attached to the underside of the gutter in alignment with the hole. A 70mm length of 32mm diameter PVC pipe was cut and used as a standpipe for each gutter to maintain the water level in the gutter. The gutter section of the system was attached to a wooden frame using fascia brackets (Marley SK501), and these were attached to each of the frames supporting arms.

A length of three-millimetre-thick mild steel section was cut to the width of 45mm and heated using a blow-torch, the heated end was then used to melt 30 pairs of grooves into the strips of plastic which connected the sides of the gutter to the bottom. These grooves were spaced in three-centimetre intervals and served as slots for the base of the settlement plates to slide into, enabling them to stand vertically (Figure 3.1.n). Four plate conditioning trough systems were built and placed in a well-lit area of the farm (Figure 3.1.o).

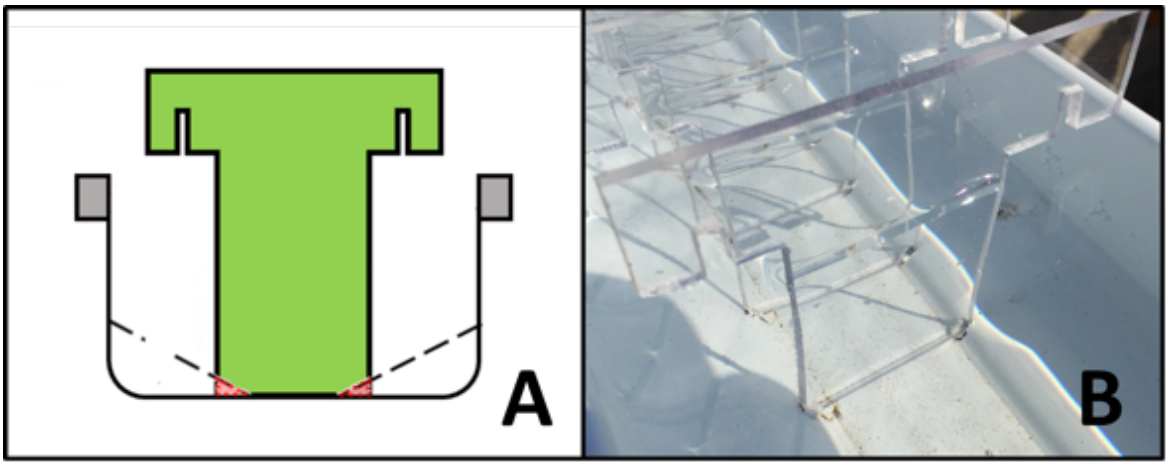


Figure 3.1.n: (A) Diagrammatic sketch illustrating settlement plate positioning within the gutter portion of the biofilm conditioning systems; black dashed lines indicate the strips connecting the gutter sidewalls to the bottom, and red markings indicate the slots melted into these strips. (B) A photograph of the transparent experimental settlement plate placed in the white gutter at the start of a biofilm conditioning period. Transparent plates make it possible to co-culture algae after abalone have settled (i.e. IMTA).

Figure 3.1o: The ambient biofilm conditioning troughs were placed in a well-lit area of the farm to facilitate diatom growth. The shade cloth covers had not yet been attached to the frames at the time this photograph was taken.

A standard, commercial abalone grow-out tank (3.95m x 0.875m x 0.75m) was used as a sump for the plate conditioning systems. Water was supplied to this tank from the farm's main header tank, where ambient seawater was filtered to 90µm by two drum filters (Gamco Marine Engineering Services, Gansbaai, South Africa). A 50µm filter bag (Aquamarine Water Treatment, PP-50-P02E) was hung under the canvas tanks inlet value to prevent any large particles from fouling the settlement plates. Water was pumped to the conditioning systems using a submersible pump (Sunsun HQB-2500 55W, SunsunGroup Co. Ltd, Zhejiang, China), via 32mm polyethylene pipe.

While the production tank was filling after being scrubbed, 30 experimental settlement plates were placed into each conditioning gutter (Figure 3.1.p). The submersible pump within the production tank was turned on and the valves to each conditioning trough set to a flow rate of 10l/min. While this flow rate may seem high considering the volume of each gutter, lower flow rates would result in the temperatures within the troughs to rise and kill off diatom growth. The settlement plates were then left in these flow-through gutters, allowing for inoculation and growth of ambient benthic diatom films to be used in settlement trials. The conditioning period varied from 10-14 days to account for the changes in photoperiod throughout the entire trial period; plates required 14-days of conditioning to develop adequate biofilms in winter when daylength was reduced. The time required to grow a comparative biofilm during summer months was reduced to 10-days.

Figure 3.1.p: Experimental-scale settlement plates at the start of a conditioning period, that ultimately allowed for the IMTA of abalone and diatoms. Shade cloth covers were used in summer, but removed during winter to control diatom growth since diatom overgrowth was less likely to occur during periods of shorter daylength in winter.

During the conditioning period, rinsing of the settlement plates was required to wash off any particles adhering to the biofilm and to rinse off filamentous diatom growths, both of which may negatively affect the settlement of Haliotis larvae. Smothering of biofilms by sediment inhibits also light penetration and most likely negatively affects gaseous exchange during photosynthesis; which left "untreated/unrinsed" leads to the sloughing off of the diatom biofilms (Jefferson van Stadens pers. obs.)

The design had to take the following factors into consideration; space constraints, number of replicates required, similarity to commercial systems, sampling methodology and the provision of environmental conditions suitable for settlement and metamorphosis of abalone larvae to occur. The experimental system was modelled in Google SketchUp Pro 2016 before final construction (Figure 3.1.q).

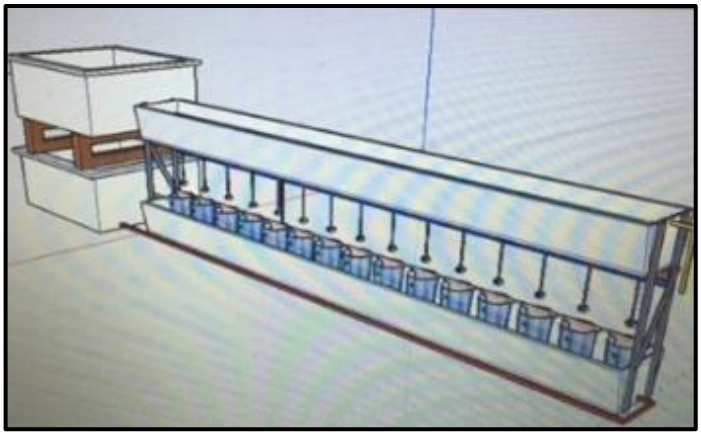


Figure 3.1.q: Concept design of the systems to be built for the settlement induction trials.

The system consisted of an upper and lower level (Figure 3.1.q). The lower level acted as a semi recirculating water bath, which would hold the settlement containers and buffer them from rapid ambient air temperature changes.

A polypropylene tray (495mm x 350mm x 178mm) acted as a sump on the lower level, holding a submersible aquarium pump (Sunsun HJ-542 5W, Sunsun Group Co. Ltd, Zhejiang, China) and an aquarium heater (Eheim 3612 Aquarium Heater 50W, Eheim GmbH & Co., Deizisau, Germany). The sump was connected to a rectangular gutter via 25mm PVC plumbing. The gutter was sealed at each end using the appropriate stop ends and marine silicone.

The strip of plastic connecting the gutters front side-wall to the bottom was removed, thereby allowing sufficient space for the settlement cups to stand level when placed inside the gutter. The strip joining the gutters rear side wall and bottom was left intact to support the 25mm PVC spray bar, which fed water into the gutter. The spray bar had two-millimetre holes (n=18) drilled evenly along its length, to provide even distribution of incoming water within the water bath gutter.

The gutter drained via a 32mm stand pipe positioned at its centre, which redirected water back to the sump. The gutter part of the systems were raised on fibre glass frames to allow for gravity feeding of water back to the sump.

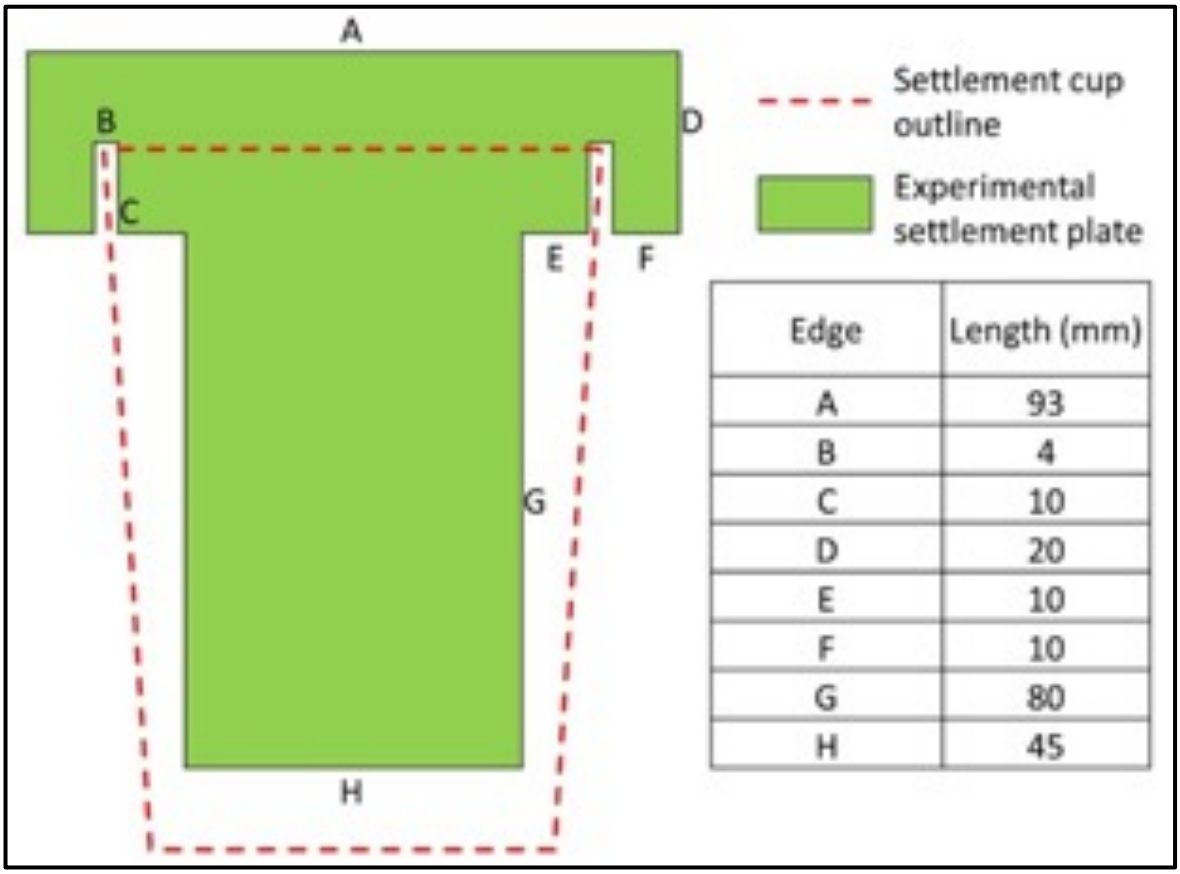
A second meat tray was positioned above the first using wooden supports. The upper meat tray served as a reservoir for the top gutter, supplying water to it via 25mm PVC piping. Any excess water flowing into the top gutter drained through an overflow pipe attached to the opposite end. The reservoir was supplied 26µm filtered ambient seawater via a pre-existing supply line at the experimental site. The top gutter was fastened to the frame 18.5 cm above the bottom gutter, and would function as a water supply source to the settlement cups.

Four-millimetre diameter holes were drilled nine centimetres apart in the upper gutter, each hole was threaded and had a threaded barb inserted. A piece of four-millimetre silicone fish tank pipe was attached to the barbs allowing water to travel from the top gutter to each of the 16 cups standing in the bottom gutter. To regulate the flow of water into each cup, an intravenous drip flow regulator (Surgi-Plus I.V Flow Control Regulator 0-250 ml/h, ISO 13485:2003) was attached to the end of each fish tank pipe. Five systems were constructed allowing for 80 settlement cups to act as experimental units (Figure 3.1.r).

Figure 3.1.r: Four of the five experimental systems designed and built for the trials carried out for this task. This figure depicts the system prior to larval inoculation, when plates were being treated – as indicated by the multicoloured pegs which were used to mark plates and their respective treatments after randomisation had been carried out.

Small acrylic containers were used as settlement vessels; in which a settlement plate could be placed, similar that observed in a commercial-scale settlement tank. The containers were filled with 200 ml of water and a mark was made at the meniscus of this water level. A 6.5mm diameter hole was drilled at this mark using a wood drill bit to allow for drainage when water was turned on. A 6.5 mm hole was the smallest diameter that allowed water to escape the cup at a steady rate by overcoming surface tension. Smaller diameter holes resulted in irregular drainage as the surface tension couldn't be overcome by the mass of the water between the rim of the cup and the hole. Any bit type other than a "wood bit" cracked the acrylic cups.

Experimental settlement plates were designed to be easily removable and hang vertically within the settlement cups. They were designed this way to fulfil the requirement of removal for sampling; without dislodging attached larvae through excessive disturbance of the water within the cup. The conditioned section of the plate was supported in the middle of the cup; two notches cut out of the wider area of the plate slid over the rim of the cup, securing the plate in place. The plates were cut out of three-millimetre thick transparent polycarbonate plastic, using a jigsaw, to the specifications seen in Figure 3.1.s.



Edge	Length (mm)
A	93
B	4
C	10
D	20
E	10
F	10
G	80
H	45

Figure 3.1.s: Experimental settlement plate design and positioning within an acrylic cup, providing a scaled down vertically oriented settlement substrate once conditioned.

Results

More larvae settled onto algal plates that had been treated with conspecific mucous than untreated diatom plates (p=0.017; Table 3.1.a). After six days there were significantly more larvae observed on the mucous-treated diatom plates (p<0.001; Table 3.1.a).

Table 3.1.a: Mean (± standard error) settlement rate, number of larvae observed, proportion of settled larvae which had metamorphosed and water quality parameters in tanks where abalone larvae were settled on either a diatom biofilm (D) or a diatom biofilm inoculated with adult abalone mucous (MD) after either one- or six-days post settlement during (Independent t-test, p<0.05; preliminary data and analysis subject to change).

	Time (d)	**D**	**MD**	**$t_{(24)}$**	**p**
Settlement (%)	1	30.01±1.68	37.48±2.36	2.58	0.017
No. Larvae observed (Total)	6	53.15±2.53	74.08±3.28	5.05	p<0.001
Metamorphosis of settled larvae (%)	6	95.13±0.88	96.59±0.67	1.33	0.196
Oxygen saturation (%)	1	94.97±0.58	94.41±0.39		
	6	95.27±0.40	95.84±0.38		
Temperature (°C)	1	16.43±0,06	16.39±0,04		
	6	16.55±0,01	16.54±0,02		
pH	1	7.86±0,01	7.87±0,01		
	6	7.85±0,01	7.85±0,01		

Similar results were obtained initially when the trial was repeated twice (Tables 3.1.a and 3.1.b respectively), in which data were collected every day for the duration go the trial. While the number of larvae that settled on the mucous-treated plates was initially higher, this increase became less noticeable over time due to increase variance (Tables 3.1.b).

Table 3.1.b: Mean (± standard error) settlement, number of larvae observed, proportion of settled larvae which had metamorphosed and water quality parameters in tanks where abalone larvae were settled on either a diatom biofilm (D) or a diatom biofilm inoculated with adult abalone mucous (MD) one to six days post settlement during trial 3 (Independent t-test, p<0.05; preliminary data and analysis subject to change).

	Time (d)	D	MD	$t_{(18)}$	p
Settlement (%)	1	9.59±1.45	22.62±1.89	-5.47	p<0.001
No. Larvae observed (Total)	1	20.4±3.09	48.1±4.02	-5.47	p<0.001
	2	12.9±2.39	33.4±3.47	-4.87	p<0.001
	3	12.2±2.89	33±8.47	-2.33	0.049
	4	12.8±2.27	17.4±2.86	-1.26	0.243
	5	6.4±1.97	20±4.51	-0.28	0.024
	6	8.8±2.25	15±6.03	-0.96	0.378
Metamorphosis of settled larvae (%)	1	00.00±00.00	00.00±00.00	*N/A*	*N/A*
	2	1.04±0.70	4.99±1.91	-1.94	0.078
	3	31.10±6.70	41.77±6.74	-1.12	0.294
	4	46.59±9.31	87.38±5.48	-3.78	0.005
	5	71.67±8.16	85.76±4.94	-1.48	0.178
	6	75.83±11.37	68.24±17.47	0.36	0.725
Oxygen saturation (%)	1	88.73±01.03	85.87±02.28		
	2	90.03±00.94	90.34±01.24		
	3	90.72±02.34	89.78±01.90		
	4	92.88±00.96	92.98±01.20		
	5	94.80±01.06	95.74±01.15		
	6	97.22±00.54	96.74±00.93		
Temperature (°C)	1	18.89±00.17	18.92±00.17		
	2	16.05±00.07	16.05±00.08		
	3	16.24±00.19	16.18±00.20		
	4	18.16±00.15	18.16±00.17		
	5	18.60±00.34	18.52±00.38		
	6	18.72±00.35	18.74±00.34		
pH	1	07.88±00.01	07.85±00.02		
	2	08.02±00.01	08.00±00.02		
	3	07.91±00.04	07.92±00.02		
	4	07.86±00.01	07.87±00.02		
	5	07.85±00.01	07.86±00.02		
	6	07.86±00.02	07.87±00.01		

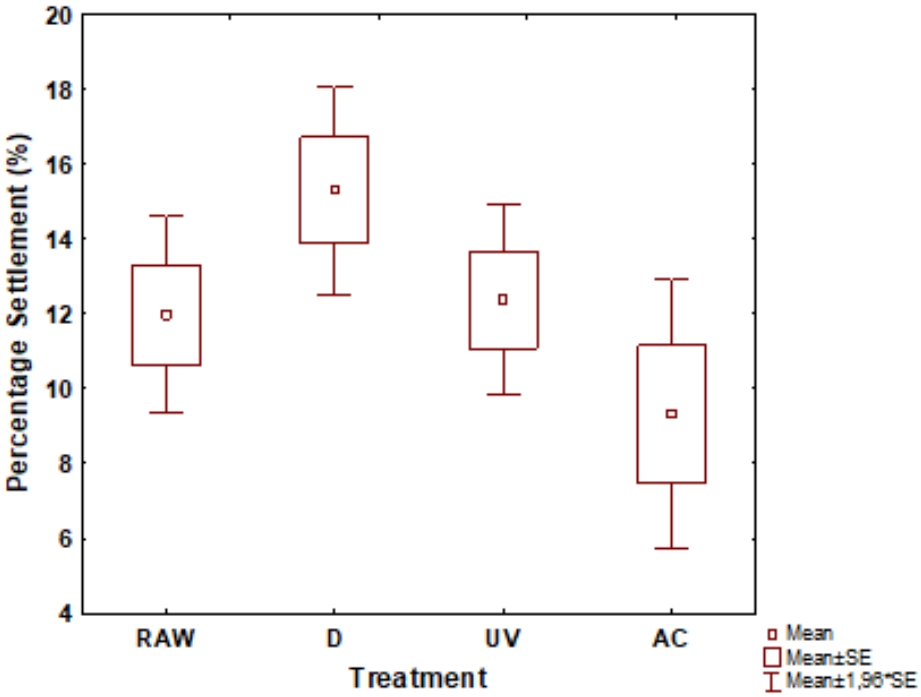


Figure 3.1.t: Settlement of abalone larvae onto diatoms (RAW) or diatoms treated with mucous (D) or diatoms treated with mucous that had been subject to ultra violet light (UV) or it was subject to heat in an autoclave (AC) (one-way ANOVA, p=0.001; preliminary data and analysis subject to change).

Settlement increased when diatoms were inoculated with conspecific mucous (Tables 3.1.a and 3.1.b; Figure 3.1.t). However, when that mucous was subject to either UV or AC (in an attempt to make it bio-secure) there were no differences in settlement rate compared with untreated diatoms (Figure 3.1.t).

Discussion

A method to increase consistent settlement of South African abalone onto diatom cultures using conspecific mucous was developed on an experimental-scale and it increased the rate of settlement of abalone larvae on to microalgae cultures, and these methods were refined in these early experimental-scale experiments. However, the increase in settlement rate did not consistently result in an increase in the number of larvae that metamorphosed, which cast initial doubt on the value of this procedure on a commercial-scale. Furthermore, there does not seem to be a way to sterilise the mucous and make it bio-secure, without it losing its effectiveness as an attractant. Abalone hatcheries cannot take the risk associated with biosecurity in introducing conspecific mucous into the hatchery. For these reasons, industry decided to discontinue this aspect of the project and did not proceed with the scale-up part of this work that was initially intended and it this scale-up was thus never included in from the AquaVitae work plan form the start.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained, the activity has been completed at 90% and the results will be exchanged with the partners collaborating in this task to implement abalone hatchery production processes that would increase settlement consistency and survival in juvenile abalone production.

Deviations & Problems: none

Outlook: It should be noted that although it was industry's initial intention to scale this work up as part of the AquaVitae Project (i.e. before the project started), and the results of these laboratory-scale trials showed potential for scale-up, it was decided that the biosecurity risk associated with bringing conspecific mucous into the hatchery was too great; and for that reason, industry partner was not prepared to take this risk and it was decided not to include the scale up in the proposed AquaVitae work plan when the work plan was first drafted.

CST 3.2: Hatchery temperature

Responsible CS Task Leader: Sylvain Huchette, FrHa

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
3.2	Abalone hatchery temperature	T1.2	FrHa	25%	!	M7	M7	M13	M24	5	7

Introduction

The rationale behind this task is to obtain crucial information about the future impact of global warming on abalone and its farm production. Global warming will lead abalone farm population to encounter extreme changes in temperature mainly during summer. These summer extremes are known to cause severe mortalities in land-based farms in Australia (Dr Rob Day, pers. Comm.). European farms need to learn how to cope with these thermal stresses before they become frequent and need to establish strategies that could contribute to mitigate the effect. In that sense, IMTA systems present an interest as the integration of macroalgae within production systems have demonstrated to have an impact on pH and nutrient values within the systems that could counter balance the effect of variations of other environmental parameters. Therefore, the results of this experiment are of interest and complementary to the information to be obtained by the IMTA nursery trial.

Methods

A first experiment was started at the end of 2019 comparing production tanks heated (+2-3°C) or kept at ambient temperature. However due to technical problems (insufficient heating potential during winter) followed by the lockdown due to COVID 19, primary results from this first experiment were obtained and the experiment was finalised. The results will be obtained from another experiment to be further pursued during 2021 when the spawning season starts.

Results and discussion

Preliminary results testing for the impact of heating on growth and survival of European abalone juveniles were obtained. Unfortunately, the experiment could not be pursued due to technical difficulties and its interruption due to COVID-19. It was observed that higher temperatures induced

higher mortality and faster growth (Table 3.2.a.) (Figure. 3.2.a). However, the growth results are biased due to lower densities at higher temperatures. The experiment has been reviewed, together with the existing literature and another experiment has been planned to test the effect of temperature on abalone during the nursery stage as part of an IMTA system and propose strategies for the production process that could mitigate the effects, especially considering the pH.

Figure 3.2.a: Experimental set up for the temperature experiments.

Table 3.2.a: Growth and survival of Haliotis tuberculata at different temperatures.

	Ambient temperature	T°C +3.5°C
Initial shell length (mm)	8,1±2,1	8,1±2,1
Final shell length (mm)	12,1±3,2 [a]	15,2±3,9 [b]
SGR (µm/day)	43,9±3,5 [a]	77,2±3,7[b]
Mortality (%)	17,6±3,3[a]	55,7±5,9[b]

Progress, deviations, problems & next 12M

Progress: After the difficulties encountered during the 1st experiment in the winter 2019-2020 another experiment was planned to start in 2021. Based on the activities performed up to date we estimated that only 25% completeness was achieved for this task.

The 2nd experiment is now designed and equipment are being purchased. The key exploitable result of this task will be an improved knowledge for abalone stress due to high temperature level that will allow to review the production processes and propose mitigation alternatives so that extreme summer temperature that we are expecting to occur in the future with global warming may be better apprehended.

Deviations & Problems: Following the technical problems with the initial experiment (insufficient heating power to reach targeted temperature in production tanks and restrictions due to COVID 19), France Haliotis decided to abandon, re-design and re-program the experiment. This task has thus been delayed for about 1 year.

Outlook: This second experiment should be carried out during the first semester of 2021 and first results should be acquired by M30.

CST 3.3: IMTA nursery trial

Responsible CS Task Leader: Sylvain Huchette, FrHa

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
3.3	IMTA nursery trial	T1.2, T3.2	FrHa	80%	!	M7	M4	M14	M19	3 or 4	7

Introduction

The experiment to establish integrated multi-trophic aquaculture in abalone nursery: abalone (*Haliotis tuberculata*) -*Ulvella lens*- Anemones-Butter fish started on track at M7 and is actually running. The methods and results will therefore be reported in the next reporting period.

Methods

The experiments will be performed in twelve 1000L experimental tanks using 4000 4-month old juveniles per tank. The experiments will be performed comparing six control tanks with *Ulvella lens* settlement plates, with complementary feeding of *Ulva rigida,* integrating only abalone, and six tanks with *Ulvella lens* plates, with complementary feeding of *Ulva rigida,* to which locally sourced specimens will placed in the integrated system, in the nursery tanks, with abalone juveniles. The introduced specimens consisting in: three specimens of *Holothuria forskali* juveniles, 20 specimens of *Anemonia sulcate,* five specimens of *Gaidropsaris vulgaris*. During the entire experimental period growth and survival of abalone juveniles, anemones, sea cucumbers and fish will be monitored, as well as the water quality and waste management within the system to estimate nutrient fluxes and nutrient's budget in the production system.

Results

Actually, the experiments are running and the data will be collected once the experiments will be finalised. A visual difference has been observed between IMTA and non-IMTA nursery tanks (Fig 3.3.a).

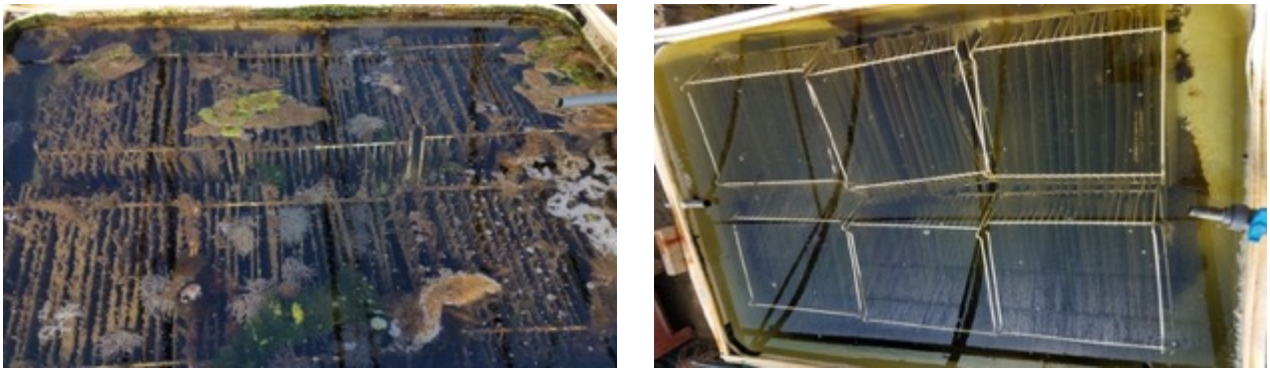


Figure 3.3.a: Photographs of the experimental tanks (On the left: "AMTI" system and on the right – control).

Discussion

To be described in the next report from M13-18.

Progress, deviations, problems & next 12M

Progress: Based on the current status of the experiment, the task is 80% completed. The experiment is running and the results are being collected and analysed. The restrictions due to the COVID crisis did not affect the collection of data in terms of growth, survival and biomass production.

Deviations & Problems: The COVID crisis did not allow the collection of information about the nutrient cycle in the nursery production system to obtain information about nutrient fluxes of interest for environmental evaluation of IMTA systems. The experiment would need to be repeated to obtain the information and is not considered within the time frame of the project. However, such information is expected to be obtained during the experiments performed for IMTA grow-out processes.

Outlook: Results to be described in the next report from M13-18.

CST 3.4: Land Based IMTA European abalone, sea cucumber and macroalgae

Responsible CS Task Leader: Gercende Courtois de Viçose, ULPGC

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
3.4	IMTA ab/sea cucumber/algae	T2.2	ULPGC	30%	✓	M8	M9	M18	M19	3-4	6-7

Introduction[26]

Various studies have demonstrated the interest of including deposit feeders, as an integrated element of IMTA systems devoted to the consumption of organic materials (Cubillo et al., 2016), and specifically sea cucumbers (Emiroğlu and Günay, 2007; Chary et al., 2019). Sea cucumbers are of interest as deposit feeder, for their environmental value that serves the goal of sustainability and their nature, which enables cultivation associated with good growth (Zhou et al., 2006; Paltzat et al., 2008) and low mortality rates (Slater and Carton, 2009; Yuan et al., 2006; Zamora and Jeffs, 2012). Various sea

[26] (Ahlgren, 1998; Beltran-Gutierrez et al., 2016; Chary et al., 2019; Cubillo et al., 2016; Emiroğlu and Günay, 2007; Hannah et al., 2013; Kang et al., 2003; Paltzat et al., 2008; Pitt et al., 2004; Reid et al., 2013; Slater and Carton, 2009; Yuan et al., 2006; Zamora and Jeffs, 2012; Zhou et al., 2006)

cucumber species within IMTA systems have been tested for co-culture with species including, salmon (Ahlgren, 1998), charm abalone (*Haliotis discus hannai*) (Kang et al., 2003), shrimp (Pitt et al., 2004), bivalves (Zhou, 2006), Pacific oyster (Paltzat et al., 2008), sablefish (Hannah et al., 2013), and seaweed (Beltran-Gutierrez et al., 2016). The interest of Holothurians as deposit feeders was demonstrated in these studies and led to the realisation of other studies investigating their mitigation ability within different IMTA systems suggesting that sea cucumbers can optimise the net use of wastes (Reid et al., 2013).

Given the existing research, the current study investigates the potential for the integrated culture of the sea cucumber *H. sanctori* with the abalone *Haliotis tuberculata coccinea* in order to enhance the integrated Land Based IMTA production of both species.

Methods

***H. sanctori* specimen recollection**

Young individuals of *H. sanctori* (mean weight: 31.07 ± 14.51 g) (Figure 3.4.a) were collected from Taliarte (27.9892° N, 15.3753° W; Gran Canaria, Canarias, Spain) at a depth of 0-10 m, by scuba diving, and maintained and acclimated in the installations of the ECOAQUA institute, university of Las Palmas de Gran Canaria, placing them under abalone (H. tuberculata coccinea) baskets (in which abalone were fed macroalgae). After their indoor acclimation, they were then respectively allocated to the experimental IMTA units.

Figure 3.4.a: Collected specimens of Holothuria sanctori.

Abalone specimens

Adult abalone of *H. tuberculata coccinea* (mean weight: 37.32 ± 9.01g and mean size: 60.83 ± 5.30mm) were obtained from the abalone production unit of the ECOAQUA institute, University of Las Palmas de Gran Canaria.

IMTA experimental setup

Six replicates of experimental IMTA systems (Figure 3.4.b; Figure 3.4.e) were designed to provide two levels compartments; one for the fed abalone (*H. tuberculata coccinea*) (Figure 3.4.c) of 50L capacity and the other for sea cucumber (*H. sanctori*) (Figure 3.4.d) located below the abalone compartment.

Together located in 300L tank capacity. The abalone compartment was perforated on the bottom and on the sides (Figure 3.4.c) to allow water exchange and release of abalone wastes to reach the sea cucumber located below.

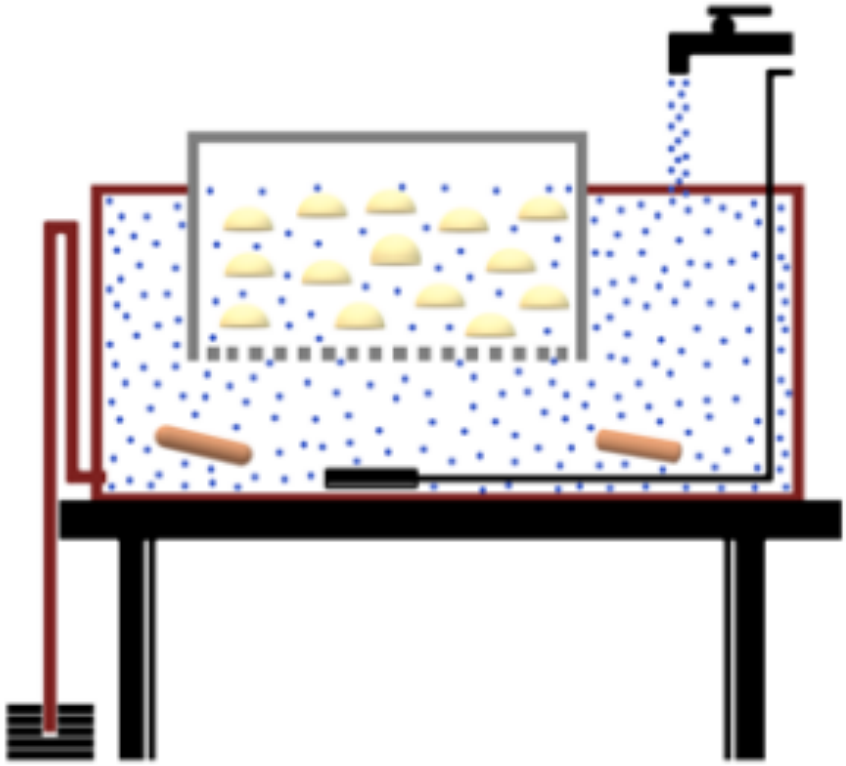


Figure 3.4.b: Structure of the IMTA system.

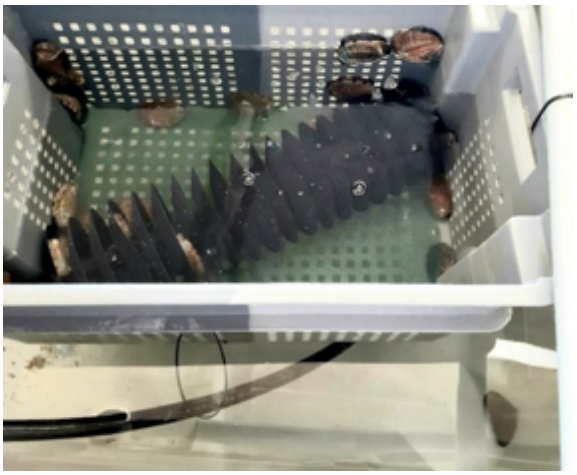


Figure 3.4.c: Abalone compartment.

Figure 3.4.d: Sea cucumber and abalone unit.

Figure 3.4.e: Sea cucumber and abalone unit.

Stocking density

Due to the limited information on the adequate stocking density of *H. sanctori*, different densities of specimens were tested in the experimental units to establish the adequate density. Tests were performed in triplicates in the IMTA experimental units with two and three sea cucumber specimens (2.5 Specimen.m^{-2} and 3.75 Specimen.m^{-2}) that were placed under abalone baskets stocked at 50 Specimen/m^{-2} and weekly fed 0.8kg of IMTA produced macroalgae (*Ulva rigida* and *Hydropuntia cornea*) (Figure 3.4.f). Specimens were distributed in groups of 2 or 3 specimens ensuring that the overall average weight of all groups was similar in all treatments. Sea cucumber growth performance was reviewed after 4 and 8 weeks to estimate the best stocking density needed for sea cucumber growth.

Control treatments including only abalone baskets, stocked at 50 Specimen/m^{-2} and weekly fed 0.8kg of IMTA produced macroalgae, were simultaneously tested to demonstrate that the feed inputs between the experimental units, throughout the weeks, were not statistically different.

Figure 3.4.f: Ulva rigida and Hydropuntia cornea used as feed source.

Sampling and data analysis

Growth performance: Sea cucumbers were individually identified through photographs. The specimens were placed in tissue to blot before being weighed. Weight Gain (WG), and Specific Growth Rate (SGR) were estimated according to the formulas:

WG (%) = 100 * (Wf − Wi) / Wi

SGR (%.day-1) = 100 * (lnWf − _lnWi) / number of days

Where Wi is the sea cucumber initial wet weight (g) and Wf is the sea cucumber final wet weight (g).

To determine the stocking density of sea cucumber to be selected for experimental purposes, the initial, intermediate (week-4), and final (week-8) weights of sea cucumbers were recorded to estimate growth performance (specific growth rate and weight gain). The increase or decrease in the sea cucumber initial weight was considered an indication of the ideal stocking density.

Results

Stocking density determination

Growth performance represented by sea cucumbers' SGR and WG was estimated after 4 and 8 weeks to evaluate the best stocking density suitable for sea cucumber growth. Overall, the mean SGR and WG values differed significantly ($p < 0.05$) among high density and low-density treatments after the experimental period. All sea cucumber specimens within the density 3.75 Sp.m^{-2}, were found to lose weights and exhibit negative SGR and WG values. On the contrary, for specimens stocked at a density of 2.5 Sp.m^{-2}, their growth performance improved as all specimens could gain weight and exhibit positive SGR and WG values (Figure 3.4.g; Figure 3.4.h).

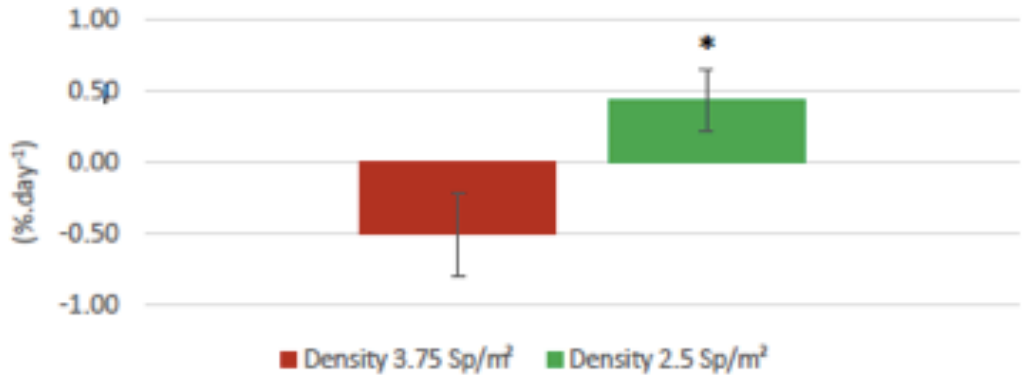


Figure 3.4.g: Specific growth rate (SGR) values (Mean ± SD) of sea cucumbers in the different stocking densities. (*) Indicate significant difference (p < 0.05).

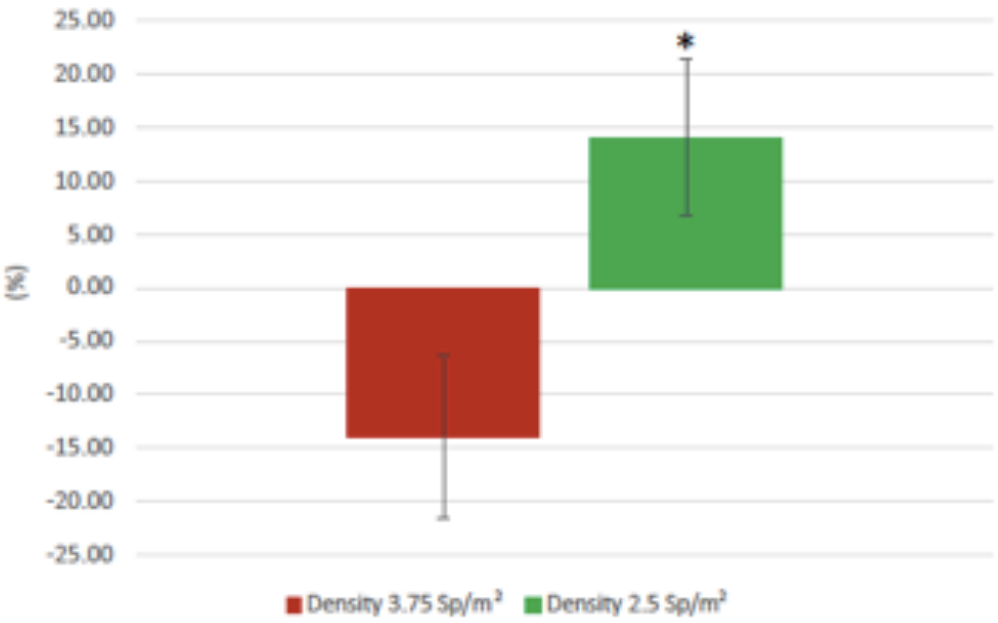


Figure 3.4.h: Weight gain (WG) values (Mean ± SD) of sea cucumbers in the different stocking densities. (*) Indicate significant difference ($p < 0.05$).

Throughout the trial, wastes produced by abalone, fed IMTA produced fresh macroalgae, were consumed as feed by sea cucumbers and no significant difference ($p > 0.05$) were observed in the quantity of abalone wastes between tanks presenting weekly quantity of 11.99 ± 2.16 g $tank^{-1}$ (DW) of abalone wastes.

Discussion

Growth performance in sea cucumbers can be attributed to three main factors; water quality, food availability, and stocking density of sea cucumber (Slater and Carton, 2007; Pei *et al*., 2012). Considering that all sea cucumbers in the tanks were subjected to the same water quality conditions and no significant difference ($p > 0.05$) was observed in the feed quantities between the tanks, the only variable that could influence the sea cucumber growth performance is the stocking density factor. Several authors have reported an inverse proportionality between sea cucumber stocking density and their growth rates (Dong *et al*., 2010; Pei *et al*., 2012; Xia *et al*., 2017). The loss of condition amongst sea cucumber fed abalone faeces at high density in the current experiment indicates that the increase in the stocking density reduces the growth of sea cucumbers, as all specimens within density 3.75 $Sp.m^{-2}$ showed negative SGR and WG values, and specimens stocked at 2.5 $Sp.m^{-2}$ showed positive SGR and WG values (Figure 3.4.g; Figure 3.4.h). A similar study on *A. mollis* sea cucumber within an IMTA system showed that sea cucumber growth is density-dependent, with the best growth performance observed at the lowest density (Slater and Carton, 2007). Considering the weight of the specimens, the stocking density in the current study was lower than the one reported by Slater *et al*. (2009) that found a loss of condition at a stocking density of 543 $g.m^{-2}$. Based on these results, a stocking density of 2.5 $sp.m^{-2}$ was selected for *H. sanctori* under the applied conditions to obtain optimal growth in further feeding trial experiments.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained so far, the task is 30% completed and will contribute to develop new production processes for the Land Based IMTA production of low trophic species.

Deviations & Problems: none

Outlook: The feeding trials experiments are ongoing to determine the response of *H. sanctori* to different feeding strategies within Land Based IMTA systems in order to establish innovative production processes.

CST 3.5: Land Based IMTA South African abalone, sea cucumber and algae

Responsible CS Task Leader: Peter Britz, RhU

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
3.5	IMTA of abalone/seacucumber/algae	T3.2	WiCoAb, RhU, AWI, MFeed	20%	✓	M8	M1	M20	0	3-4	6-7

Introduction

Life cycle analysis (LCA) has never been carried out on a commercial scale IMTA system used to produce abalone. This approach will measure the energy flow (input and output and that retained in the system) using carbon, nitrogen and phosphorus budgets of a non-IMTA system used to produce abalone and compare it to alternative systems that include various IMTA loops, e.g. the IMTA of abalone where *Ulva* is produced using the dissolved wasted from abalone production, the inclusion of IMTA and non-IMTA produced algae in the abalone feed, and the possibly the inclusion of sea cucumber in the IMTA system to remove solid waste from abalone tanks. This task will draw on data from CS Task 3.7 and from data produced in CS7 (sea cucumber aquaculture technology development), and it will develop an LCA that demonstrates the environmental and possibly economic saving of a commercial-scale IMTA, thus developing the commercial-case for the use of IMTA in the future.

Methods

Life cycle analysis of land based IMTA

It was hypothesised that the nutrient utilisation and production efficiency of the farming systems is affected by seasonal changes. Also, it was hypothesised that the abalone / *Ulva* Spp. IMTA biofilter system performed differently and better than the abalone and *Ulva* Spp. monoculture (non-IMTA) systems on the farm. Therefore, sampling methodology was defined and planned to run from April /May 2020 up to January (Summer) of 2021. Water samples for nutrient analysis will be collected at different points (incoming seawater, post abalone effluent, post seaweed biofilter effluent) in the IMTA and non-IMTA systems (Figure 3.5.a). Physiochemical parameters which included temperature, pH and dissolved oxygen will be recorded in-situ at ponds inlet and outlet every week, to cover 30 days Ulva production cycle on the farm. During this period, triplicate water samples for total ammonia nitrogen (TAN), nitrite nitrogen (NO_2^--N), nitrate nitrogen (NO_3^--N), phosphate (PO_4^-), total nitrogen (TN), total phosphorus (TP), chemical oxygen demand (COD) and total organic carbon (TOC) and suspended solid (Table 3.5.a) will be collected. Samples of macroalgae, abalone, nutrients and abalone tank sediments will be collected and analysed for their nitrogen, phosphorus, and carbon.

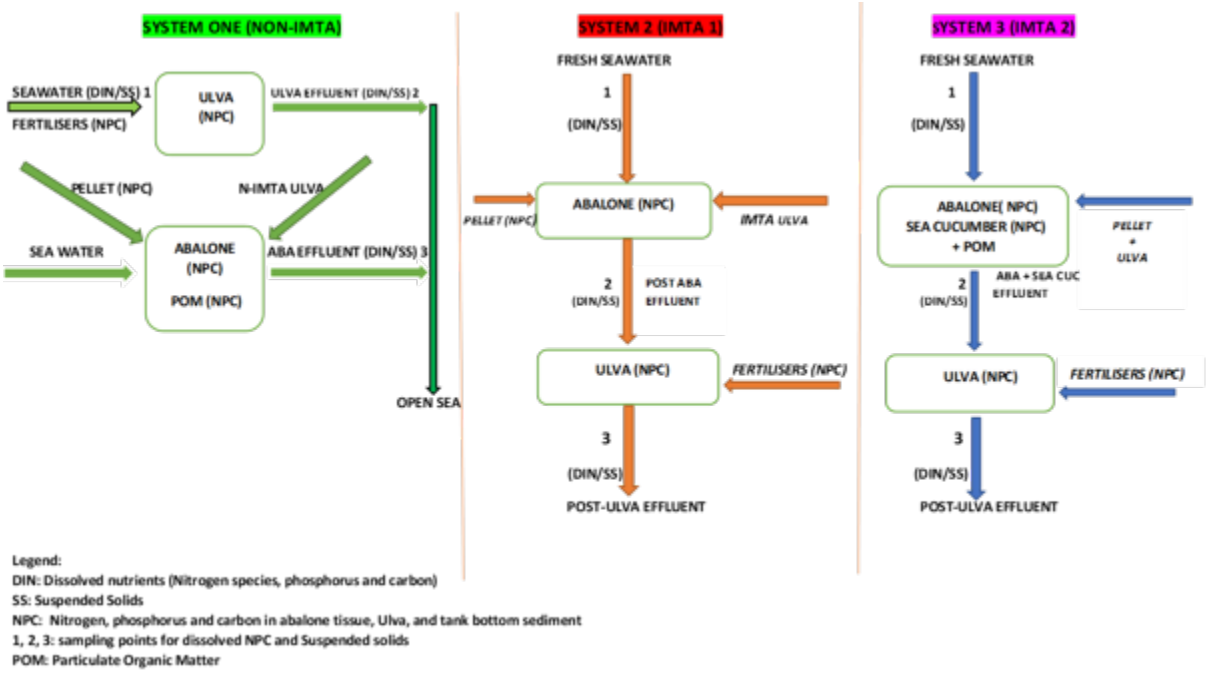


Figure 3.5.a: Schematic of the experimental design comparing IMTA systems at Wildcoast Abalone Farm Pty Ltd to determine environmental cost differences in IMTA and non-IMTA systems using life cycle analysis (LCA).

Table 3.5.a: Experimental sampling points.

PARAMETERS	SAMPLING POINTS NON-IMTA	SAMPLING POINTS IMTA	FREQUENCY
pH	1, 2, 3	1, 2, 3	Every sampling period
Temperature	1, 2, 3	1, 2, 3	Every sampling period
Dissolved oxygen	1, 2, 3	1, 2, 3	Every sampling period
Dissolved and particulate nutrients NH_3, NH_4, NO_2, NO3, PO_4, TN, TP, TOC	1, 2, 3	1, 2, 3	Weekly per Ulva cycle per season
Suspended solids	1, 2, 3	1, 2, 3	weekly
Flowrate	1, 2, 3	1, 2, 3	Every sampling period
Abalone tissue N P C	4	4	Every season
Abalone stocking biomass			
Abalone sediment quantity	4	4	Twice
Abalone Sediment N P C			
Ulva biomass	5	5	At stocking and after 4 weeks of Ulva production cycle per season
Ulva N P C	5	5	At stocking and after 4 weeks of Ulva production cycle per season
Feed pellet quantity			
Feed N P C	4	4	
Fertilizer quantity	5	5	Every production cycle
Nutrients N P C composition			Every season

Pre-trial - Diurnal water quality dynamics on South African abalone farm

This study was aimed at investigating the diurnal variations in dissolved nutrients (nitrogen, phosphorus, and carbon) on land-based abalone farm and to document the period of peak concentrations of major water quality parameter. The experiment started at 1600 h, one-hour post-feeding period for cultured abalones at Wild Coast Abalone (Pty) Ltd. Three raceways which housed grow-out (65-75g) abalones were selected and fed (at 1500h) a combination diet of compounded feed pellet (Abfeed® S34) and *Ulva* Spp. according to the standard feeding time and practice on the farm. Each raceway contained sixteen Ivey Blue up™ baskets with an average biomass of (± standard deviation S.D.) 200 ± 0.11 kg, Abalone tanks were gravity fed with 1000 µm filtered seawater at an average flowrate at 1.01 ± 0.12 L s^{-1}, and mean temperature of 15.4 ± 0.7°C. Water samples from the inlet and outlet of the three (3) raceways were monitored every 4-hour over a period of 20-hours from 1600-h till 1200-h of the next day. Temperature, pH and dissolved oxygen, of the inflow and outflow water of abalone raceways were measured at every sampling period (total n=6 per sampling time) using Ebro temperature logger (model EBI 300, serial number 73268609 by Xylem Analytics, Germany), thermo Scientific Eutech Expert pH meter (serial no. 2913115, Singapore) and Oxyguard handy Polaris DO meter (Model v. 2.51, serial no. 015BODE0140000 by Oxyguard International A/S, Denmark) respectively. The pH and DO meters were calibrated in their respective buffer solutions before sampling. During the same sampling hours, water samples for dissolved nutrients, unionised ammonia (NH3), ammonium nitrogen (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-), and orthophosphate (PO_4) were collected in triplicate from the inlet and the outlet of each replicate abalone raceway using 500ml plastic bottles. Water samples were filtered through 0.45µm (25cm diameter) Whatman GF/F glass fibre filters and analysed with the Palintest automatic wavelength photometer (model 7100, Palintest Ltd, England) and test kits following standard methods and procedures for the examination of natural and wastewater (APHA 2005).

Results

Life cycle analysis in a commercial IMTA abalone farm

Data collection for the main LCA task has started and is currently being reported in CS Task 3.7. No data have been analysis for the LCA to date (month-12). These analyses will only be carried out after all data needed for the LCS have been collected and this will take place towards the end of 2021.

Pre-trial - Diurnal water quality dynamics on South African abalone farm:

We carried out a pre-trail data collection to determine when best to collect daily water quality data on the abalone farm. There were distinct diurnal variations of some of the water quality parameters tested. Highest mean concentrations of total ammonia nitrogen (TAN) exiting abalone raceways (0.47 ± 0.07 mg/L) was recorded at 04h00 which was similar to 0.45 ± 0.13 mg/L recorded at 08h00. The TAN values at 04h00 and 08h00 were significantly higher than concentrations at 12h00, 16h00, 20h00 and 24h00 (ANOVA $F_{(5, 12)}$=14.391, p=0.0001). Nitrate nitrogen concentration also showed a diurnal pattern with significantly higher nitrate value (0.46± 0.02 mg/L) recorded at 08h00. However, significantly highest mean phosphorus concentration of 0.53 ± 0.04 mg/L exiting abalone raceways was recorded at 04h00 (Kruskal-Wallis test: $H_{(5, 18)}$ =16.17789, p=0.006). This could be as a result of post-feeding phosphorus leaching from abalone feed.

Discussion

Data collected in the pretrial will be used in the experimental design of the main trial that will be reported on after the LCA has been carried out.

Progress, deviations, problems & next 12M

Progress: Part of the data (both from CS Task 3.5 and CS7) for the LCA was collected and based on the current status and development of the trial, it is estimated that the task is 20% completed.

Deviations & Problems: Some of these data have already been collected, but delays due to labour restrictions on the farm (50% labour allowed on the farm for six months) resulted in a delay in establishing the sea cucumber work; although data collection for the non-IMTA and IMTA with abalone and *Ulva* Spp. continued through the Covid-19 related delay it started late, but we will still complete the within the period of the AquaVitae project. An LCA specialist has been identified and employed to review our method and design ahead of data collection. We will expose the PhD student to an LCA training course that would be outsourced and paid for by the industry partner so that the student can implement the instruction provided by the LCA specialist. The student who will carry out the LCA, could not participate to the planned LCA training course, since it was cancelled due to Covid-19. The data for the LCA has been collected ahead of the student becoming familiar with the LCA. Therefore, once he will have done the course, that has been rescheduled once Covid-19 restrictions are lifted, he will be able to process and analyse the data.

Outlook: The LCA analysis will take place when all data are collected and the training has been completed. The abalone/*Ulva* Spp./IMTA data needed for the LCA will conclude by June 2021. The sea cucumber work will start in 2021 and these data collected during the course of 2021.

CST 3.6: Land Based IMTA abalone/algae/innovative feed/Systems

Responsible CS Task Leader: Gercende Courtois de Viçose, ULPGC

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
3.6	IMTA ab/algae/feed/Sys	T2.2, T2.4	ULPGC	30%	✓	M7	M9	M23	M24	3-4	6-7

Introduction

The objective of this task is to improve grow out methods of *Haliotis tuberculata coccinea* through different feeding strategies and production systems in order to develop innovative production processes for this low trophic species. It is therefore necessary to test the adaptability of the feeding strategies in Land Based IMTA systems and RAS systems. In order to perform the experiments of task 3.6 it was necessary to produce batch of animals to obtain sufficient quantities of juveniles and it was also necessary to set up the experimental facilities.

Methods

Spawning induction

The broodstock selected to be induced were specimens belonging to the IU-ECOAQUA facilities located at the Science and Technology Park of Taliarte (Canary Islands, Spain). The animals selected presented a gonadal index between 2 and 3) and were induced to spawn, separated by sex, by exposure to 1µm filtered and UV sterilised seawater (Wang *et al.*, 2010; Courtois de Viçose *et al.*, 2012).

Post-larval and juvenile culture

Post-larvae were produced in order to obtain the juveniles required to perform the grow- out experiments.

In the post-larval tanks (Figure 3.6.a and Figure 3.6.b) the post-larvae were fed the diatoms *Navicula incerta, Amphora* spp. and *Cylindroteca fusiformis* (Figure 3.6.c)

Figure 3.6.a: abalone Post-larval (PL) tank.

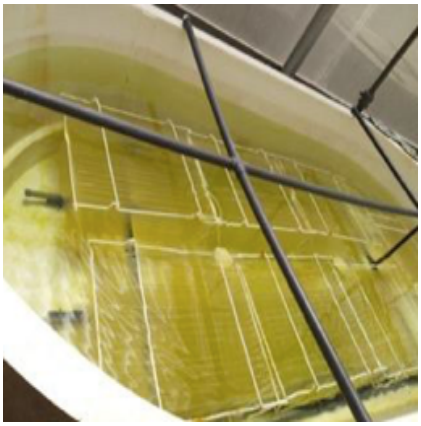


Figure 3.6.b: Settlement plates in PL tank.

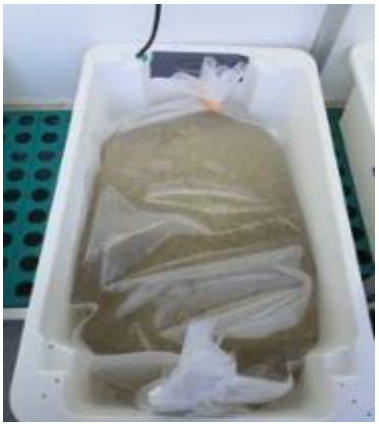


Figure 3.6.c: Diatom production.

Figure 3.6.d: Macroalgae production in IMTA system.

Post-larvae were kept in post-larval tanks for about 5 months until they developed the capacity to feed on macroalgae. At this point, the juveniles were moved to nursery tanks and were fed with macroalgae produced in IMTA systems (Figure 3.6.d)

Experimental set up

The experimental facilities were set up to allow simultaneous abalone production in both Land Based IMTA and RAS systems. The grow-out units consisted in 100Ltanks fitted with two baskets, in which the animals are placed (Figure 3.6.e).

Figure 3.6.e: Abalone grow-out unit.

The recirculation system (Figure 3.6.f) set up consisted of a water storage tank and a pump that conducted the water from the storage tank to the recirculation tanks, passing through a water temperature controller, a sand filter to remove solid and particulate materials and a biological filter to control the nutrients accumulated in the system. The recirculation tanks were connected by a joint drainage system to returned the water to the storage tank. The system also included a skimmer to prevent the accumulation of proteins and a pH meter associated with a buffer tank filled with basic solution, to control pH levels in the system.

Figure 3.6.f: Set up of the recirculated grow-out systems and units.

Results

To be described in the next deliverable on month 30.

Discussion

To be described in the next deliverable on month 30.

Progress, deviations, problems & next 12M

Progress: Based on the current status and development of the experiment, it is estimated that the task is 30% completed. The experiment is running and the results are being collected and analysed. The restrictions due to the COVID crisis did not affect the collection of data in terms of biological parameters.

Deviations & Problems: None

Outlook: During the next 12M the specimens will be placed in Land Based IMTA and RAS systems for grow-out and fed different nutritional sources. Their biological parameters will be monitored to evaluate the effect of the production systems and feeding sources.

CST 3.7: Land Based IMTA abalone/algae/innovative feed

Responsible CS Task Leader: Peter Britz, RhU

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
3.7	IMTA ab/algae/feed	T2.2, T2.4	Mfeed, RhU, WiCoAb	10%	✓	M8	M8	M21	M21	3	7

Introduction

Land-based aquaculture farms discharge waste nutrient and organic matter into coastal waters. This makes the potential environmental threat associated with this system of aquaculture a global issue of concern. Research efforts in many regions of the world are now channelled towards finding potential ways of decreasing the level of discharged nutrient waste from aquaculture sites. In this preliminary study, numerous trials were carried out to investigate how nutrients are produced on land-based abalone farms in South Africa and how the production vary diurnally. Two possible ways in which nutrients end up in aquaculture effluents are through leaching of nutrients from formulated feed pellets and secondly from degradation of waste materials produced from uneaten fed feed and faecal waste. These were examined and finally an experiment was carried out to monitor the variation in concentration of nutrients exiting abalone raceways over 24-hour period.

Figure 3.7.a: Abalone farm production system where the data was collected.

Figure 3.7.b: Land-based IMTA production Ulva Spp. at Wildcoast Abalone Farm (Pty) Ltd fed with waste water from abalone production tanks.

Methods

Testing pelletised abalone feed containing land-based IMTA grown seaweed

The methods for this trial are detailed in the work plan for CS3 in annex 3 of Deliverable 1.1.

Pre-trial - Solid waste production pattern on commercial abalone farm in South Africa

This trial was conducted for two groups of abalone: the IMTA abalone (fed pellets with effluent grown *Ulva Spp.* and *Gracilaria gracilis*) and non-IMTA abalone (fed pellets with fresh seawater cultured Ulva and *G. gracilis*). Six standard abalone production tanks (Figure 3.7.a) of the same surface area, volume and stocking density were selected to determine the quantity and the nutrient composition of waste generated from *H. midae* tanks during culture conditions over three days period (WCA number of days between cleaning). Prior to this trial, 45 sediment traps 30cm height and volume of 4618.74cm^3 were constructed onsite from schedule 40 C-PVC pipes and 2mm thick grey sheet (Figure 3.7.c). Fifteen (15) sediment traps were arranged underneath three rows of abalone baskets i.e. five traps per row to ensure that one-third of each raceway was covered. After the setup, each group was fed their respective diets for three days before the next cleaning event (standard feeding and abalone raceways cleaning procedures at WCA). At the end of the three days, all experimental raceways were drained, and sediment traps carefully retrieved. The traps were left to stand for 5-minutes before recording their weights. The contents (settled solids and water) of all traps were filtered with pre-weighed filter papers and the residue rinsed with deionised water. Filter papers with their residues were oven-dried at 105°C for 48h and reweighed (final weight) after they have cooled off. The recorded weights were used to calculate for the total suspended solids (TSS in mg/l) and the mass of settled solids (mg/l) in the traps (APHA 2005, EPA 2007). Mass of solids in sediment trap = TSS value x vol. of sediment trap. To quantify the volume of abalone waste generated per m^2 of a tank, the total mass of solids in the trap was divided by the surface area of the sediment trap. Sub-samples of the resulting dry materials from each replicate tank were pulled together, ground in a mortar and analysed for carbon, phosphorus, and nitrogen composition. The result of this trial is needed to conduct a nutrient mass balance and Life Cycle Analysis of the IMTA and non-IMTA systems at Wild Coast Abalone.

Figure 3.7.c: Construction of the solid waste sedimentation traps.

Results

Testing pelletised abalone feed containing land-based IMTA grown seaweed

Trial setup started in M8 and data collection is in progress. Results will be reported in the annex of deliverable D1.4: Report on second developmental phase in case studies.

Pre-trial - Solid waste production pattern on commercial abalone farm in South Africa:

The non-IMTA abalone system produce more solid waste (921.83 ± 148.47 gm^{-2}) than IMTA abalone (498.92 ± 22.84 gm^{-2}) at Wild Coast Abalone. However, there was no clear distinction in the N and C of the solid waste from the two production systems. The IMTA abalone solid waste N, P, C (1.45 ± 0.04; 0.44 ± 0.01; 9.30 ± 0.12 % and non-IMTA N, P & C 1.42 ± 0.37; 0.32 ± 0.07; 9.23 ± 2.92 %.

Discussion

Data collected in the pre-trial will be used in the design of the main trail, that will be reported on in the annex of deliverable D1.4: Report on second developmental phase in case studies.

Progress, deviations, problems & next 12M

Progress: Based on the current status and development of the experiment, it is estimated that the task is 10% completed.

Deviations & Problems: The start of data collection in this task was delayed due to Covid-19 as it was not possible for researchers to obtain the ingredients needed of the abalone feed and the transport of ingredients was also not possible. Luckily the student that had been engaged to collet these data as part of his PhD research moved his permanent work-base from Rhodes University to Wildcoast Abalone Farm ahead of Covid-19 lockdown; as such he spent lockdown on the abalone farm and was able to prepare and eventually start his research during lock-down. However, the farm was subject to 50% of its labour force and the student was not able to be on site daily as result of these restrictions and this delayed progress substantially; the delay was reduced by the student access site after hours for much of lock down.

Outlook: In the next 12 Months feeding trials will be performed in the Land based IMTA systems to evaluate the effects of IMTA and non-IMTA grown macroalgae (*Ulva lactuca*) meal as a feed ingredient.

CST 3.8: Vegetal/animal screening for new and/or improved IMTA products

Responsible CS Task Leader: Gercende Courtois de Viçose, ULPGC

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
3.8	animal/vegetal screening	T3.2	ULPGC, WiCoAb, FrHa, MFeed, RhU	0%		M25	0	M36	0	4-5	7-8

Introduction

This task is scheduled to start in M25. Task 3.8, is designed to assess the potential of Land based IMTA production for new and improved products and/or applications; the land-based IMTA produced species and their by-products will be screened through the performance of nutritional and quality analysis and the results will contribute to determine the valorisation of the products in the production systems tested. Animal and vegetal species produced in Land based IMTA systems in experiments (case study tasks 3.3, 3.4, 3.5, 3.6 and 3.7) in WP2, will be screened and their chemical/biochemical, physical and biological properties and will be analysed during year 3&4 of the project.

Methods

Analysis will be performed through the evaluation of chemical/biochemical, physical and biological properties of the products to provide an assessment of land based produced IMTA products in terms of quality, sustainability and nutritional value (see also work plan of CS. Detailed methods to be described in the reports of the corresponding periods.

Results

None yet. Task is scheduled to start in M25.

Discussion

Task is scheduled to start in M25.

Progress, deviations, problems & next 12M

Progress: Task is scheduled to start in M25 and therefore 0% completed.

Deviations & Problems: none

Outlook: not applicable

Summary of progress report for Case Study **4**

Date of report:	**1 April 2020**
Case Study name:	**Seabased IMTA**
of relevane for WPs	**1, 2, 3**

Abstract/Summary

Case study 4 takes place in South Africa, Sweden, Faroe Islands and France. The tasks in this Case Study aim to develop and refine sea-based IMTA systems by developing processes for specific species and conditions in different regions.

In South Africa, work is being carried out to assess methods to ensure the biosecure inclusion of algae into abalone feeds, without compromising abalone growth. The potential for growing seaweed on existing mussel rafts and the effects of this IMTA-grown seaweed on abalone grown is also being assessed. The three tasks in South Africa are currently between 8 – 80% complete, with some deviations experienced in all tasks, but solutions have been suggested and implemented.

In Sweden, the potential and benefits of integrating European lobster and flat oyster in a sea-based IMTA system is being tested and evaluated. This task in on track and at 2% complete with no deviations experienced thus far.

On the Faroe Islands, the mitigation effect by blue mussels and seaweed will be investigated. Sampling has progressed well with approximately 25% of the task completed. Deviations experienced are a result of increased efforts in improving the methodology.

The two Case Study Tasks in France will assess the IMTA of seaweed and abalone on the same sea-based concession, as well as assessing the integration of queen scallop and flat oysters into existing IMTA system with abalone and seaweed. The work in France are between 10 – 40% complete. One task is on schedule, with no deviations, while the other has been delayed due to Covid-19.

CST 4.1 Biosecure integration of algae into abalone culture

Cliff Jones and PhD student Petronilla Mwangudza, Rhodes University

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
4.1	Biosecure integration of algae into abalone culture	T2.2, T2.4	MFeed, RhU	15%		M7	M9	M44	M44	4	8

Introduction

The provision of biosecure feed is essential in intensive aquaculture conditions. This reduces the risk of introducing potential pathogens to the stock which could lead to infections and disease outbreaks. Macroalgae is recognized as an excellent source of bioactive compounds and have been associated with metabolic regulation, resulting in improved health and growth of different cultured species including abalone. Despite these benefits, the biosecurity implications surrounding macroalgae inclusion in abalone diets are not fully understood. Fresh macroalgae are considered a potential vector for parasites, pests, diseases and associated organisms and therefore presents a risk to cultured abalone stock. In addition, feeding abalone macroalgae grown in effluent water, as in the case on farms using recirculating system, or where macroalgae are produced in integrated multitrophic aquaculture

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement No 818173. This report reflects only the authors' view and that the Commission is not responsible for any use that may be made of the information it contains.

(IMTA) or systems where farm effluent is treated using algae before the water is returned to the ocean, also pose a risk.

The overall aim of this study is to assess biosecurity risks associated with macroalgae inclusion in abalone diets and the development of biosecurity measures to mitigate these risks.

Specifically, this study is set to addresses the following objectives:

1. To assess the efficacy of potential biosecurity measures on the inactivation of macroalgae-transmitted abalone pathogens;
2. To assess the effects of biosecurity processing treatments on the biochemical composition of macroalgae; and
3. To determine the effects of biosecure macroalgae on the health and growth performance of abalone.

Methods

No data were collected in this period for this task, so no methods have been presented here. The period was spent carrying out a literature review, project planning with partners, developing methods and training.

Results

Not applicable.

Progress, deviations, problems & next 12M

Progress: PhD student Ms Petronilla Mwangudza was engaged to work on Case Study Task 4.1 on a full-time basis since, registered with Rhodes University, South Africa. This work will constitute the research component of her PhD by thesis. Assisted by her project supervisors Prof. Cliff Jones (Rhodes University) and Dr Brett Macey (Department of Environment, Forestry and Fisheries, South African National Government), the student was involved in literature review and project proposal development. This review and proposal were presented in orally for review by the Department of Ichthyology and Fisheries Science at Rhodes University and have been documented in the students PhD proposal.

The research team had various meetings with South African industry partners (Wildcoast Abalone (Pty) Ltd. and Marifeed (Pty) Ltd.), as well as European industry partner (France Haliotis, France), where different approaches on the methodology and experimental design were discussed, so as to fully align the work for application in industry. Meetings were also attended with AquaVitae research partners from Germany (AWI), Spain (University of the Grand Canary Islands) to ensure that our research approach was well aligned with similar research taking place across the project (Figure 4.1.a).

Figure 4.1.a: Researchers and industry partners from South Africa, Spain, France and Germany met during the kick off meeting of AquaVitae CS3, CS4 and CS7 to coordinate the research of this and other aspects of the project. PhD student Ms Mwangudza participated in this meeting (seated center) to coordinate this task with other partners.

In this reporting period the student was also sent on a one-week training course to develop various skills in microbiological and molecular techniques (Figure 4.1.b). This took place at the Department of Environment, Forestry and Fisheries (DEFF) in Cape Town, and among them techniques that were covered included: preparation of culture media for microbial growth, serial dilution and basic PCR techniques.

Figure 4.1.b: PhD student engaged full time on this aspect of the AquaVitae project, seen here developing laboratory skills needed for data collection later in the project.

Based on the progress described above, this task is estimated to be 15% complete. The compilation of literature, meetings with research partners as well as skills development has been vital for the development of this task. Laboratory work and growth trial are still to be done.

Deviations & Problems: A major challenge was finding an appropriate laboratory to carry out the experiments which led to the delay in starting the work. However, after a few enquiries, the laboratory at the Marine Research Aquarium (Department of Agriculture, Forestry and Fisheries) in Cape Town was made available for the experiments.

A virus strain was required as part of the test pathogens, but sourcing this was unsuccessful since no viral diseases of abalone have been reported here in South Africa, combined with the risks involved in importing the virus. Therefore, instead of using a virus strain, bacteriophage lambda as proxy for abalone herpesvirus was decided upon.

Outlook: Macroalgae samples will be collected and laboratory work will begin in July 2020.

CST 4.2 Seaweed production in Saldanha Bay

Dirk Weich, Marifeed (Pty) Ltd.

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
4.2	Seaweed production in Saldanha Bay	T2.2, T2.4, T3.2	Mfeed, RhU	80%		M10	M0	M24	M24	3	7

Introduction

Case study Task 4.2 investigated the potential of culturing seaweed on existing mussel rafts in Saldanha Bay, South Africa. the inclusion of macroalgae (i.e. an alternative aquaculture product from a different trophic level form that of mussel) that better utilises the existing aquaculture facilities on the mussel farm, creates the opportunity for sea-based integrated multitrophic aquaculture (IMTA) at this facility. If the existing infrastructure can be utilized with little modification, the growth of a seaweed will make this a more economically sustainable operation, which fits the definition of IMTA[27][28]. Phase 1 of this task was a preliminary trial conducted by industry partner, Marifeed (Pty) Ltd. Following the results obtained from the pilot study, a larger scale trial was conducted in order to refine the methods for the co-production of Gracilaria and mussels, specifically in Saldanha Bay, South Africa.

Methods

Phase 1 – preliminary trial

The study was conducted on mussel rafts in a 50-ha mussel culture lease site in Saldanha Bay, South Africa. Gracilaria was collected from the bay and used as stocking material. The tufts of seaweed were pulled through netlon rope using looped wire (Figure 4.2.a).

[27] Chopin T, Robinson S, Mac Donald B, Haya K, Page F, Ridler N, Szemerda M, Sewuster J, Boyne-travis S. 2006. Integrated multi-trophic aquaculture: seaweeds and beyond... the need of an interdisciplinary approach to develop sustainable aquaculture. *Journal of Phycology* 42: 11-11.

[28] Chopin T. 2018 Integrated Multi-Trophic Aquaculture (IMTA): A responsible approach to farming our waters. *International Aquafeed 2018*: 16-17

Figure 4.2.a: Gracilaria gracilis pulled through netlon ropes using looped wire.

Two stocking densities of Gracilaria were tested (25 - 30 and 15 - 20g Gracilaria tufts every 30 and 50cm, respectively; Table 4.2.a).

Table 4.2.a: Mean stocking weight of two stocking densities.

Rope 1	Stocked with 25 – 30 g Gracilaria tufts every 30 cm Total stocking weight ± 4.5 kg
Rope 2	Stocked with 15 – 20 g Gracilaria tufts every 50 cm Total stocking weight ± 1.75 kg

The ropes were transported in water-filled buckets by boats to the rafts and then attached to the outside of the mussel rafts with nylon droplines every four metres (Figure 4.2.b). Weights were attached to the ends of the rope to ensure that the lines remained underwater and to prevent entanglement due to the movement of currents. After the ropes were seeded, an area on the land-based site was set up for drying by direct sunlight after harvesting. Ropes were monitored weekly by photo updates and after seven weeks, ropes were taken down and transported to shore for weight and length captures.

Figure 4.2.b: Transportation of seeded ropes and attachment of ropes to mussel rafts.

Phase 2

Based on the outcomes of Phase-1, the production of *Gracilaria gracilis* would be scaled up and the drying of the Gracilaria would be done on site. A seaweed line drying area was erected on the Blue Ocean Mussel site. A total of 25-lines were seeded with seaweed and attached to the mussel rafts.

Results

Phase 1

Photographs of the lines were captured after one week in the water (Figure 4.2.c). The tufts thickened and gained biomass.

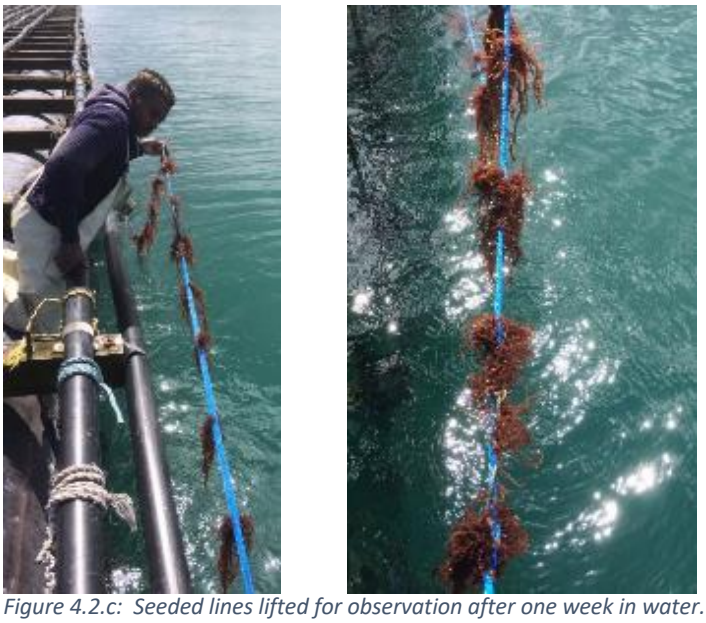


Figure 4.2.c: Seeded lines lifted for observation after one week in water.

After the first month of the trial, the seaweeds turned a yellow colour, which suggested that the Gracilaria were stressed (Figure 4.2.d). However, after six weeks the seaweed recovered well and grew significantly (Figure 4.2.e). At the end of the seven-week trial, the ropes were taken down and transported to shore for data capture (Figure 4.2.f).

Figure 4.2.d: Gracilaria gracilis after four weeks in the water.

Figure 4.2.e: Gracilaria gracilis after six weeks in the water.

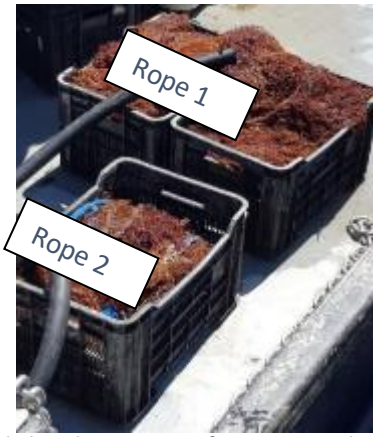


Figure 4.2.f: Ropes being removed and placed into crates after seven weeks.

Upon arrival on shore, the length and weight measurements were captured for each seaweed tuft (Figure 4.2.g). Noticeably large amounts of sea-life were observed in between the *Gracilaria* Spp., particularly amphipods (Figure 4.2.g).

Figure 4.2.g: Weigh and measure station for Gracilaria gracilis tufts (left) and amphipods within the Gracilaria (right).

Significantly better growth was observed from rope 1 (25 - 30 g) compared to rope 2 (15 – 20 g) with 2.82kg/week and 0.73kg/week, respectively (Table 4.2.b). Consequently. the estimated yield/rope/annum was found to be considerably higher for rope 1 (Table 4.2.b). Rope 2 appeared to have lost a higher percentage of its total tufts (33.7%), compared to Rope 1 (10.7%; Table 4.2.b).

Table 4.2.b: Growth parameters of seeded Gracilaria gracilis.

	Rope 1	Rope 2
Stocking weight	4.50kg	1.75kg
Final weight	24.25kg	6.83kg
Nett yield	19.75kg	5.08kg
Growth/week	2.82 kg/week	0.73 kg/week
Extended to yield/rope/annum	146.6kg	37.8kg
Total tufts	150	95
Lost tufts	16	32
% lost tufts	10.7%	33.7%
Ave tuft weight	175.7g	99.4g
Ave initial weight/tuft	27.5g	17.5g
Ave weight gain/tuft	148.2g	81.9g
Ave % gain/tuft	539%	468%
Ave % gain/day	11%	9.6%

The drying area identified previously for the seaweed was not ready, so ropes were packed in open polystyrene boxes and transported to Hermanus. The seaweed was harvested at 09h00 in the morning, and was still in good shape after the measuring and weighing – around 14h45. Unfortunately, the trip back to Hermanus was one bridge too far, and the seaweed started to rot (Figure 4.2.h), and was unusable by the time it was ready to hang it up to dry back in Hermanus. The seaweed was discarded in the municipal dumpsite (Figure 4.2.h).

The cumulative time it took harvesting, transporting back to shore, measuring and weighing the seaweed in the heat in Saldanha and the transport back to Hermanus, together with the biomass of dead amphipods was too much to keep it fresh.

This was a valuable lesson, and underscores the necessity to hang the seaweed up to dry within four hours after harvesting, especially in hot days.

Figure 4.2.h: Rotten Gracilaria gracilis (left) and disposal of unusable Gracilaria (right).

Phase 2

Twelve out of the 26 ropes that were stocked with Gracilaria were successfully dried on site in the newly erected drying area and then transported to Marifeed (Pty) Ltd. for testing, while the remaining ropes did not dry well due to poor weather conditions (misty and rain) and resulted in degradation of the *Gracilaria*. The rotten seaweed was then discarded.

Discussion

Gracilaria was successfully grown in Netlon ropes attached to a mussel raft. We also have a first indication of growth rate and yield. If the yield from Rope-1 is extended to the 25 existing rafts it comes to 7.33 tonnes production per year. Drying and processing the next harvest will give us a better indication of dried yield and quality.

There was significant better growth and yield from Rope-1 compared to Rope-2. This could be linked to a number of variables:

1. Initial stocking weight and tuft spacing

Rope 1 was stocked with tufts of 25 – 30g each, spaced 30cm apart
Rope 2 was stocked with tufts of 15 – 20g each, spaced 50cm apart

2. Relative position of ropes

The raft position was NNE to SSW as depicted below in Figure 4.2.i.

Figure 4.2.i: Aerial photograph of the site in the bay and enlargement of raft where ropes were installed.

3. There may be a difference in average sunlight exposure and shading between the two sides of the raft.
4. The current and wind direction could also be a variable between the raft's two sides. This may impact the exposure to nutrients from mussels on raft, and possible mechanical stress by rubbing against mussel raft.
5. Rope-2 also lost more tufts than Rope 1 (33.7% vs 10.7%). Most of the lost and damaged tufts seemed to be a result of the Netlon rope winding up in the current, lifting the tufts out of the water.
6. Handling stress during stocking may have also been a factor. Rope-1 was stocked by two people, while Rope-2 was stocked by mussel farm workers. When stocking the ropes, there is a risk of damaging the seaweed tufts when pulling them through the Netlon rope with the wire loop tool. Workers should be trained to stock tufts carefully to reduce damage and handling stress.

Harvested Gracilaria needs to be dried within 4 to 5 hours after harvest to stop it from starting to deteriorate. There are large populations of amphipods and some other small sea creatures present in the harvested seaweed tufts. Simple wooden structures could be erected (see proposed design; Figure 4.2.j) in the identified area in the land-based site to hang the harvested seaweed lines on. In favourable sunny weather the seaweed should dry within 1-2 days.

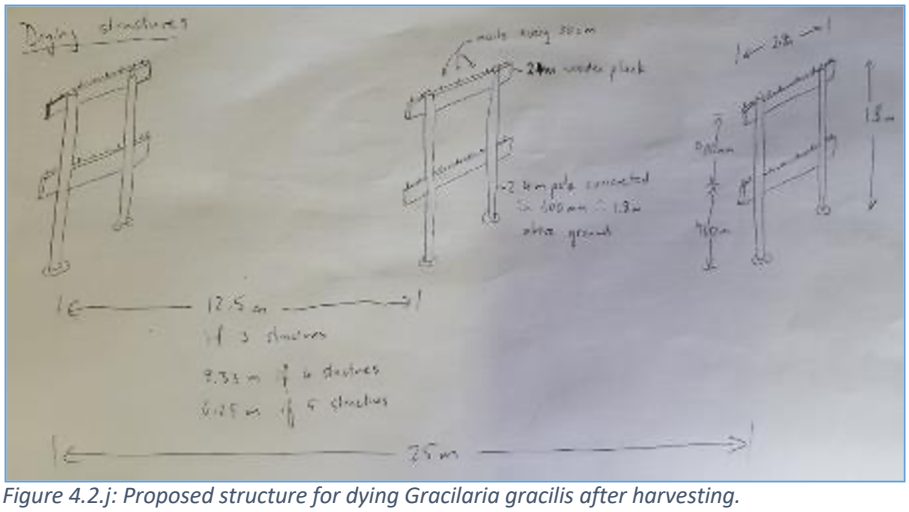


Figure 4.2.j: Proposed structure for dying Gracilaria gracilis after harvesting.

Phase two was an expansion of the preliminary trial (two ropes in Phase 1 versus 26 ropes in Phase 2). Valuable insight was gained, but the upscaling resulted in a number of challenges and will be highlighted below.

Progress, deviations, problems & next 12M

Progress: This case study task is approximately 80% complete. Phase 1 and Phase 2 of the task have been achieved ahead of plan and Phase 3 (although not part of the initial work plan – and will constitute additional value) is scheduled to begin in M13. The lessons and knowledge gained from Phase 1 and 2 of this study will be used to further develop and refine methods to test the feasibility of IMTA of Gracilaria and mussels within the unique location of Saldanha Bay, South Africa.

Deviations & Problems: A number of problems were encountered during both Phase-1 and Phase-2. The drying area identified for the seaweed was not ready for all the material returned to land. The harvested seaweed could not dry in time due to space and weather conditions. Some of the ropes were packed into polystyrene boxes and transported to Hermanus to dry in an automated system. The time taken from harvesting to arrival at destination (Hermanus) was too long and the seaweed began to rot and was no longer usable. The heat in Saldanha Bay and the biomass of dead amphipods contributed to the unsuccessful outcome of usable seaweed for drying. The time taken to seed the ropes, transport it out to sea and install it was a concern. The tufts were either lost or damaged due to the ropes winding up in the current and lifting the tufts out of the water. The on-farm labour was dedicated to the mussels, therefore little efforts were put into this important preliminary trial.

To reduce the time from seeding to installation, it was proposed that workers should be used to seed the Netlon lines while on the water, using a 2.4m x 1.2m plywood sheet to work on. In future, seaweed will need to be hung up to dry within four hours after harvesting, particularly on hot days. To mitigate lost or damaged tufts, a PVC conduit pipe may be slipped over the nylon ropes that will hold the ropes in place. To reduce handling stress, workers should be trained to stock tufts carefully to reduce damage and handling stress. To reduce deterioration of the seaweed, simple wooden structures will be erected in the identified area in the land-based site to hand the seaweed. Seeding was done by using a rope-

over-pipe method, therefore Gracilaria was dropped at a specific distance in the pipe to place Gracilaria at the correct length along the rope.

The time and skills needed to stock the ropes were challenging. A solution to reducing stocking time was implemented, which included stuffing the ropes with clumps of seaweed, but this led to reduced growth rates by 4% per day. The clumps of seaweed did not dry properly and had to be cut open to remove seaweed for drying. Wind and wave action resulted in ropes twisting and killing the seaweed tufts. The PVC conduit put in place to hold the ropes in place caused friction and cut through the ropes, resulting in lost seaweed tufts. Twelve of the lines that were harvested did not dry well and became rotten, which were then discarded. The monitoring of the lines by the staff on site was poor.

To reduce the loss of *Gracilaria* Spp. in the vertical ropes, the ropes were removed from the water at a slower speed. Although the labour needed was insufficient, an effective processing line was set up, making sampling more efficient.

Outlook: Implement and further refine the methods developed in the preliminary trial with better monitoring and increased replicates to assess the growth of *Gracilaria gracilis* and mussels in Saldanha Bay.

CST 4.3 IMTA algae into Abfeed

Cliff Jones and MSc student Njabulo Madlala, Rhodes University

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
4.3	IMTA algae into Abfeed	T2.2, T2.4	Mfeed, RhU	8%		M26	M10	M37	M37	4	8

Introduction

Abalone (*Haliotis midae*) naturally feed on kelp, and are widely distributed along coastal regions where cooler currents and brown algae predominate. Diet significantly affects their growth and since algae is not freely available and formulated feeds accelerate growth and feed conversion, commercial abalone farms rely on these formulated feeds. The inclusion of algae in the diet improves growth, health and feed conversion ratio (FCR) of the abalone and it reduces drip loss during processing. However, the kelp that is currently included in the feed is wild harvested and this resource is limited and under increasing pressure.

There is a need to develop an alternative source of algae, that will reduce the pressure on natural kelp resource with improved environmental sustainability and that is less likely to compromise the biosecurity of the abalone. Therefore, this study will investigate the inclusion of algae (*Gracilaria gracilis*) produced in sea-based integrated multitrophic aquaculture (IMTA) as a feed ingredient used to produce abalone.

This study aims to use *G. gracilis* algae produced with other organisms (i.e. mussels and not abalone) in a sea-based IMTA system, where after it will be included into abalone feeds. Its influence on abalone growth, FCR and feeding behaviour will be evaluated.

Methods

No data were collected. For more details on the envisaged methods please see work plan of CS4 in annex 3 of D1.1.

Results

Not applicable. Planned starting date is M26.

Discussion

Not applicable. Planned starting date is M26.

Progress, deviations, problems & next 12M

Progress: A full literature review, experimental design and full research proposal were prepared and submitted for review. This time was also spent planning the experimental system and training. It should be noted that although we had intended to start data collection in this period (i.e. ahead of schedule), we were not actual due to start yet. Based on the progress thus far, this task is estimated to be 8% complete and can be considered satisfactory since this task is not scheduled to begin yet.

Deviations & Problems: The start of the experiment was delayed due to Covid-related national restrictions (i.e. there was a national work and travel ban).

Outlook: A travel permit will hopefully be obtained in the next few weeks so that preparations on the farm can be started. Further activities include:

1. Harvesting Gracilaria at Saldanha Bay;
2. Diet formulation and manufacturing;
3. Growth trial;
4. Sample for proximate composition analysis;
5. Behavioural study;
6. Data analysis; and
7. Thesis write-up.

CST 4.4 IMTA of European lobster and oyster

Anna-Sara Krång, IVL / Svenska Miljöinsitutet AB

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
4.4	IMTA of European lobster & oyster	T2.2	IVL, BoHu	25%		M1	M1	M36		4	7

Introduction

European lobsters (*Homarus gammarus*) and flat oysters (*Ostrea edulis*) offer nutritious products with unique gastronomical qualities and high market values, but since wild populations are limited and declining, culturing techniques are needed for increased production. The market demand for the European lobster is high and there is a large interest from the industry in aquaculture of the species, but the lack of economically reasonable and sustainable larval rearing and grow-out systems impedes the development of commercial production. At the same time, the current oyster industry suffers from heavy fouling, hindering sufficient water exchange and thus efficient growth. Initial trials in Spain, Ireland and the UK have shown potential for sea-based cultivation of lobster juveniles in submerged oyster cages without feed supplementation and with minimal human intervention. These studies indicate that fouling organisms along with filter feeding sustain good survival and growth for the lobsters, compared to land-based hatchery controls[29][30].

[29] Daniels CL, Wills B, Ruiz-Perez M, Miles E, Wilson RW, Boothroyd D. 2015. Development of sea-based container culture for rearing European lobster (*Homarus gammarus*) around South West England. *Aquaculture* 448: 186-195.

[30] Halswell P, Daniels CL, Johanning L. 2018. Evaluation framework for external and internal parameters associated with Sea Based Container Culture (SBCC): Towards understanding rearing success in European lobsters (*Homarus gammarus*). *Aquacultural Engineering* 83: 109–119.

In this project we will provide proof of concept of sea-based systems for co-cultivation of these high quality and most valuable species and evaluate the benefits of integration of the two species in an IMTA system, that sees (a) oyster production benefit from utilising nitrogenous waste produced by the lobsters as a feed source and (b) the lobsters utilising by-products of oyster production, i.e. feeding on organisms that seed naturally onto the oyster cages, thus addressing biofouling and reducing labour costs, improving water-flow and oyster feeding rates. In summary, we will provide knowledge and techniques valuable for the development of commercial and sustainable farming of the European lobster, as well as mass-production of lobster juveniles for restocking and enhancement purposes. Furthermore, the results can offer improvements for oyster industry with regards to reduced biofouling.

The aim of this task is to test and evaluate the potential and benefits with integrating lobster and oyster in a sea-based IMTA system adapted to local environmental conditions.

To address this aim, this case study task will develop the following processes:

4.4.1: New prototype and protocol for co-cultivation of lobsters and oysters, for increased food production and restocking purposes.

4.4.2: Adaptation of the culture system for Swedish environmental conditions, with stratified waters and large fluctuations in temperature, salinity and plankton availability.

4.4.3: Evaluation of low tech and relatively inexpensive method for on growth of lobster juveniles in sea-based systems that request minimized rearing and no additional food supply.

During the first 12 months (M1-12), the goal has been:

- To develop and prepare the IMTA system.
- To conduct pilot tests in land-based tanks for evaluation and optimization of the system.
- To rear juvenile lobsters from locally caught females and to collect local juvenile oysters, to be used in the sea-based IMTA system.

Methods

Planning and preparation of the IMTA system

Planning, ordering and preparation of the cultivation system, including applying for permits from local authorities, took place during the first phase of the project. Planning of the system was greatly supported by Dr Carly Daniels, researcher at the National Lobster Hatchery in Padstow, UK, who is in charge of their sea-based lobster rearing. Also, input from local oyster farmers (e.g. Kent Berntsson, Ostrea Aquaculture, Sweden and Katrin Persson, Bohus havsbruk, Sweden) and lobster fishermen (via the Swedish Lobster Academy) guided the development of the IMTA system. The aquaculture system was ordered from Padstow.

Pilot tests in land-based tanks for evaluation and optimization of the system

Through collaboration with ongoing research projects at Kristineberg Marine Research station, there was an opportunity to receive oyster and lobster juveniles for a pilot test to be run already during the summer and autumn 2019, to test and optimize the system. Lobster juveniles reared until stage V (i.e. second postlarval stage after metamorphosis) in controlled conditions at an aquaculture facility at Kristineberg, were provided via Per-Olof Samuelsson through a restocking project between Sotenäs municipality and University of Gothenburg. Oyster juveniles were provided from local oyster farms. Since the lobsters did not originate from locally caught females, the pilot test had to be conducted in land-based tanks (outside the aquaculture facility).

The pilot test started 20th June 2019, culturing oysters or lobster juveniles in round oyster baskets with four inner compartments (18×6cm, mesh size 2mm × 2mm for lobsters or 4mm × 4mm for oysters). The inner containers contained either one lobster or 5-10 oysters (depending on size). Five baskets were stacked into a unit, plus one extra, empty basket acting as a lid (Figure 4.4.a). Each unit contained i) either oysters in all inner compartments (treatment 1 – oysters only); or ii) lobsters in all inner compartments (treatment-2 – lobsters only); or the baskets contained a mixture of both species; either iii) altering oysters and lobsters between inner compartments within each basket layers (treatment 3 – oysters and lobsters mixed within baskets); or iv) with oysters and lobster separated between basket layers (treatment 4 – oysters and lobsters in separate basket layers) (Table 4.4.a). There were two units per treatment, submerged into a large tank with flow-through of ambient deep seawater, temperature and salinity monitored continuously.

Figure 4.4.a: Cultivation system for lobster and oyster culturing. One lobster or 5-10 oysters placed into inner compartments of an oyster baskets, stacked into units of five (plus one extra as lid). Basket units submerged in an outdoor tank with flow-through of ambient deep seawater.

Lobsters and oysters were monitored periodically for five months (every second week during the first month and then every second month), to evaluate differences in survival and growth between treatments. No food was supplied during the test.

Local oyster juveniles (ca 2 years old, 30-45 mm) were collected and measured, ready to be used in the sea-based systems.

Hatchery rearing of juvenile lobster larvae from locally caught females

Three egg-berried female lobsters were caught with creels (lobster pots) by local fishermen on the 22nd October 2020, at a location in the Gullmarsfjord (58° 18',37 N; 11° 31',15 E; ca. 18-20m depth) close to Kristineberg Marine Research station. They were kept in individual tanks with flow-through of ambient seawater at the lobster rearing facility at Kristineberg until hatching was initiated. To increase egg development and synchronise hatching, seawater temperature was raised by one degree Celsius per day until 18°C (i.e. optimal rearing temperature), starting in January 2020. To be able to follow egg development and plan hatching, egg development was evaluated for all females once per week, which was done by removing and photographing ca. 10-eggs per female, measuring embryo eye size (maximum length and diagonal). Larvae from all females hatched in March and were reared in controlled conditions according to established protocols until stage V (i.e. second postlarval stage after metamorphosis).

Results

Planning and preparation of the IMTA system, including pilot test

Planning, ordering and preparation of the cultivation system was successfully conducted during the first 12-months of the project. Furthermore, permits for sea-based aquaculture of local lobsters and oysters were ordered and received from local authorities.

The results of the pilot test showed on high initial survival of both species (97% survival of lobsters and 98% of oysters for the first two weeks), but wile survival remained high for oysters, the survival of lobsters sharply declined after the summer period (27% survival in September and only 6% in November), indicating insufficient food supply and/or contamination of the tank. Nevertheless, the experience gained from the pilot test assisted the final planning and helped optimizing the design of the field implementation of the sea-based IMTA system, to be initiated in the next phase of the project.

Hatchery rearing of juvenile lobster larvae from locally caught females

Hatching of the three locally caught lobster females was successfully synchronised for March 2020 and lobster juveniles of similar age and size were produced in excess, to be used in the field implementation of the sea-based IMTA system. In addition, local oyster juveniles have been collected and measured, ready to be used in the sea-based systems.

Discussion

There are no main results of the task to this date, since the field implementation of the IMTA system has not yet started. This is however in accordance with the work plan. All three goal of the first 12 months have been achieved, i.e. to develop and prepare the IMTA system, to conduct a pilot test in land-based tanks for evaluation and optimization of the system, as well as to rear juvenile lobsters from locally caught females and to collect local juvenile oysters, to be used in the sea-based IMTA system to be set up in the Gullmarsfjord on the Swedish west coast.

Progress, deviations, problems & next 12M

Progress: CST4.4 is on track and all major goals for the first 12-months have been achieved. Based on this, it is estimated that a 25% completeness of the task has been obtained so far.

Deviations & Problems: There are no significant deviations or matter of concerns for this task.

Outlook: Everything is prepared and ready to start the field implementation, to be initiated within the next few weeks. Thus, during the next 12-months, a pilot-scale co-culturing system will be set up in the Gullmarsfjord on the Swedish west coast, testing different designs of co-cultivation of juvenile lobsters and oysters at two depths. Lobsters and oysters will be monitored regularly for up to 1.5 years, to evaluate differences in survival and growth between different designs of co-cultivation and between different depths. The goal is to provide new knowledge and techniques for ongrowing cultivation methods for lobster juveniles with respect to cost efficiency, sustainability and quality of animals, while at the same time improving oyster production by managing biofouling during the grow out stage.

CST 4.5 Mitigation effect by two various approaches of salmon-mussel-macroalgae aquaculture

Gunnvør á Norði, Fiskaaling

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
4.5	Mitigation effect by two various approaches of salmon-mussel-macroalgae aquaculture	T1.5, T2.2, T6.2	Fisk, StellU, P/F Luna	25%	✓	M1	M1	M41	M41	3	7

Introduction

Until recently the legislations in the Faroe Islands have prevented farming of multiple species in the same fjord. In 2019 the legislation was changed and opened up for multiple species in the same fjord and hence also the possibility for IMTA. However, the possible mitigation effect by IMTA is highly dependent on the local environment and the specie that are cultured.

Salmon farming is the major aquaculture in the Faro islands. Blue mussels have been farmed on trials basis, and seaweed farming is and upcoming industry in the Faroe Islands. Both seaweed and blue

mussels have shown a great growth potential and since salmon farming is highly established, the IMTA concept that is most promising evolve into an industry is the salmon- mussel-macroalgae concept.

This task will investigate the mitigation effect by blue mussels and seaweed, where the local physical biological and spatial constraints are taken into consideration. IMTA will be approached in 1) the traditional manner where blue mussels are expected to assimilate waste from the farm directly, and 2) from a fjord ecology perspective, investigating the possible mitigation effect on the carbon and nitrogen load in the fjord.

The study is conducted in Sørvágsfjørður in collaboration with the local fish farming company Luna, who have invested in the blue mussel and seaweed lines, and provide data on currents and fish farming activity. In addition, the seaweed growth trials and some other data collection is funded by the Nordic Centre of Excellence Sureaqua (www.sureaqua.no).

In February 2020 Stellenbosch University chose to join the task and conduct hydrodynamic modelling of the area based on the best available data.

One of the original goals for the first 12-months was to calculate the water exchange rate in the fjord system based on previously conducted current measurements and/or freshwater runoff and salinity balance. This goal has significant expanded after Stellenbosch University entered the task and will conduct detailed hydrodynamic modelling of the fjord. That is, we are delivering substantially more than was planned in the initial work plan as a result of this new collaboration.

In addition to hydrodynamic investigations, the goal for the first 12-months was to collect data for the task. Establish and test blue mussel long lines and seaweed lines, investigate blue mussel larval availability and collect basic ecosystem data, such as seasonal changes in nutrients, phytoplankton and zooplankton in the fjord.

Modelling of the fish waste assimilation by blue mussels connected to the fish farm has been initiated although it was scheduled later in the project.

Methods

Blue mussel long lines and seaweed growth lines

Two kinds of blue mussel long lines were deployed in the fjord in May 2019 (Figure 4.5.a). In addition, two one-year old lines were available for sample collection. Since the fish farming company is primarily interested in the mitigation effect by the mussels, the long lines will be self-regulating since they are low labour intensive. Macroalgae lines seeded with *Saccarina latissima* were also deployed in January 2019 and January 2020. Lines were deployed at the fish farm and 800m away from the farm. Macroalgae growth rates were investigated once a month during the growth season, while the blue mussel lines were investigated once during the first 12-months. Samples were frozen for later analysis for carbon and nitrogen content. Seasonal availability of blue mussel spat was investigated as a part of the water quality survey.

Water quality survey

In order to evaluate the possible mitigation effect by blue mussel and seaweed on the fjord ecosystem, some basic knowledge on the ecosystem is needed, and thus the lowest trophic levels in the food chain were investigated. Weekly surveys were conducted from May to September 2019, investigating temperature and salinity, nutrients, Chl-*a* and zooplankton abundance. See figure text below for details.

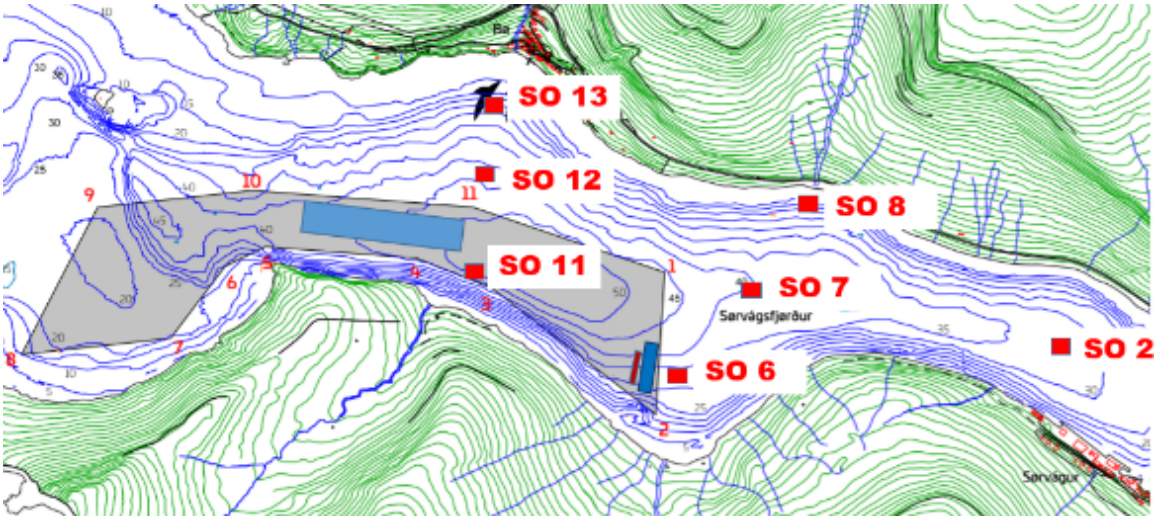


Figure 4.5.a: Map showing fish farm, (light blue), blue mussel farm (dark blue) and seaweed farm (red line) and the survey stations in Sørvágsfjørður. Temperature, salinity and fluorescence was investigated at all stations with a CTD. Zooplankton was investigated at stations, SO2, SO6, SO8, SO 11 and SO 13. Nutrients and Chl a were investigated at station SO 6 and SO 8. Chl a samples were taken at 4, 8 and 25 m depth while, nitrate, phosphate and silicate was investigated at 4, 8, 12 and 25 m depth.

Hydrodynamic modelling

Hydrodynamics in the fjord and surrounding region was modelled with Delft3D-FLOW. This is a three-dimensional, finite volume hydrodynamic and transport model which simulates flow and transport resulting from tidal and meteorological forcing. The model allows for modelling by means of domain decomposition which implies that, in this case, there are two simulation setups with the smaller domain nested in the larger domain.

Modelling fish waste uptake by blue mussels

According to the work plan investigations on the direct uptake of fish waste by blue mussel should be initiated in M30. But the contribution by due Stellenbosch University implied changes in the workflow and timing of data requirements which made it more meaningful to initiate the subtask considerably earlier. There are three steps in the modelling: (1) Evaluation of the availability of fish farm derived particulate organic matter; (2) dispersal of waste while it sinks through the water column; and (3) incorporation of fish farm waste in the blue mussels.

The total production of particulate waste at the fish farm was calculated from production data from the fish farm company. It was assumed that the mussels were able to incorporate faeces while feed loss was too large for the blue mussels to assimilate. The dispersion of waste was modelled according to the particle tracking module in DEPOMOD assuming various settling velocities for the various portions of the fish waste. Two setups of blue mussel farms were modelled, one with the mussels adjacent to the fish farm and one unrealistic scenario with mussels below the fish farm.

Results

First year field sampling of water parameters is completed. One seaweed growth season is completed and semiquantitative sampling of mussel settlement and growth have been conducted. However, most of the data has not been thoroughly processed yet.

Blue mussel long lines and seaweed growth lines

Initial examinations on the seaweed growth trial data, show that the growth conditions are quite good in the area and that there was a considerable production of biomass both at the fish farm and at the growth site away from the farm. The trials with blue mussel long lines showed low blue mussel densities 6-months after deployment (277 ± 100 mussels/m) while the density at the 1.5-year-old lines was considerably higher. The mussel spat availability was quite reasonable during summer with mussel larvae densities up to 4000/m^3.

Water quality survey

The primary production started in early May and Chl-*a* continued to increase until early June where after there was a decline followed by a second peak in late July (Figure 4.5.c). The nutrient availability changed according to the changes in Chl *a*.

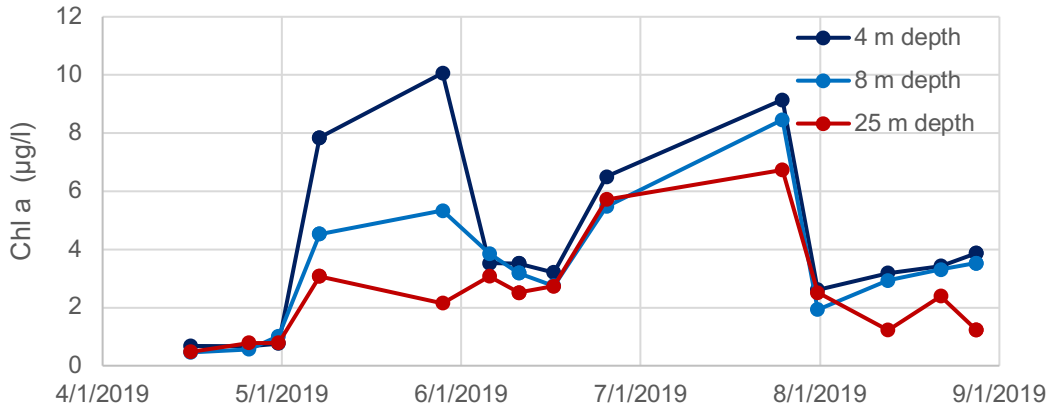


Figure 4.5.b: Seasonal changes in Chl a concentration at 4, 8 and 25 m depth.

Hydrodynamic modelling

The initial input data is bathymetry and water level constituents from the TPXO tidal model. On completion of the water level calculations, winds and surface runoff will be added to the model.

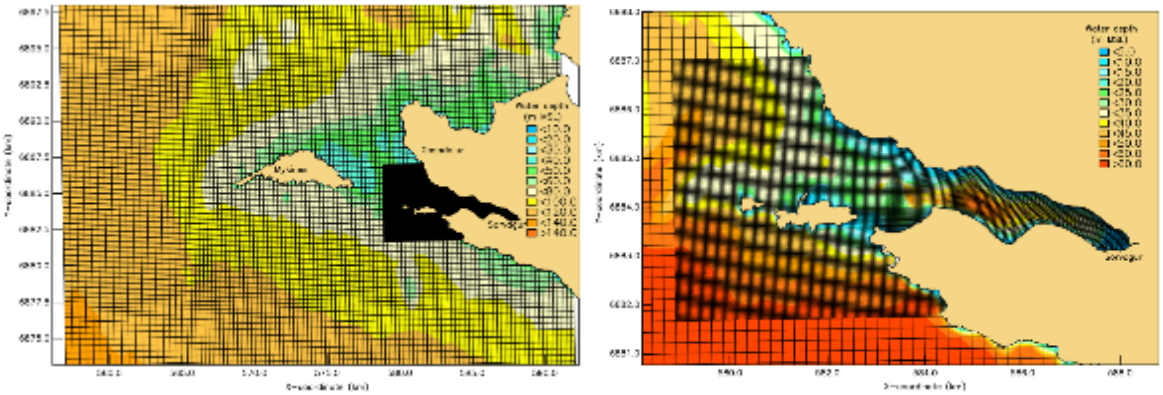


Figure 4.5.c: Computational grids and bathymetry of the modelling domains.

Modelling of fish waste uptake by blue mussels

Initial results from the modelling show that the blue mussels only assimilate a small fraction of the waste, regardless of the setup. Only a small fraction of the fish waste reaches the mussel farm adjacent to the fish farm while the majority of the waste passes the mussel farm below the fish farm (Fig. 4.5.d).

However, the residence time in the mussel farm implies that only a small fraction of the waste is assimilated by the blue mussels.

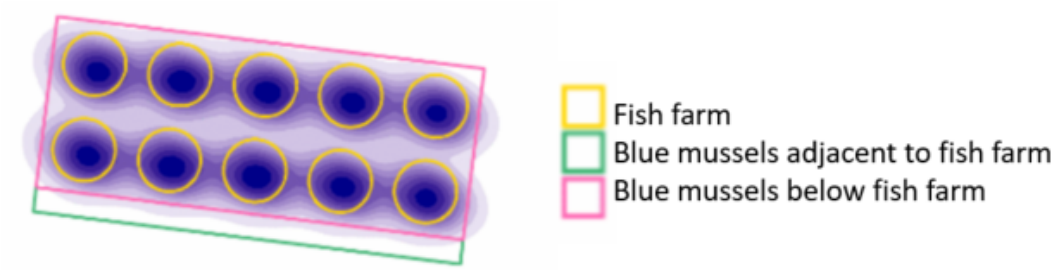


Figure 4.5.d: Dispersion of fish faeces.

Discussion

The field surveys show good growth conditions for macroalgae, and the mussel spat availability and growth conditions for blue mussels should also be acceptable, however the 6M survey showed that the settlement success was in the lower end of what has been observed in other areas.

Initial modelling of the direct assimilation of fish waste by the blue mussels indicate that this setup probably will not have a significant higher mitigation effect compared to what could be expected if the fish farm and blue mussels are not linked directly.

Progress, deviations, problems & next 12M

Progress: The field sampling has progressed as planed and data has been stored internally. Interpretation of the results has been postponed somewhat since the modelling of fish waste was prioritized. Based on the results obtained and discussed above, this task is estimated to be 25% complete. Surveys, field work and modelling still need to be completed.

Deviations & Problems: Deviations from the original work plan are mostly a result of the increased effort on the hydrodynamics, which on the long term will strengthen this study considerably.

Outlook: In the next 12-months field surveys will continue. Field work regarding seaweed growth trials and water quality will be completed in September 2020. Initial runs with the 3D hydrodynamic model will be conducted where only tidal forces have been included, and if that proves valuable the model will be extended to include meteorological forcing and runoff, based on local data. Modelling fish waste uptake by blue mussels will be finetuned and a manuscript for a scientific paper will be prepared.

CST 4.6 Seaweed production as feed for abalone: production of kombu on long lines above abalone benthic cages.

Sylvain Huchette, France Haliotis

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
4.6	Seaweed production as feed for abalone	T2.2, T2.4, T3.2	FrHa	40%	✓	M9	M6	M14	0	5	8

Introduction

As planned in the Case Study work plan, CS Task 4.6 involved the assessment of sea-based multitrophic production of seaweed and abalone on the same sea-based concession. The production of seaweed mostly aims at generating healthy food for abalone. The longlines were deployed on France Haliotis concessions at the mouth of the river Aber Wrac'h located at about 30km North-West of Brest in

Brittany. The seaweeds are deployed along the abalone benthic sea-cages. Initially programmed to test only one seaweed species (*Saccharina latissima*), France Haliotis decided to widen the scope of this work within AquaVitae to include at least three other species in the multitrophic experiment. *Ulva* Spp., *Alaria esculente* and *Palmaria palmata* were also tested.

Methods

The first step in order to assess *Saccharina latissima* culture at sea was to secure the supply of young seaweed plantules at reasonable price. Plantules were obtained from various suppliers in order to test their quality. Production of seed was done on site or seeded longlines were purchased. In 2019, there were two hatcheries for *Saccharina latissima* in France: Algolesko/Aleor and C-Weed aquaculture. The first one was far too expensive and not willing to collaborate. Some plantules on rope were purchased from the second. Considering the difficulties to obtain such plantules, France Haliotis' team decided to produce their own plantules in Plouguerneau's hatchery so they could be tested alongside C-Weed's.

Alaria esculente plantules were obtained from the Station Biologique of Roscoff and were deployed at the same time.

Palmaria palmata plantules, although at very early stage of development, were supplied on thin ropes by Agrocampus Beg Meil to be tested.

Several methods for deployment were tested (on horizontal and vertical long line, directly on the cage). A batch of seed was produced under organic standard specifications using organic nutrients Geogreen™.

A) Reproduction

1) Harvest of broodstock

For this first year of growing algae we decided not to reproduce, but to buy ready-made seedlings. However, given the price of the seeded string and the difficulties in obtaining it, we changed our minds and decided to try to master the entire algae cycle. Mature sporophytes (Figure 4.6.a) were collected from the infralittoral at Plouguerneau. A mature *Saccharina latissima* is recognizable by its colour. In fact, on its distal part there is a more or less thick and dark line. This line is dark because the sporocysts (cells containing the spores) are filled with spores. The maturity period of this seaweed is extended from mid-November to the end of February in Brittany. It is preferable to select the broodstock directly in its natural habitat (lower part of the tidal swing zone). But this area is not always accessible. Stranded algae can also serve as broodstock, but in general the choice of the individual cannot be made and the quality is not optimal. France Haliotis was fortunate to have a natural growth on the abalone cages and this is where the harvest and selection of the broodstock was mainly carried out. The quantity of parents depends on the quantity of twine to be seeded and the quality of maturation, but generally it is necessary to take the maximum of sporophytes to increase the density of meiospores during the phase of fixation. During harvest, try to keep the algae in water at the same temperature as the water from which it was taken in order to avoid stress. In fact, stress will induce sporulation and the goal is to control this stage of sporulation. You should try to prepare them directly after harvest or if necessary keep them in a pond with a high flow of water to once again avoid stress.

Figure 4.6.a: Mature Saccharina latissima sporophyte.

2) Cleaning of spores

The cleaning protocol was found during bibliographic searches in the book entitled: Protocols for Macroalgae Research (Charrier et al. 2018) and "Improvement of seaweed culture techniques: in vitro culture of long-stemmed kelp seeds and seeding ropes of culture" (Leblanc et al. 2008). These techniques had to be adapted to a larger production with the equipment already present at the company. To begin with, you have to cut the part containing the sporocysts and then carefully rub each piece with absorbent paper in order to remove all the organisms present on the thalli of the algae. Then cut the pieces into 5cm squares and soak them for two minutes in a disinfection bath: a bleach solution dosed at 200ppm. This bath will make it possible to destroy all the microorganisms which are still present after cleaning by rubbing. It is imperative to then perform two baths of sterile seawater of thirty seconds each to rinse the pieces. The algae are drained on absorbent paper and placed on a tray which will be covered with aluminium foil to protect them from light before being placed in the fridge. This step makes it possible to have pieces of algae that are as clean as possible and thus avoid any external contamination during the sporulation phase.

3) Sporulation and fixation

The algae are left in the refrigerator in the dark for 24-hours to cause high stress. This stress will allow an emission of the spores by the algae. The next day, the algae are immersed in a bath of sterile moving seawater until a sufficient concentration of spores is obtained (15 to 30min) (at least 30 visible and vivid spores are required at 10 × 10 magnification). To check the concentration, it is necessary to take regular samples. When a satisfactory concentration is obtained, the algae squares are removed from the solution and several filtrations are carried out: twice at 40µm and twice at 20µm to remove a maximum of contaminants. The spores measuring less than 10 µm then pass through. Then the sterilized collectors are immersed for 5-hours in the solution in order to fix the spores on the twine.

The collectors (Figures 4.6.b) were previously designed to make a simple object to produce in large quantities and reusable from one year to the next. They are made of PVC, measure 40cm high and 10cm in circumference. They need to be very ventilated to prevent the twine from coming into contact with the "skeleton" of the collector so that the seedlings develop over the largest possible area and that light and water circulate at best. The twine on which the spores will attach is a 1.2 mm cotton twine. The capacity of the twine collectors is about 100 m.

Figure 4.6.b: Twine collector before and after.

B) Preculture in a controlled environment

1) Production with chemical fertilizer

Once the spores are attached to the twine (about 5 hours), they must be passed through their 1st culture medium. This medium will be richer in nutrients than the following ones. The preculture tanks are white 170L tanks. The medium is made from sea water filtered at 1µm and sterilized by U.V. Again, water with a temperature of around 10°C is needed.

For an alga to develop, it takes mainly thee compounds already present in our water but enriched to promote the growth of seedlings.

Nitrogen is added as sodium nitrate (NaNo3) at a concentration of 17mg/ml.

The phosphorus is added in the form of sodium dihygenophosphate (NaH2PO4) at a concentration of 2 mg/ml.

The iron is added in the form of chelated iron (Fe-EDTA) for better assimilation by the algae at a concentration of 0.2 mg / ml.

An LED ramp is placed 50 cm above the bins and is programmed for a day / night cycle of 14/10.

Aeration is also present from 5-days to create a movement of water and prevent the formation of biofilms.

A water change will be made once a week. The concentrations for the second culture medium being:

NaNo3: 10mg/ml

NaH2PO4: 2mg/ml

Fe-EDTA 0.04mg/ml

This preculture can last from 4 to 6 weeks depending on temperature and lighting to obtain seedlings close to a millimetre in size. In France, we tend to deploy the twine with small seedlings unlike Asians or Americans

2) Nutrient comparison test

The goal is to compare three different preculture media (Tables 1 and 2) in order to optimise the growth of the seedlings but also for ecological and practical purposes. Current growing media are based on chemical fertilizers, but seaweed farming tends to turn to organic and even organic nutrients (Organic Agriculture Certification). Even if the volumes are negligible, finding an alternative to chemical fertilizers for more responsible fertilizers would be preferable for the cultivation of *Saccharina latissima*.

For this experiment, nine aquariums of 12L will be placed, each containing two collectors inoculated with *Saccharina latissima* spores. Sporulation and fixation are carried out under the same conditions as for production. The water is filtered (1µm) and U.V. sterilised. Aquariums are equipped with ventilation.

The experiment began on Thursday, February 13th, 2020. The spore concentration during the adhesion bath was 65.42 million per litre.

C) Longline culture at sea

Once past the 4-week mark in preculture in a controlled environment, the seedlings will be able to be transferred to sea. The twine will be wound around larger ropes so that the algae crampon develops on this larger rope. The strings are transported to the boat in damp towels to preserve them as much as possible.

We studied three deployment systems (Figure 4.6.c) and we only retained the horizontal deployment technique. Firstly, this technique is easier to perform because it is only a question of a 100-meter rope stretched between two anchors and floats. In addition, almost all the material necessary to carry out these sectors is already present in the company so there will be no big investment to plan.

The other interesting system was the vertical die which makes it possible to increase the length of cultivation because we add vertical lines but the concession does not allow to put lines of more than 3m because beyond this length the bottom of the lines rubs on the sand at low tides. However, the maximum number of lines per line is 36, i.e. a linear meter length of 108m.

In addition, as each suspension is weighted to keep it vertical, a float purchase for a sum of €600 is necessary. The vertical longline technique will therefore not be retained for economic reasons. It is better to use horizontal dies instead as the total crop length is 90m which is only 18m less than vertical and the investment is less.

The last technique considered was to seed ends connected to the line and attached to a float, but the Aber Wrac'h being a major tourist spot and frequented by many boats, this could have hampered navigation. And even if navigation remains prohibited on the concession, the company tolerates it. The ends could also have hampered the manoeuvres of the boats of France Haliotis.

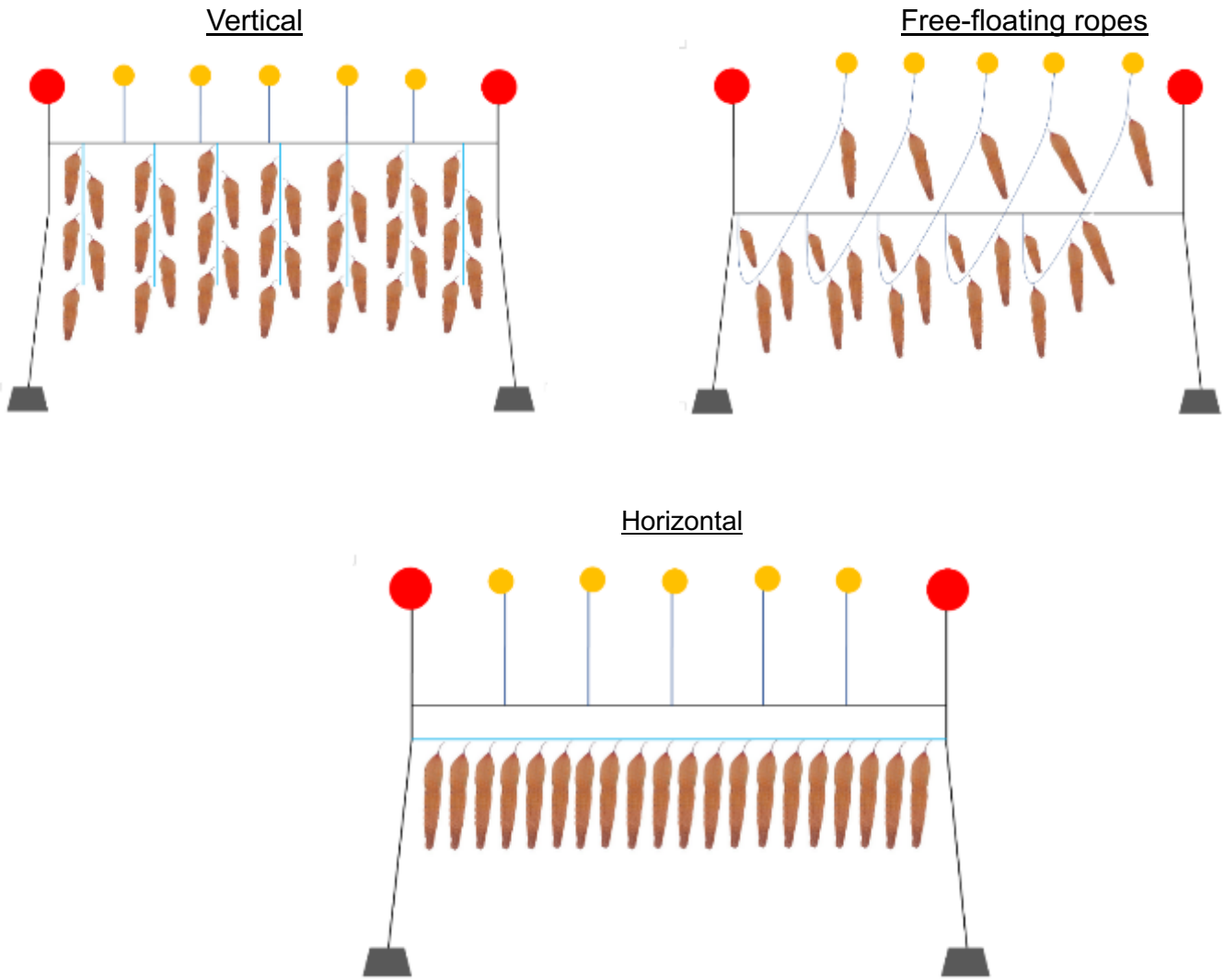


Figure 4.6.c: Types of longlines tested (Schéma G. Gouadec).

Biological material:

We have three batches of *Saccharina latissima* collectors which were inoculated on three different dates at France Haliotis (FH) and also collectors from an external company (c-Weed) and a batch of *Alaria esculenta* laboratory of research (Roscoff SBR biological station).

Batch 1 FH: 27/11/19 (720m) Batch C-Weed (200m *Saccharina latissima*)

Batch 2 FH: 12/17/19 (960m)

Batch 3 FH: 19/12/19 (800m) Batch SBR (300m)

Production material:

There are five lines including three horizontal lines (Figure 4.6.d) of 100m which have 90m recoverable on which 108m of twine can be wound.

That is to say a total of 450 linear meters of longline for 540m of twine.

Concession at sea:

The 3 Horizontal longlines were organised with the 3 batches as follow:

Batch 1 FH Batch C-Weed Batch SBR

Toward shore (shallower) toward channel (deeper)

- Longline 3B placed on the 10/01/2020
- Longline 5B placed on the 21/01/2020
- Longline 14 placed on the 21/01/2020

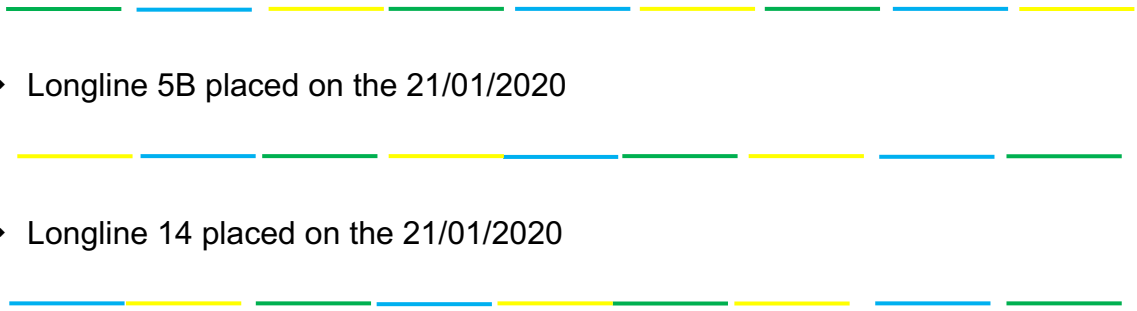


Figure 4.6.d: Organisation of the 3 batches on the various longlines.

Measurement protocol:

The lines are each divided into nine sections of 10m with an alternation of algae from three different origins. A measurement will therefore be taken on each section.

For the number of seedlings, the number of individuals over 50cm will be counted. The 50cm will be chosen randomly on the section.

For the size of the seedlings, a measurement of the thallus on 30 individuals selected at random will be carried out per 10m section.

D) Deployment of the seeded twine directly on the outside of abalone cage culture at sea

Abalone cages represent a large area on the concession. The exterior of the cages is not valued and a great diversity of algae naturally attaches itself to them. It is interesting to try to seed algae on the cages. In addition, the proximity of abalone could promote the growth of algae thanks to organic waste from animals.

However, care must be taken to inoculate cages that will stay at least a year at sea without being too much handled. Mandatory handling is the feeding of abalone (about once a month) but this could be an opportunity to monitor and measure the growth of algae.

Biological material

We have batches of collectors of *Saccharina latissima* from France Haliotis (FH) and also a batch outsourced from an external company (c-Weed) or from a research laboratory (Roscoff SBR biological station and Agrocampus Beg-Meil AC).

Batch 1 FH: 27/11/19 (720 m) Batch C-Weed (200m *Saccharina latissima*)

Batch 2 FH: 17/12/19 (960 m) Batch AC (200m *Palmaria palmata*)

Batch 3 FH: 19/12/19 (800 m) Batch SBR (300m) *Alaria esculenta*)

Production material

The cages on which the twine will be deployed contain only abalone from the 2017 and 2018 generations because these cages will stay the longest at sea. They are located in a shallow zone (line 2 and line 3) and in a deeper zone (sector 9 and 10)

Deployment of the twine

Screws will be added on the outer faces of the cages in order to fix the twine in contact with the surface of the cage so that the algae crampon attaches to the latter. Approx. 20m of twine can be deployed on a cage (Figure 4.6.e).

Figure 4.6.e: Deployment of seeded twine on the outside of abalone cage.

Results

A) Reproduction

The last sporulation protocol was very satisfactory. In the bibliography, Acseeded twineing to IFREMER, the fact that a drop of solution containing the spores (under a microscope at 10 x 10 magnification) must have a minimum of 15 to 25 biflagellate spores in the ocular field to ensure sufficient inoculation (Figure 4.6.f). The cleaning of the sores and the dosage of the bleach bath at 200ppm and the filtration were satisfactory because the final solution contained only spores in good condition and very mobile.

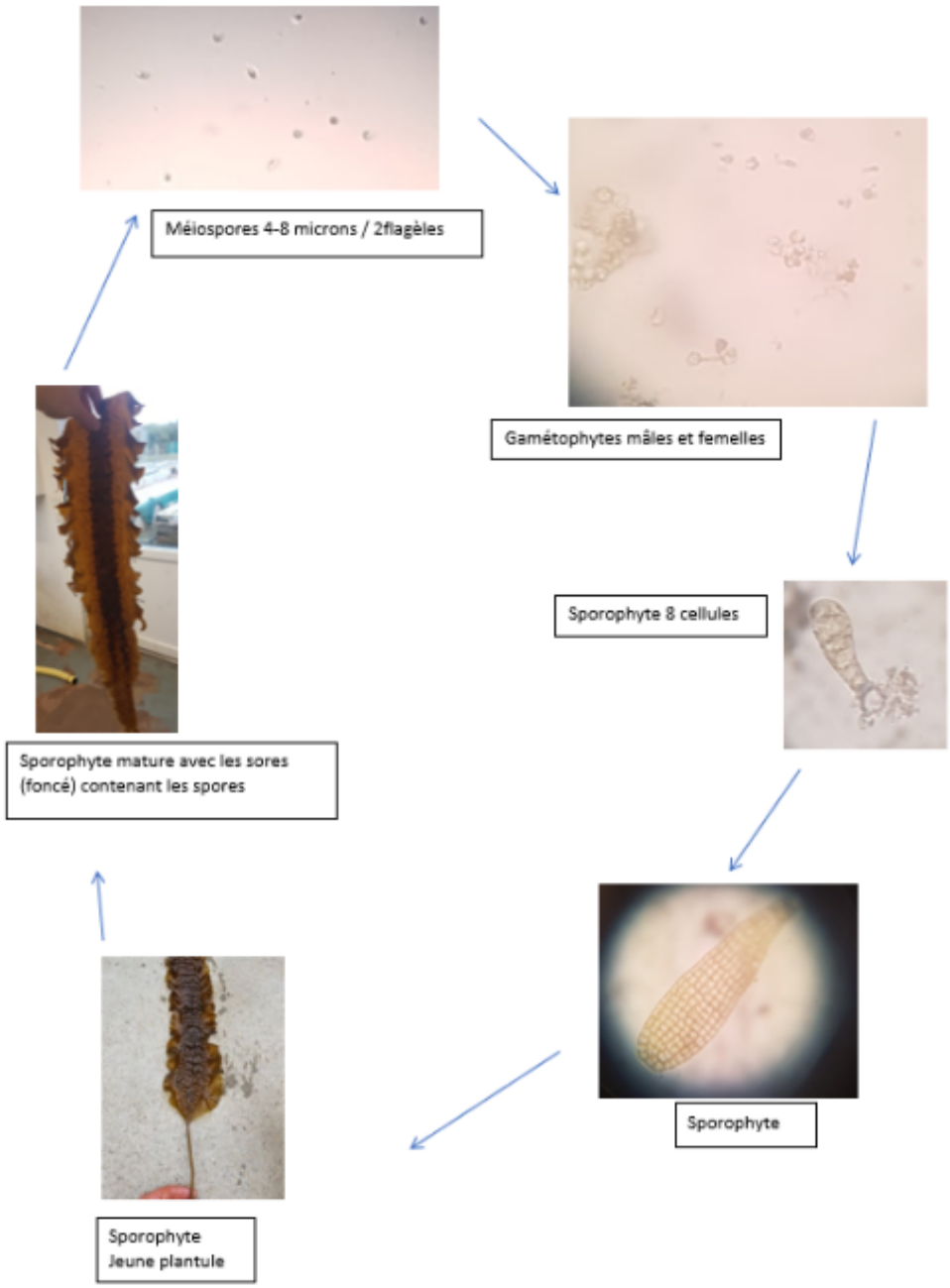


Figure 4.6.f: Saccharina latissima life cycle as obtained in France Haliotis hatchery (schema and photo G. Gouadec).

Regarding the solution obtained by France Haliotis, there were more than 50 spores in the ocular field, which is very good because the more spores there are, the more efficient the fixation on the support will be.

The selection of broodstock is essential because it is necessary that they have reached an optimal maturity. The difficulty was to harvest mature broodstock because the harvest was dictated by the winter Breton climatic conditions. The storm succession was a real problem in the harvest.

For the years to come it is absolutely essential to find an alternative to harvesting mature broodstock. The France Haliotis team intends to induce the maturation of the parents. With a harvest of sporophytes starting in August, we overcome the winter weather conditions. With management of the photoperiod and temperatures via LED ramps and a heat pump (equipment already present at the

company for the maturation of abalone), it is possible to cause the maturation of the broodstock and thus no longer depend on the maturation. natural. Thus, it is therefore possible to advance reproduction and allow better time management for the cultivation of algae.

Maturation only lasts about 50-days. It will therefore be possible to proceed with the sporulation protocol from the beginning of October, and therefore gain two months compared to natural maturation.

These two months will be welcome during the passage at sea because the algae which colonize the sectors to the detriment of *Saccharina latissima* are mainly *Saccorhiza polyschides*, which have a reproduction period in the natural environment similar to that of *Saccharina latissima*. The two months will therefore allow the seedlings to develop and it will therefore be less easy for *Saccorhiza polyschides* to colonise an end on which algae are already attached.

In addition, the two months will allow an earlier harvest of algae and therefore less degradation by aquatic fauna (developed later in the thesis) because it takes place during the summer period.

B) Preculture

Preculture was also a success, as evidenced by the collectors:

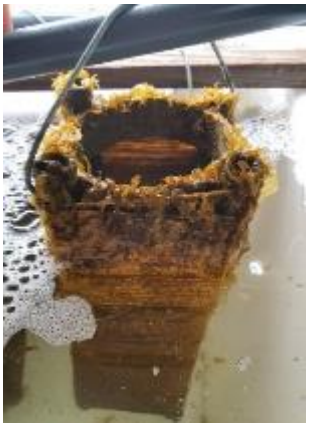


Figure 4.6.g: Collector (photo G. Gouadec).

The preculture medium with chemical fertilization is therefore effective (Figure 4.6.g). The dosage of brightness and temperature also seems to be under control. On the other hand, on the top of the collector (closer to the light source) the algae developed faster. We will therefore have to think about turning the collectors regularly in the future to obtain more homogeneous growth.

The collectors were contaminated with diatoms as well as small ciliated organisms. It had little impact on the crop, but there are techniques to avoid these contaminations such as adding Germanium dioxide GeO_2.

Regarding the experience of different fertilizers, unfortunately no quantitative analysis could be carried out. However, this experience revealed qualitative information. At the start of preculture, addition of chemical nutrients was more effective than bioavailable nutrients and control. The growth was faster. Quickly a bacterial biofilm was created on the surface of the collectors with the biological nutrients (Figure 4.6.h) killing any *Saccharina latissima* seedling.

As for the control without nutrients, growth was very slow and at the end of the experiment there were no more seedlings.


a)

b)

Figure 4.6.h: Collector with biological fertilization (a) without fertilization (b).

This small volume experiment (10L) showed another problem that can occur during preculture in a closed environment, a contamination called rosification. This disease is not known, but infected collectors must be removed very quickly to prevent contamination. If the disease develops, no seedlings will survive. "Protocols for the culture of the long-stemmed kelp (*Saccharina longicruris*) and the sweet kelp (*Saccharina latissima*) in the context of Quebec" (Tamigneaux et al. 2013)

C) Longline culture

Longline culture has obtained mixed results.

Passage of the seeded twine at sea

Several methods were used for the transfer of collectors at sea. The collectors were stored in wet towels and in basins full of water. Basins full of water preserve the seedlings better but are less easy to handle than basins with only a cloth because they are heavier. But this is the technique that was chosen provided the water temperature does not rise too much.

For the deployment of the seeded twine, the method has not yet been developed. Indeed, the initial idea was to insert the end into the collector to deploy it easily or the floats being already installed and changing collector every 10m to have sections distributed over all the dies. it was impossible.

The collector had to be turned around the industry, which took time and labour.

For the years to come, it is necessary to imagine collectors capable of sowing a complete line, i.e. (120m of seeded twine) but also of being able to fix the floats after the deployment of the seeded twine so as not to hinder the passage of the collector on the end

Vertical sector

The experience of vertical dies was a failure because the weight of the lines, the sinkers and the algae were too great for the floats. The lines therefore dragged on the ground and handling was too difficult given their weight.

The shallow depth was also a negative point for this technique.

The dies were very quickly colonized by *Saccorhiza polyschides* and as a result became even heavier.

To be able to cultivate the *Saccharina latissima* in a vertical way it is necessary to have either more depth but the concession does not allow it or to shorten the vertical lines but this is of no interest if the length of the lines does not allow to increase the total cultivable length.

It is better to favour horizontal dies which are easier to handle and sow.

Horizontal sector

Several observations were made on the horizontal sectors. First, it was the culture at sea technique that worked best. They are the easiest to seed and handle either by hand with the small barge or with the crane of the larger boat.

Figure 4.6.i: S. Latissima longlines (right) and Alaria esculente longlines (left) at sea.

The results were positive with the *Saccharina latissima* seedlings (Figure 4.6.i) produced at France Haliotis as well as *Alaria esculenta* from the Roscoff biological station.

However, the seeded twine purchased from the producer of seaweed C-Weed did not yield any results. The sections where C-Weed's *Saccharina latissima* was deployed remained pristine and no seedlings developed there. It seems that the seedlings were still too young when they went to sea.

For the yields of *Saccharina latissima* from France Haliotis they reached 14kg per meter of end (with an average of 30 plants/meter) during the month of August. Which is very encouraging for a first year of culture. The health situation and the partial unemployment of part of the team limited the number of average weight checks carried out. The first was carried out at the beginning of June (3kg per meter). Most of the growth took place during the summer (Figure 4.6.j).

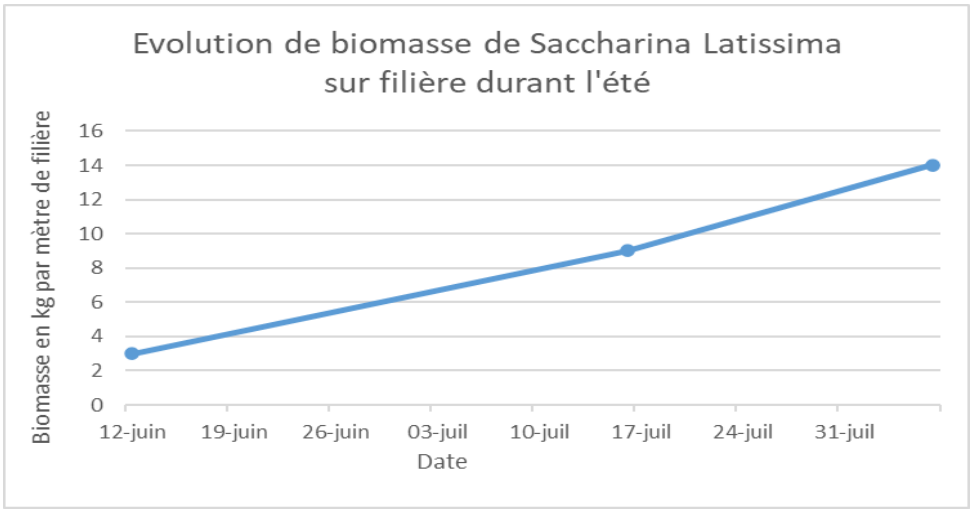


Figure 4.6.j: Growth curve of Saccharina latissima on horizontal longlines in kg per meter.

Regarding the maximum yield of Alaria esculenta it was measured in June and amounts to 1.5 kg per meter (on average 75 holdfasts per meter). *Alaria esculenta* being very fragile, it was mostly destroyed by summer colonisers (crustaceans, ascidians, and epiphytic algae).

Whether on *Saccharina latissima* or *Alaria esculenta* at the beginning of July, these colonisers were observed, which gradually deteriorated the algae. (Figure 4.6.k)

To fight against this phenomenon of summer contaminations, artificial maturation and the delay in reproduction of about 2 months is a good solution because it allows to gain two months of growth and thus allow a harvest of algae before the summer period during which the opportunistic organisms thrive.

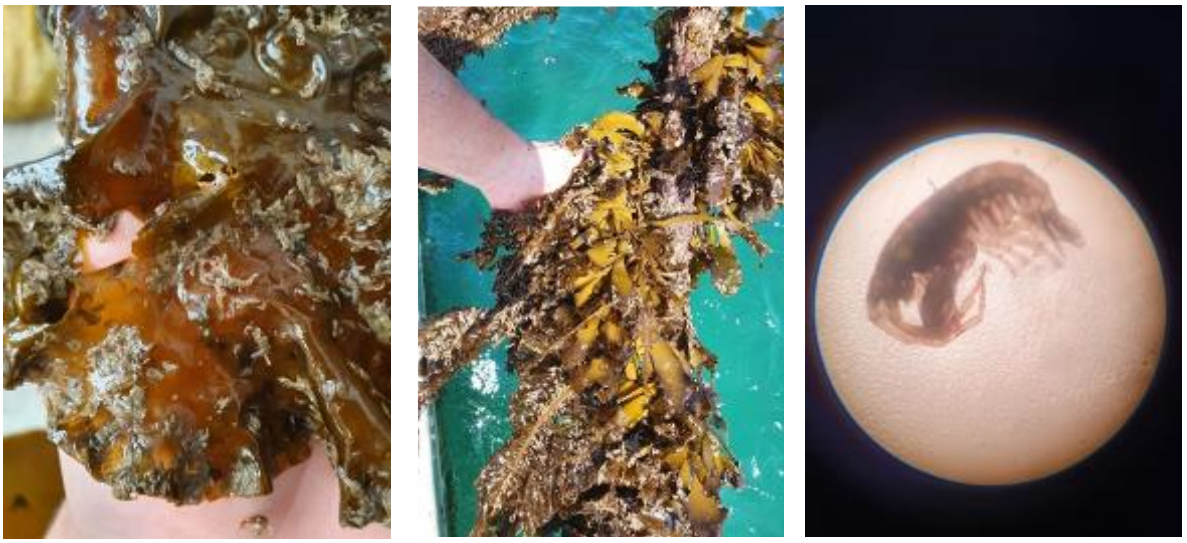


Figure 4.6.k: a) Colonization of Saccharina latissimi; b) Alaria esculenta; and c) crustacean.

D) Cage culture

Cultivation on the outer sides of abalone cages was unsuccessful. As much for the seeded twine of *Saccharina latissima* from France Haliotis as for the seeded twine of *Palmaria palmata* from agro-campus. The reason is quite simple, in winter during storms, a phenomenon of silting up of the cages occurs. This silting must be stopped to save the abalone and the material.

Thanks to the power of the boat, the cages can be raised but this operation is very difficult. The cages are dragged and turned to take them out of the sand, which destroys the seeded twine and some of the algae.

In addition, when releasing the cage after feeding for example, one of the four sides of the cage rubs against the hull of the boat to allow the employee to unhook the cage from the crane. Finally, the pieces of seeded twine which were spared were quickly colonised by competing algae.

The installation of the seeded twine on the cages was very time-consuming (two full days for three employees for around 30 cages) which is not economically viable given the results obtained.

No cultured algae therefore developed on the surface of the cages. Natural seeding on the cages seems to be the best alternative to exploit this surface. At the beginning we notice a colonization by green algae of the *Ulva* type then comes *Saccharina Latissima* as well as *Saccorhiza polyschides* which must be removed because not very nourishing for abalone but competitive for *Saccharina latissima*. In addition, these studs prevent good water circulation in the cage (Figure 4.6.l).

Figure 4.6.l: State of the twine on the cages after the winter.

Artificial seeding is therefore of little interest in view of the diversity and the algal biomass naturally present.

Discussion

This first year of cultivation of *Saccharina latissima* is encouraging for the years to come, as the company could very soon rely on seaweed cultivation to replace some of the harvested algae. The goal is not to depend entirely on cultured algae but to produce about a third of the algae used as abalone feed.

This year has shown that an induction of maturation of the parents would have facilitated and improved the culture. Indeed, this would have made it possible to overcome winter weather conditions but also to be able to harvest the algae earlier before it was colonised and degraded by organisms present in the summer.

The technique of sporulation and seeding of the collectors is efficient so few corrections to be made in the future except the design of new collectors which would facilitate the deployment of the seeded twine around the main end.

The quantity of seeded twine will not be a limit because the production capacity is very large (2500 meters for this year). The limit will be the length of lines that can be deployed at sea in the event that the company does not buy an additional concession.

Cultivation must be done exclusively on horizontal channels for practical and technical reasons. Today there is the possibility of deploying 8 channels with the current configuration of the concession. With an average of 14kg per meter of end like this year, this represents 11.2-tonnes of algae or 13.6% of the annual algae requirement. But one can easily imagine a new layout of the park to free up space in order to install new sectors.

Subsequently, it would be interesting to carry out analyses of the protein level as well as comparisons of the growth of abalone fed with cultured algae and wild algae to verify the impact of these cultured algae on the performance of the abalone farming which remains the main activity and source of income of the company. It would also be interesting to look at the economic benefits of this culture.

It would also be interesting to assess the impact of the proximity to the abalone cages on this algae culture as was planned this year but which unfortunately could not be done. Because it could be considered multi-trophic aquaculture.

Key exploitable results:

Kombu production on long lines between M6 and M18:

- Successful for 2 species: *Saccharina latissima* and *Alaria esculente*;
- Best productivity obtained with *S.* Latissima (average >15kg/meter);
- Good production with A. Esculente but availability for feed too short (May & June only);
- Production of *Ulva* Spp. directly on our abalone cage with good value for juveniles;
- Failure of our trials with *Palmaria palmata* on our abalone cages.

Progress, deviations, problems & next 12M

Progress: The work achieved during the first 12-months of AquaVitae is probably going well beyond what we could expect to achieve in the first place and can be estimated to be 40% complete. The progress towards the main key exploitable result of this task is well on its way: a seaweed production at sea alongside the abalone farm should allow for the production of a significant amount of food for the abalone.

Deviations & Problems: There were no deviations or problems encountered for this task.

Outlook: The next step for France Haliotis will be to scale up the production of seaweed and to test for the seaweed quality as food for abalone.

CST 4.7 Integrating queen scallop or flat oysters into existing IMTA system with abalone and seaweed culture in sea-based concession

Sylvain HUCHETTE, France Haliotis

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
4.7	Integrating queen scallop or flat oysters into existing IMTA system	T3.2	FrHal	10%		M6	M12	M42		4	8

Introduction

Filter feeders are low trophic species that would use a different ecological niche than the abalone. Growing filter feeding bivalves inside abalone benthic cages would allow France Haliotis to diversify its production and make its concession more productive. By increasing the shellfish biomass on the

concessions, the excreted nutrients would benefit the associated seaweed production. During M6 to M12, shellfish spat had to be found and negotiated with the local hatcheries.

Methods

Experiments will be designed when the spat become available. Proposed methods can be found in the work plan for CS4 in annex 3 of Deliverable 1.1.

Results

Not applicable.

Discussion

Not applicable.

Progress, deviations, problems & next 12M

Progress: This task started a little behind schedule. Scoping and planning were done. Structures have been prepared, so that we assume the task to be complete by 10%. But since then, the task has been delayed as seed-stock cannot be obtained due to confinement as a result of COVID-19. There is sufficient time to catch up on the progress that has been lost here.

Deviations & Problems: Hatcheries could not supply spat on time because of the pandemic. The delay in the beginning of CST4.7 due to COVID-19 should not hinder the work for the task.

Outlook: Spat should become available during summer 2020.

CS5

Summary of progress report for Case Study 5

Date of report:	**6.4.2020**
Case Study name:	**Biofloc and pond-based IMTA**
of relevane for WPs	**2, 3**

Abstract/Summary

This case study will work in three main fronts: 1) Improve biofloc technology by adjustments of production parameters like aeration, use of biofilms and carbon:nitrogen ratio. 2) Develop a biofloc system using IMTA concepts, using diferent species like shrimp (*Litopenaeus vannamei*), fish (tilapia and mullet), moluscs (oyster) and seaweed (*Ulva* Spp.), 3) Design and validate a shrimp - seaweed - oyster IMTA production system on a commercial scale to enhance profitability, ensure sustainability and promote the circular economy of aquaculture enterprises besides conserving natural resources. For the first task, it was established that the nitrification process was more efficient in the treatments with biofilm and bigger air flow rate, presenting smaller concentrations of ammonia and nitrite in comparison to the BFT treatment. Similarly, treatments with biofilm and stronger flow rate showed better zootechnical performance of the shrimp. For second task, it was stablished that the presence of mullets, independent of the IMTA system used, resulted in lower TSS concentrations, compared to the monoculture of shrimp, thus confirming mullet can be used to control TSS concentrations originated from shrimp production in BFT system. Also, it was demonstrated that *U. ohnoi* performed significantly better than *U. fasciata* when cultured using effluent from a biofloc shrimp tank and the in the assessment of different stocking densities (2 and 4 g L^{-1}), the lower one proved to be more efficient. For the third task, it was demonstrated that *Ulva lactuca*, *Gracilaria caudata*, and *Hypnea pseudomusciformis* have no adaption to earth ponds.

CST 5.1 Improve shrimp production in biofloc by adjusting production parameters

Wilson Wasielesky, FURG

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
5.1	Improve shrimp production in biofloc by adjusting production parameters	T2.2, T3.2	FURG, UFSC	40%	✓	M1	M1	M40	0	5	9

Introduction

Nitrogen is an important nutrient for living organisms, as it is an essential component for the constitution of proteins and nucleic acids. It may be a limiting factor for primary production in these ecosystems, but can also be toxic for aquatic organisms when present in higher concentrations (Vieira, 2017).

In the nitrification process, the successive oxidation of ammonia to nitrite and subsequently to nitrate is mainly made by autochemolitotrophic microorganisms (Ebeling et al., 2006) belonging to two groups of bacteria. The first, the ammonia-oxidizing bacteria (AOB) are responsible for the oxidation of ammonia to nitrite. Most of these organisms belong to the genera *Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosolobus and Nitrosovibrio*. The second group, the nitrite-oxidizing bacteria (NOB), performs the conversion of nitrite to nitrate, and the majority of these microorganisms belong to the *genera Nitrobacter, Nitrococcus, Nitrospira* and *Nitrospina* (Ebeling et al., 2006; Madigan et al., 2016). Recent studies have demonstrated that microorganisms of the Archaea domain also participate in the nitrification process (Ward, 2013).

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In the production of aquatic organisms, like fish and shrimp, higher concentrations of nitrogen compounds as ammonia and nitrite can become a problem, when they accumulate in the aquatic environment due to the excreta of the produced organisms, decomposition of unconsumed foods and organic waste (Timmons & Ebeling, 2010). Therefore, the control of nitrogen elements within the breeding environment is important, since they can cause damage to the produced shrimp and fish (Lin and Chen, 2003, 2001)

The concentration of ammonium (TAN) in the medium increases with increasing pH and water temperature, and reduces with increasing salinity (Boyd & Tucker, 2012) Exposure to inadequate concentrations of these compounds can cause stress, triggering various physiological changes, and compromising performance leading to death, thereby impairing production (Girotto, 2010). Nitrite, on the other hand, binds to hemocyanin, transforming it into metahemocyanin, preventing the transport of oxygen to tissues and reducing the amount of oxygen available for metabolism (Tahon et al., 1988). This process can lead to hypoxia and, consequently, mortality of organisms produced (Chen et al., 1986).

An efficient aeration system is important for the oxygen supply to produce animals, and to keep the flocs in suspension in the BFT system. However, low concentrations of dissolved oxygen limit or suppress nitrification (Avnimelech, 2009; Zhu et al., 2008), since the nitrifying bacteria, AOB and NOB present a demand for oxygen for cellular activity, growth and reproduction. Thus, in order to carry out the nitrification process it is essential that these microorganisms settle in the growing environment like bioflocs and biofilm, but also have ideal environmental conditions to absorb and transform nitrogen compounds.

The biofilm can be defined as an organic matrix adhered to any submerged substrate, which is colonized by a microbial community composed of bacteria, protozoa, fungi and algae (Ramesh et al., 1999). It has been shown that biofilm is responsible for removing nitrogenous compounds from water, especially ammonia and nitrite. Thompson et al. (2002), evaluated the biofilm efficiency in the maintenance of water quality through the absorption of dissolved inorganic nutrients (ammonia and phosphate). Moreover, Ballester et al. (2003) evaluating the influence of biofilm on *Farfantepenaeus paulensis* production, concluded that the biofilm positively influenced shrimp growth, especially by providing an alternative food source.

In order to increase efficiency in shrimp production, the use of artificial substrates for biofilm fixation in the Biofloc Technologic system has already been carried out by Ferreira et al. (2016). However, these authors concluded that biofilm served only as a source of complementary food, but did not observe any difference in the metabolization of nitrogen compounds in comparison to bioflocs alone. Thus, in BFT systems the use of substrates for biofilm development would not be necessary to keep water quality in good standards, since the bacteria present in the bioflocs would be enough to keep ammonia and nitrite at low levels and also represent an extra food source.

However, it is likely that biofilm in aquaculture systems was not analyzed in all its dimensions. For instance, there is little information in the literature on the lower efficiency of the nitrifying bacteria in the biofilm in BFT systems and its relationship with the oxygen limitation. In BFT systems, aeration must be maintained at lower rates in order to keep the flocs in suspension without causing their rupture and to guarantee the nitrification by the bacteria present in the biofloc (Lara et al., 2017; Souza et al., 2019). However, this low aeration rate may be a problem to nitrifying bacteria present in the biofilm. Thus, the objective of this study is to determine the response of nitrifying bacteria present in the artificial substrate biofilm submitted to different aeration intensities in the production of *Litopenaeus vannamei* (Boone, 1931) in systems with clear water and also with bioflocs.

Methods

Location and facilities

The study was carried out at the Marine Aquaculture Station (EMA / FURG) - Institute of Oceanography of the Federal University of Rio Grande - FURG, located in the city of Rio Grande, RS, Brazil (32º 19 'S, 52º 15 'W).

In both experiments, 800L tanks of useful volume were filled with seawater, chlorinated with 10ppm of sodium hypochlorite, later de-chlorinated with 1ppm of ascorbic acid. Water temperature was maintained with submerged electric heaters (Hydor Theo 200W). The aeration system was composed of a 4 HP blower and Aerotubes® microperforated hoses, to maintain constant aeration. The aeration rate of 20.00L/min is normally used in the aquaculture systems of EMA / FURG, but other rates were tested. In order to measure the airflow, individual rotormeters (TRP-255-H-7 1 POL NPT-Tecnofluid®) were coupled to the aeration inlet of each experimental unit and regulated in the flow according to the treatment.

The artificial substrates used for colonisation of the biofilm were non-floating Needlona®, in a proportion of 200% of the lateral area of the tank. Needlona® used comprised of 100% polyester fiber; 250g/m^2 weight; 1.4mm thickness, 0.18g/cm^3 density. Before the beginning of the experiments, the substrates were kept for 30-days in a biofloc system.

Experiment 1: Biofilm in clear water

The experiment consisted of four treatments with three replicates each, denominated: 1) W/AIR: without aeration; 2) V7.5: flow rate 7.5L/min; 3) V33.75: flow rate 33.75L/min and 4) V75: flow rate 75L/min in a clear water system, without shrimp. To determine the efficiency of the nitrifying bacteria, 7mgL^{-1} ammonium chloride was added representing the safety limit for the *L. vannamei* species at salinity 35 (Lin and Chen, 2001). The study lasted for 10-days.

Samples were collected every four hours for analysis of ammonia (TAN), nitrite (N-NO_2), alkalinity ($CaCO_3$) following methodologies UNESCO (1983), Aminot e Chaussepied (1983) e APHA (2012) respectively and dissolved oxygen with Multiparameter.

Experiment 2: Biofilm and Biofloc

The experiment performed during 47-days used the best aeration rate (33.75L/min) determined in experiment 01. The experimental consisted of three treatments with three triplicates, being: 1) BFT - Biofloc with 20.00L/min flow rate; 2) BFT+BF - Bioflocs and biofilm with flow rate 33.75L/min and 3) BF - clear water and biofilm with flow rate 33.75L/min. The shrimp (7.81 ± 0.24g) were stocked at a density of 500m^{-3} and fed with Guabi® 1.6mm commercial feed with 40% crude protein. The food was supplied twice a day (08:00 and 16:00) after samples according to the methodology of Garça de Yta et al. (2004).

To begin the biofloc formation, organic fertilizations were carried out with the addition of sugar cane molasses (37% of carbon) when the concentrations of TAN reached 1.0mgL^{-1} to maintain the relation C:N 15:1.

During the experiment, the dissolved oxygen, temperature and pH were monitored twice a day using a multiparameter (YSI PRO 20). The alkalinity was analysed three times a week. When pH and alkalinity values were below 7.3 and 150mgL^{-1} respectively, there was the addition of hydrated lime as described by Furtado et al. (2011). Salinity and total suspended solids (SST) were measured on a weekly basis,

according to AOAC (1999). When SST concentrations exceeded 500mg/L, clarifiers (biofloc settling tank) were used in order to remove surplus solids, as recommended by Gaona et al. (2011).

Nitrogen compounds such as total ammonium nitrogen (TAN) and nitrite ($N\text{-}NO_2$) were analyzed daily, and nitrate ($N\text{-}NO_3$) once a week according to Aminot e Chaussepied (1983). Every time that the concentration of nitrite reached 26 $mgL^{-1,}$ the safety level for the salinity employed, 30% of water renewal was done (Lin and Chen, 2003).

Sampling of microorganisms

To characterize the microbial community of the second experiment, weekly samples of 18ml of water were collected from each experimental unit and fixed in formalin at the final concentration of 4% for subsequent identification of the microorganisms in the Laboratory of Phytoplankton and Marine Microorganisms of the FURG, among the treatments with and without substrate, so the CW+BF treatment was not analysed. According to the methodology of Utermöhl (1958). Bacterial abundance analysis was performed on days 0, 17, 26 and 47 for BFT and BFT+BF treatments.

Zootechnical performance

In the second experiment, the zootechnical performance of the animals was determined after weekly samples of 30 animals, using a digital scale with an accuracy of 0.01g.

Statistical analysis

Data were expressed as mean ± standard deviation. Undergo tests of normality (Shapiro-Wilk) and homoscedasticity (Levene), with the proof of these premises. Analysis of multiple variables was conducted with the One-way Analysis of Variance (ANOVA) and post-hoc Tukey test. Data that did not satisfy the assumptions for ANOVA were submitted to the non-parametric test of Kruskall-Wallis followed by a multiple comparison test (Zar 2010). The level of significance was 5% in all cases ($p < 0.05$).

Results

Experiment 1 - Biofilm in clear water

There was no significant difference for the ammonia and nitrate parameters among treatments. The nitrite of treatments V7.5, V33.75 and V75 showed similar values throughout the study, but were lower than concentration measured in W/AIR, which reached 0.17 mgL^{-1} (Table 5.1.a).

For the alkalinity values the V33.75 treatment had the lowest mean value, significantly different from the other treatments. The values of dissolved oxygen were higher in the three aeration intensities tested than in the without aeration treatment.

*Table 5.1.a – Mean, standard deviation (**overall mean**) minimum and maximum of the physical and chemical parameters over the 10-day study of different aeration intensity with different flow rates (tree replicates). Different letters on the same line represent statistical difference p <0.05. W/AIR: without aeration contribution; 2) V7.5: flow rate 7.5 L min-1; 3) V33.75: flow rate 33.75 L/min and 4) V75: flow rate 75 L/min in a clear water system.*

Treatment	W/AIR	V7.5	V33.75	V75
Total ammonia nitrogen (mg L^{-1})	3.07 ± 0.49 (0.30 – 7.53)	2.60 ± 0.57 (0.06 – 7.53)	2.52 ± 1.03 (0.06 – 7.43)	2.65 ± 0.37 (0.06 – 7.67)
Nitrite (mg L^{-1})	0.17 ± 0.08[a] (0.00 – 0.30)	0.05 ± 0.03[b] (0.00 – 0.14)	0.08 ± 0.08[b] (0.00 – 0.49)	0.05 ± 0.03[b] (0.00 – 0.13)
Nitrate (mg L^{-1})	2.21 ± 0.04 (0.00 – 4.83)	2.85 ± 0.61 (0.00 – 8.67)	3.32 ± 0.96 (0.00 – 6.67)	3.55 ± 0.97 (0.00 – 6.26)
Alkalinity (mg L^{-1})	153 ± 27 (107 – 250)	149 ± 25 (103 – 225)	145 ± 25 (100 – 237)	155 ± 24 (105 – 232)
Dissolved oxygen (mg L^{-1})	6.23 ± 0.03[a] (6.20 – 6.25)	6.33 ± 0.03[b] (6.30 – 6.35)	6.34 ± 0.04[b] (6.35 – 6.38)	6.40 ± 0.02[b] (6.40 – 6.48)

Experiment 2 - Biofilm in clear water and biofloc

The products (molasses, hydrated lime) used to control the amount of floc and the settling time, water exchange during the study and amount of water to produce 1kg of shrimp is represented in the table 5.1.b. The temperature, salinity, pH, nitrate and SST did not show significant difference ($p > 0.05$) between treatments during the experimental period. There was a significant difference ($p < 0.05$) for ammonia, nitrite, dissolved oxygen and alkalinity. The BFT treatment presented higher values of ammonia, dissolved oxygen and alkalinity than BF, whereas the $N\text{-}NO_2$ of the BFT+BF and CW+BF treatments were significantly lower than the BFT treatment. The dissolved oxygen levels in the BFT+BF and CW+BF treatments were statistically higher than those of the BFT treatment. This pattern was demonstrated throughout the experiment. The alkalinity presented lower values in the treatments BFT+BF and CW+BF, when compared to the BFT treatment (Table 5.1.c).

Table 5.1.b – Mean and standard deviation of molasses, hydrated lime, settling hours, water exchange and amount of water to produce 1 kg of shrimp over the 47-day study.

Treatment	BFT	BFT+BF	BF
Total of Molasses (g)	2507.34 ± 22.06	380.01 ± 8.50	-
Hydrated lime (g)	1280.07 ± 22.58	1521.59 ± 24.73	1282.46 ± 24.34
Settling hours (h)	10.00 ± 0.58	40.00 ± 1.51	-
Water Exchange (L)	4480.00l ± 66.03	-	-
Water Use (L kg^{-1} shrimp)	719.69 ± 46.72	174.64 ± 6.93	167.65 ± 10.29

Bacterial abundance analysis was performed on days 0, 17, 26 and 47 for BFT and BFT+BF treatments due to the high concentrations of ammonia and nitrite. The total of free bacteria did not present significant differences ($p > 0.05$) among treatments. However, there were statistical differences for the groups of analyzed bacteria.

The BFT treatment differed from the BFT+BF treatment for bacilli and filamentous, bacteria, with higher abundances of bacteria. It is possible to observe the gradual increase of microorganisms in the system as time passes, only on day 26 the number of organisms decreases and increases again as shown on day 47. The BFT+BF treatment has a stability in the organisms from start to finish of the experiment having an increase for filamentous and amoebae over time.

*Table 5.2.c – Mean, standard deviation overall mean and minimum and maximum of the physical and chemical parameters of the water over the 47-day study. *Different letters on the same line represent statistical difference p <0.05. * 500 m^{-3} Litopenaeus vannamei (7.81 ± 0.24g) in BFT with different aeration intensity (Biofloc with flow rate of 20.00L/min, BFT+BF: Bioflocs and biofilm with flow rate of 33.75L/min and BF: biofilm with flow rate of 33.75L/min) with tree replicates.*

Treatment	**BFT**	**BFT+BF**	**BF**
Total ammonia nitrogen (mg L^{-1})	1.73 ± 0.41[a] (0.00 – 13.20)	0.51 ± 0.11[b] (0.00 – 5.20)	0.70 ± 0.54[b] (0.00 – 6.30)
Nitrite (mg L^{-1})	15.36 ± 5.03[a] (0.00 – 57.00)	1.13 ± 0.56[b] (0.00 – 28.00)	1.11 ± 0.44[b] (0.00 – 3.50)
Nitrate (mg L^{-1})	43.91 ± 9.02 (1.70 – 124.00)	73.85 ± 8.25 (4.79 – 204.00)	52.61 ± 17.78 (4.79 – 124.00)
Dissolved oxygen (mg L^{-1})	5.02 ± 0.22[a] (3.75 – 6.15)	5.18 ± 0.16[b] (4.30 – 6.30)	5.22 ± 0.14[b] (3.95 – 6.20)
pH	7.55 ± 0.11 (7.07 – 8.05)	7.55 ± 0.09 (6.96 – 8.06)	7.58 ± 0.07 (7.17 – 8.12)
Temperature (°C)	29.13 ± 0.76 (24.55 – 32.40)	29.44 ± 0.83 (24.70 – 34.40)	29.02 ± 0.31 (25.00 – 32.05)
Salinity	30.80 ± 0.90 (26.00 – 33.3)	31.41 ± 1.89 (27.10 – 35.00)	30.14 ± 1.30 (26.00 – 35.00)
Total suspended solids (mg L^{-1})	298.19 ± 88.73 (0.00 – 665)	346.30 ± 57.28 (0.00 – 665)	332.56 ± 73.18 (0.00 – 575)
Alkalinity (mg L^{-1})	154.75 ± 15.10[a] (95.00 – 250)	135.92 ± 22.11[b] (55.00 – 185)	136.17 ± 22.14[b] (55 – 270)

The results of the zootechnical performance of the shrimp at the end of the experiment are presented in Table 5.1.c. There was no significant difference between the treatments (p <0.05) in relation to the final weight. Survival and final biomass were significantly higher in the biofilm treatments (BFT+BF and BF) than in the biofloc treatment (BFT) alone (Figure 5.1.d).

Table 5.1.d - Mean and standard deviation of the zootechnical performance of the L. vannamei over the 47-day study. Different letters on the same line represent statistical difference p <0.05. BFT: Biofloc without flow control, BFT+BF: Biofloc and biofilm with flow rate 33.75L/min and CW+BF: Clear water and biofilm with flow rate 33.75L/min.

Treatment	BFT	BFT+BF	CW+BF
Initial weight (g)	7.81 ± 0.24	7.81 ± 0.24	7.81 ± 0.24
Final weight (g)	13.50 ± 0.40	13.14 ± 0.19	13.63 ± 0.63
Survival (%)	62 ± 41.49[a]	87 ± 9.54[b]	88 ± 6.93[b]
Biomass final (m^3)	3998.40 ± 490[a]	5732.02 ± 180. 13[b]	5979.47 ± 289.47[b]

Discussion

The first experiment demonstrated the importance of the aeration in the production system for a greater efficiency of the nitrification process by biofilm, especially for NOB bacteria. This was evidenced by the higher concentrations of nitrite in the treatment without aeration when compared to the others. The lack of water movement and the consequent limitation of the oxygen transfer can generate a gradient in the concentration of this gas along the biofilm, with the presence of hypoxic or anoxic areas in the innermost regions of the biofilm (Vlaeminck et al., 2010). In this case, nitrite oxidizing bacteria will be less active and this nitrogen compound will accumulate in the water.

In general, AOB are found in the outermost part of the biofilm, whereas NOB are present in the deepest region (Gieseke et al., 2003). Therefore, NOB is likely to be more subject to the decrease in dissolved oxygen concentration than AOB. This is evident in this experiment, since nitrite variation was more influenced by the oxygen concentration than ammonia. Ammonia levels did not present significant differences among treatments, indicating that AOB were not affected by the oxygen concentration. On

the other hand, the nitrification process did not seem to be affected by the intensity of the aeration, since no differences were observed in the three aeration intensities tested. However, for the second experiment we have chosen the aeration rate of 33.75L/min, which is bigger than the aeration rates of 20.00L/min, normally employed in our production systems, but not as strong as 75.00L/min, which is leads to a high energy consumption.

The water quality parameters of the second experiment, such as temperature, pH, salinity, nitrate and total suspended solids, remained throughout the experiment under ideal conditions for the production of *L. vannamei* (Furtado et al., 2014; Gaona et al., 2011; Van Wyk and Scarpa, 1999), except for dissolved oxygen that presented lower values in the BFT treatment. However, mean concentrations of dissolved oxygen were greater than 5 mgL^{-1} required for bacteria in the nitrification process, as well as for shrimp requirements (Timmons & Ebeling, 2010; Van Wyk and Scarpa, 1999).

In the second experiment it is possible to observe the benefits of the incorporation of the artificial substrate colonized with biofilm, since the biofilm treatments presented lower ammonia and nitrite values, indicating a faster and more efficient removal of nitrogen compounds when compared to bioflocs alone. Holl et al. (2011), also consider that the nitrifying community fixed in the substrate is capable of completely performing the nitrification of the system, even if there is no activity of the bacteria present in the water. On the other hand, in a similar study Ferreira et al. (2016) observed that the biofilm had no positive effect on the removal of the nitrogen compounds in comparison to bioflocs.

It is possible to observe a greater abundance of bacteria in the BFT treatment compared to the BFT+BF treatment, where the BFT+BF treatment remains more stable throughout the period, different from the BFT that presents an increase of bacteria over time. This difference of bacteria between the treatments is related to the formation of the microbial community in the BFT treatment and the presence of the substrate colonized in the BFT+BF. The different amounts of bacteria indicate that great part of these microbes, especially the nitrifying ones, would be attached to the biofilm and not on the flocs, or free in the water. In our study, the lower abundances of free bacteria in the biofilm treatment compared to the treatment with only bioflocs may have resulted from a transfer of bacteria from the water column to the substrate which, as observed by Oliveira et al. (2006).

There were no differences in growth among the shrimp submitted to different treatments, but there was a significant difference in survival rate, with higher values in treatments with biofilm. In addition, the inclusion of the substrate resulted in higher biomass due to higher survivals in these treatments. This result may be a consequence of the higher levels of nitrite in the BFT treatment, which remained about 24 days with concentrations above the safety level proposed by Lin and Chen (2003). However, it is possible that the high concentrations of nitrite, the stocking density and the initial size of the animals may have negatively influenced survival in this treatment, mainly resulting in a high standard deviation of survival data in this treatment Since the treatments that had the artificial substrate with biofilm show an increase of area decreasing the relative density. Otoshi et al. (2006) evaluated the growth and survival of juveniles of *L. vannamei* produced with and without artificial substrate observing an increase in growth and survival in substrate treatments. Similarly, Schveitzer et al. (2013) studied the use of artificial substrates in a biofloc system with different storage densities, concluding that the best survival was in the treatments with the presence of the substrates.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained and discussed above we consider this task to be complete by about 40%. We executed two experiment and one more experiment and the evaluation of the prototype are still to be done.

Deviations & Problems: nothing declare

Outlook: next tasks are evaluating the reuse of water in biofloc system and validate the methodology in a pilot scale.

CST 5.2 Develop a biofloc system using IMTA concepts, improving efficiency in the use of natural resources and increased quality and value of the final products

Felipe do Nascimento Vieira, UFSC

CST #	Name	Reports to task(s)	Partners involved	complete d by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
5.2	Develop a biofloc system using IMTA concepts, improving efficiency in the use of natural resources and increased quality and value of the final products	T2.2, T3.2	UFSC, FURG	35%	✓	M1	M1	M40	0	5	9

Introduction

The continuous growth of aquaculture, including production of shrimp and carnivorous fish in intensive systems, lead to questions related to its sustainability (Naylor et al., 2009; Perdikaris et al., 2016). The Biofloc Technology (BFT) has been developed and adopted mainly by shrimp and tilapia farmers bearing in mind increased productivity coupled to a greater concern with the environmental impacts of aquaculture through nutrient recycling and minimizing water use (Wasielesky et al., 2006; (Azim and Little, 2008). The reduction in the emission of effluents with the utilization of BFT system, reduces the risks of contamination and dissemination of diseases (Ekasari et al., 2014). The microbial community that contributes to the maintenance of adequate water quality, is also a source of supplementary food for the cultivated animals (Lara et al., 2017). The formation of the microbial community occurs due to the alteration of the carbon:nitrogen ratio in the water (elevation to 15:1) due to the accumulation of nutrients from the excretion of cultured animals, and decomposition of uneaten food in the system, coupled to intense and constant aeration (Ebeling et al., 2006). This way, organic matter and nutrients that would be disposed of to the environment in conventional farms, through water renewal, now remain in the system and are recycled by microorganisms (Krummenauer et al., 2014).

Despite the benefits of BFT system, limited or no water renewal results in the accumulation of total suspended solids (TSS) and nutrients (especially nitrate and phosphate), which in high levels can be harmful to the cultured animals (Ray et al., 2010; Gaona et al., 2011). The constant increase of TSS in a BFT system is a consequence of the increase of microbial biomass, which takes advantage of the carbon and the nitrogenous compounds from organic fertilizations and excreta of the animals. During the pacific white shrimp *L. vannamei* production, the recommended concentration of TSS must remain between 100 and 300mgL^{-1} (Gaona et al., 2017). These solids can turn into an environmental problem if not properly discarded. The excess of suspended material deteriorates water quality, can cause occlusion of the gills inducing the animals to hypoxia and consequently harming shrimp growth. It also raises energy cost to keep the levels of dissolved oxygen in limits appropriate for the species (Avnimelech, 1999; Crab et al., 2012; Gaona et al., 2017). Therefore, in order to keep TSS in adequate levels, they are commonly removed from the tanks by mechanical clarifiers, which act as particle decanters (Gaona et al., 2011).

The control of TSS can also by performed by adoption of Integrated Multi-Trophic Aquaculture (IMTA), since this method integrates several species of organisms that can use the solid or dissolved residues present in the production system (Chopin et al., 2001; Troell et al., 2009; Martínez-Espiñeira et al., 2015; Legarda et al., 2019; Poli et al., 2019). In general, IMTA include inorganic extractive species (macro or microalgae) and/or organic extractive species (mollusks and low trophic level fish) in the production of carnivorous fish and shrimp (Neori et al., 2007; Ekasari et al., 2014; Granada et al., 2016; David et al., 2017). With the appropriate composition of species in an IMTA, reduction of excess nutrients and organic matter generated in the system can be achieved.

The mullet (*M. liza*) is a promising species to IMTA: being for iliophagic, consuming mainly the vegetal matter removed from effluent or sand (Oliveira and Soares, 1996), these fish are able to convert the potential energy of the waste into energy that It can be used at other trophic levels and are also

potential consumers of excess solids from the BFT system, acting as a filter for the system. Some studies have already reported the production of Mugilidae in a BFT system (Rocha et al., 2012; Da Silva et al., 2013) as well as their use in integrated production with other species of fish (Melo et al., 2016; Shpigel et al., 2016), and even with shrimp *L. vannamei* (Hoang et al., 2018), and in floating cages (Ghosh et al., 2016). Therefore, the present study aimed to evaluate the feasibility of IMTA composed by mullet and shrimp grown in a BFT system, evaluating the best spatial arrangement for both species, if reared together in the same tank or alone in separate tanks.

Taking into account the potential of seaweeds, in general, and *Ulva* Spp., in particular, to take advantage of excess dissolved inorganic nutrients generated in biofloc systems, as well as their economic relevance, this work aimed to evaluate two aspects of *Ulva* Spp. cultivation using water from a shrimp biofloc rearing system as a nutrient source. First, we assessed the growth of two different species of *Ulva* Spp., *U. fasciata* and *U. ohnoi*, collected from sea and lagoon, respectively, in the city of Florianópolis, SC, Brazil. Next, the best-performing seaweed was submitted to another experiment to assess its cultivation under two stocking densities (2 gL^{-1} and 4 gL^{-1}), in which both the productivity and nutrient uptake rates were assessed. In both cases, environmental variables were also monitored.

Methods

Experiment 1: Shrimp and mullet

This study was conducted at the Marine Aquaculture Station (EMA) from Institute of Oceanography of Federal University of Rio Grande - FURG, located at Cassino Beach in Rio Grande, RS, Southern Brazil. Juvenile mullet used in this experiment were produced at the Laboratory of Marine Fish Culture from the Federal University of Santa Catarina (Brazil) accordingly to the protocol described in Cerqueira et al. (2017). Prior to the experiment, juveniles (8.8 ± 2.1g) were acclimated in the BFT system for two weeks. Juvenile shrimp (1.2 ± 0.52g) were reared in a greenhouse using the BFT system at the Laboratory of Shrimp Culture (FURG). This experiment was approved by the FURG's Ethical and Animal Welfare Committee (Process number 23116.005895/2016-42).

Systems and experimental design

The experiment lasted for 31-days. The experimental design consisted of three treatments in triplicate (3×3): shrimp monoculture (MONO), IMTA single tank (SING) -shrimp and mullet cultured in the same tank; and IMTA multi tank (MULT) - shrimp and mullet cultured in different tanks. Stocking densities were the same for all treatments: 204 shrimps m^{-3} and 100 mullets m^{-3}, however the number of animals was increased for MULT to deal with the larger water volume in the system (two tanks for each replicate, one tank for mullet and one tank for shrimp) (Figure 5.2.a). In this last treatment, the water from the mullet tanks was constantly circulated to the shrimp tanks (flow rate 720Lh^{-1}) with the aid of a pump (Sarlo Better®, SB 1000C, Brazil, 13W) and returned to the mullet tanks by gravity. Experimental tanks had bottom area 0.36 m^2 and water volume 220L). Water was constantly aerated through air stones, in order to aid the suspension of the bioflocs and to keep adequate dissolved oxygen concentration. Water temperature was kept constant using heaters with thermostats (Stealth, ETP250, USA, 250W).

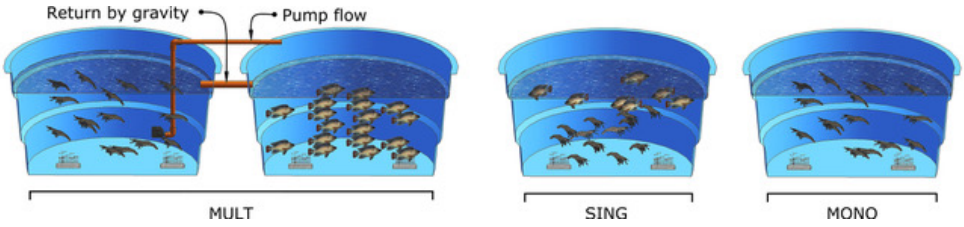

Figure 5.2.a: Diagram of the experimental units in the diferentes treatments, monoculture (Monoculture of L. vannamei); IMTA ST (Integrated culture of L. vannamei and M. liza in same tanks); and IMTA DT (Integrated culture of L. vannamei and M. liza in different tanks). Illustration: Everardo Sousa

Biological material and feeding

The experimental units were stocked with mature bioflocs corresponding to 20% of the volume of the experimental tank (initial concentration ± 80 mg L^{-1} of TSS). All animals were fed on commercial dry diets, during the first week with Potimar 40J with a 40% crude protein and later on they were fed on Potimar 38 Active with a 38% crude protein (1.6 mm), both produced by Guabi Nutrição e Saúde Animal S.A. (Brazil). Shrimp were fed twice a day (9:00 a.m. and 5:00 p.m.) following the methodology described by Jory et al. (2001), while mullets were underfed (once a day with 1% of fish biomass, stimulating the mullet to feed on the bioflocs) throughout the experimental period.

Water quality analyses and management

Dissolved oxygen, temperature and pH were monitored twice a day prior to feeding the organisms. Temperature and dissolved oxygen were measured using an oxymeter (YSI, model Pro-20, USA) and pH with a pH meter (Mettler Toledo, FEP20, Brazil). The salinity was verified with an optical refractometer (ATC, RTP-20ATC, Brazil).

Total ammonia nitrogen (TAN) and nitrite were measured daily according to the methodology described by UNESCO (1983) and Bendschneider and Robinson, (1952), respectively. Turbidity and TSS were analyzed at 4-day intervals. Turbidity was measured by reading in a portable turbidimeter (Hach®, 2100P, Portugal) and TSS following (APHA, 2017). Alkalinity was measured twice a week (APHA, 2017). Nitrate and ortophosphate were measured once a week, following methodologies of Aminot and Chaussepied (1983). Alkalinity and pH were corrected with hydrated lime [Ca $(OH)_2$] accordingly to Furtado et al., (2014). Organic fertilizations with molasses occurred whenever the concentrations of TAN exceeded 1mgL^{-1}, according to Avnimelech (1999) and Ebeling et al., (2006). Salinity was maintained at 21.

Growth performance

Shrimp growth was observed weekly, while for fish the weighings were performed at the beginning, middle and end of the experiment. using a digital scale with 0.01g precision (Marte Científica, AD 2000, Brazil). Before mullets were weighed, they were anesthetized on a benzocaine bath (50 mgL^{-1}) as described by Braz et al. (2017). At the end of the experimental period, all remaining animals (shrimp and fish) and system (shrimp plus fish) were counted for the determination of survival. The parameters analyzed were:

Survival (%) = (final number/ initial number) ×100;

Final mean weight (g): final weight of animals (g) / total number of animals;

Total biomass (g): ∑ final weight of animals (g);

Weekly growth gain (g $week^{-1}$): ((final mean weight (g) – initial mean weight (g)) / weeks of culture;

Apparent Feed conversion ratio (FCR) = offered feed (g)/(final biomass (g) - initial biomass (g));

Productivity (kg m^{-3}): [(final biomass (kg) – initial biomass (kg)) x 1000] / useful tank volume (L).

For system productivity calculations the total volume considered in the MULT treatment was 0.44m^3 and the total volume considered in the SING treatment was 0.22m^3;

Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA). Homoscedasticity and normality were analyzed by the Tests of Levene and Kolmogorov–Smirnov respectively. Tukey's test was applied when significant differences were detected ($P<0.05$). Survival results were arcsine transformed prior to the analysis (Zar, 2010).

Experiment 2: Shrimp and *Ulva* Spp.

The two experiments were conducted at the Marine Shrimp Laboratory (LCM), which is part of the Department of Aquaculture of the Federal University of Santa Catarina (UFSC).

Seaweed from different locations

U. fasciata was collected from Prainha da Barra da Lagoa (approx. 27° 34' 26.2" S, 48° 25' 15.1" W), while *U. ohnoi* was collected from a set of brackish water lagoons near LCM (approx. 27° 34' 57.2" S, 48° 26' 32.4" W). They were rinsed with water of suitable salinities (33gL^{-1} seawater in the case of the former and 25gL^{-1} brackish water for the latter) to remove associated fauna, flora and detritus, followed by stocking in 800 L tanks (useful volume) filled with the respective salinities for acclimation inside a greenhouse under natural irradiance. Daily, the water was renewed, and the salinity of the brackish water unit was adjusted to 33gL^{-1} at the rate of 1gL^{-1} per day. The tanks were equipped with 800W heaters, and the temperature was adjusted to 25 °C at the rate of 1°C per day.

Both species were evaluated in an experiment conducted in a completely randomized design in triplicate, lasting from 4th June 2019 to 25th June 2019. Experimental units consisted of U-shaped tanks with 60L of useful volume for the macroalgae culture, equipped with 100W heaters to maintain temperature at 25°C and two air stones connected to a blower aeration system to maintain water circulation and adequate dissolved oxygen concentrations at night. The units were placed inside a greenhouse under natural irradiance. During the experimental period, the approximate average daily sunlight duration was 10.4 ± 0.0h, based on data obtained through the National Oceanic and Atmospheric Administration (NOAA) Solar Calculator (https://www.esrl.noaa.gov/gmd/grad/solcalc/calcdetails.html).

Initially, 15L of water (34.5 ± 1.5gL^{-1}) from a shrimp biofloc rearing unit was filtered through a bag- type filter and mixed with 45L of seawater (33gL^{-1}) in each of the 60L tanks for a final salinity of approximately 33gL^{-1}. The seaweeds (199.57±8.30g) were then stocked and cultivated under a density of 3.3gL^{-1}. Weekly, tank water was discarded, and the same dilution procedure was performed. During this weekly procedure, biofilm present on the tanks was removed and the algae were also screened for adhered organisms.

Water temperature, dissolved oxygen concentration and illuminance were measured twice daily using an oximeter (YSI Pro20) and a digital luxmeter (Hikari HLX-881A). The values of illuminance (lux) were then converted to quantum irradiance (µmol photons m^{-2} s^{-1}) by multiplying them by 0.018 (Gensler, 1986).

Total ammonia nitrogen (TAN) (Grasshoff et al., 1983), nitrite (NO_2^-) (Strickland and Parsons, 1972), nitrate (NO_3^-) (APHA et al., 2005), orthophosphate (PO_4^{3-}) (APHA et al., 2005), alkalinity (APHA et al.,

2005), pH (pHmetro Tecnal®) and salinity (Eco-Sense YSI EC30) were measured once a week. The water samples were collected immediately after the weekly biofloc dilutions.

Macroalgae were weighed weekly until harvest after gently squeezing to remove excess water. Growth performance was assessed through the following variables:

$$Biomass\ gain\ (g) = W_t - W_0$$

$$Specific\ growth\ rate\ (SGR)\ (\%\ wet\ weight\ day^{-1}) = \left[\left(\frac{W_t}{W_0}\right)^{\frac{1}{t}} - 1\right] * 100$$

$$Yield\ (g\ wet\ weight\ m^{-3}\ day^{-1}) = \frac{\left[\frac{(W_t - W_0)}{t}\right]}{V}$$

where W_t and W_0 are the final and initial weights, respectively, t is the experimental period in days, and V is the unit volume in m^3. The specific growth rate was calculated according to Yong et al. (2013).

Different algae densities

U. ohnoi specimens were collected again from a set of small lagoons near LCM, rinsed with brackish water to remove associated fauna, flora and detritus, and stocked in 800L tanks (useful volume) in an acclimation room under a 12/12h photoperiod. Daily, the water was renewed, and salinity was gradually raised from 25gL^{-1} to 33 L^{-1} at the rate of 1gL^{-1}. The tanks were equipped with 800W heaters to allow an increase in temperature to 28.5°C at the rate of 1°C per day.

Two densities (2gL^{-1} and 4gL^{-1}) of *U. ohnoi* were evaluated in an experiment conducted in a completely randomized design in quadruplicate, which took place between 26th September 2019 and 16th October 2019 (Figure 5.2.b). Experimental units were the same as those employed in the aforementioned experiment. The average sunlight duration during the experimental period was 12.5 ± 0.2h, based on data obtained through the NOAA Solar Calculator.

Initial seaweed biomass was 120.38±0.24g and 240.85±0.34g for the 2gL^{-1} and 4gL^{-1} treatments, respectively. They were stocked in the 60L tanks after an initial dilution. The first and then weekly dilutions were performed as in the first experiment.

Environmental variables were monitored as in the first experiment, the difference being the addition of total suspended solids (TSS) (APHA et al., 2005), which were evaluated weekly. Algae growth was assessed as in the first experiment.

In addition to the water samples collected right after the dilution, water samples were also collected right before the new dilution for the analysis of TAN (Grasshoff et al., 1983), nitrate (APHA et al., 2005) and orthophosphate (APHA et al., 2005). The results of these analyses were not used in the environmental monitoring tables, as they reflected conditions already affected by the different treatments. Instead, they were used for the result of nutrient uptake rates.

Figure 5.2.b: Experimental design used for the evaluation of two stocking densities of Ulva ohnoi (2 and 4gL^{-1}) using effluent from a biofloc shrimp rearing unit.

Statistical Analysis

All data were submitted to Shapiro-Wilk and Levene tests to assess normality and homoscedasticity, respectively. Percentage data were arcsine transformed before the normality assessment. When both assumptions were met, the Student´s t-test was employed. When the equality of variances assumption was not met, the Welch's t-test was performed. Otherwise, if the normality assumption was not met, the non-parametric Mann-Whitney U-test was used (Zar, 2010). Results were considered statistically significant when p<0.05. Data analysis was performed using *jamovi* software (Version 1.0) (The jamovi project, 2019).

Results

Experiment 1: Shrimp and mullet

Water temperature, dissolved oxygen, pH, alkalinity, salinity, TAN and orthophosphate showed no differences (P> 0.05) between treatments. On the other hand, nitrite concentrations were significantly higher (P <0.05) in SING and MULT treatments compared to monoculture treatment. While nitrate concentration was higher (P <0.05) in SING treatment compared to monoculture and MULT treatment (Table 5.2.a).

Table 5.2.a: Water quality parameters (mean ± standard deviation) during the experiment in the different treatments: shrimp monoculture (MONO), IMTA single tank (SING) -shrimp and mullet cultured in the same tank; and IMTA multi tank (MULT) - shrimp and mullet cultured in different tanks. Different letters in the same line represents significant differences (P<0.05)

among treatments after One-way ANOVA followed Tukey's Test. DO (dissolved oxygen), TSS (Total suspended solids), TAN (total ammonia).

	Monoculture	SING	MULT
TemperatureºC	28.05± 0.58	27.84±0.66	28.62±0.61
DO (mgL^{-1})	6.15±0.35	6.18±0.35	6.09±0.34
pH	8.15± 0.15	8.14±0.11	8.22±0.12
Alkalinity (mg $CaCO_3L^{-1}$)	124±11	120±10	131±9
TSS (mgL^{-1})	$190.28±103.32^a$	$142.11±63.69^b$	$89.79±18.32^c$
Turbity (NTU)	$88.04±60.94^a$	$71.54±42.59^b$	$40.9±14.14^c$
TAN (mgL^{-1})	0.07±0.06	0.08±0.13	0.05±0.03
NO_2^--N (mgL^{-1})	$0.07±0.04^a$	$0.44±0.23^b$	$0.48±0.35^b$
NO_3^--N (mgL^{-1})	$13.15±12.22^a$	$18.27±14.19^b$	$12.55±9.36^a$
PO_4^{-3}(mgL^{-1})	1.34±0.85	1.75±1.33	1.94±1.69

Total suspended solids

Throughout the trial period, TSS and turbidity values were higher ($P<0.05$) in monoculture treatment when compared to SING and MULT procedures, with lower values in MULT treatment compared to the other levels (Figure 5.2.c).

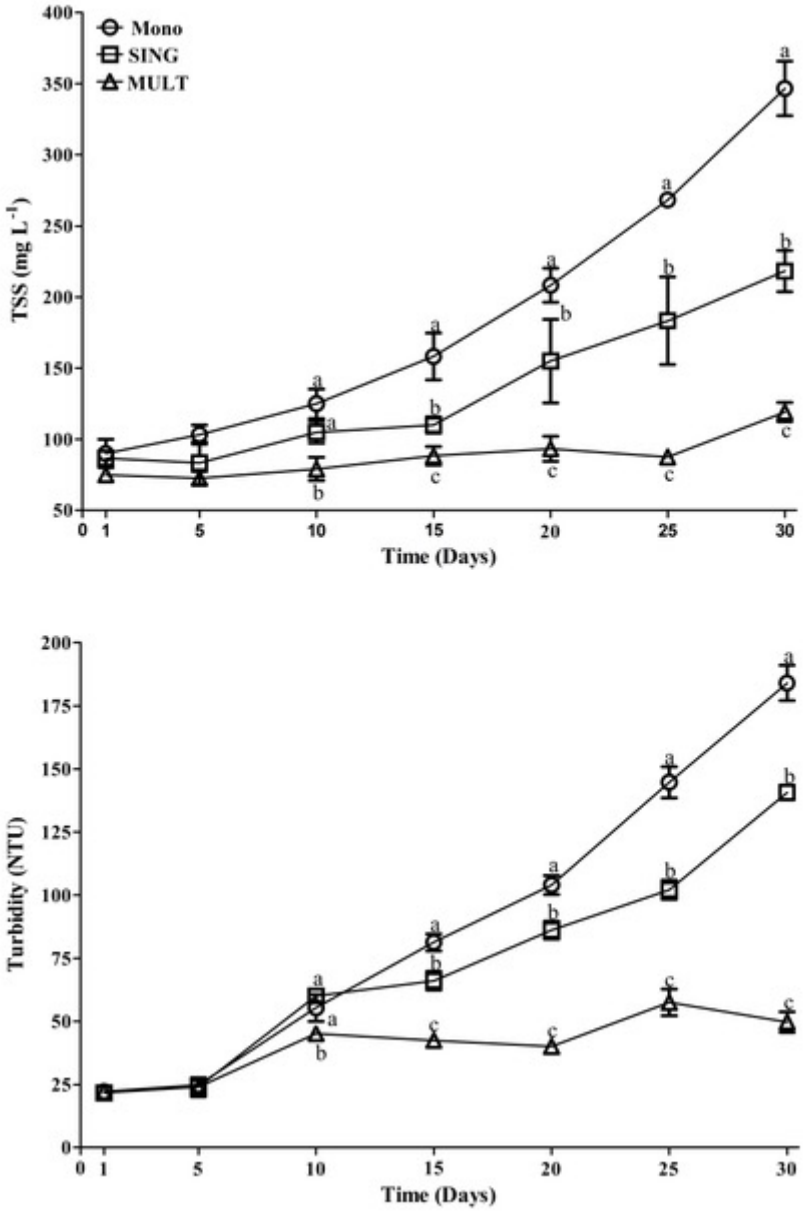

Figure 5.2.c: Mean concentrations of TSS (mg L^{-1}) and turbidity (NTU), over the 31-day experimental period, in the different treatments: monoculture (Monoculture of L. vannamei); IMTA ST (Integrated culture of L. vannamei and M. liza in same tanks); and IMTA DT (Integrated culture of L. vannamei and M. liza in different tanks). Data are presented as mean ± standard error (n = 3).

Zootechnical performance

Survival of shrimp and fish did not show significant differences (P>0.05) among treatments. All zootechnical performance parameters were lower in the SING treatment when compared to the monoculture and the MULT treatments (Table 5.2.b). Final shrimp biomass was significantly higher (P<0.05) in the treatment MULT in comparison to the monoculture and the SING treatments. The growth and final weight of the shrimp in the MULT treatment was equal to that of the monoculture treatment, but the zootechnical performance of *L. vannamei* was impaired in the SING, in which the final weight was twice lower (Table 2), proving that the integrated cultivation of *L. vannamei* and *M. liza* in the same tank, under the experimental conditions tested, impairs the zootechnical performance of the shrimp. On the other hand, as the mullets showed a growth of 7.17g in the SING treatment and 2.27g in the MULT treatment during the 31 days of experiment, once again demonstrating the mullet

consumption of bioflocs. Legarda et al. (2019) observed a similar result in an integrated cultivation of *L. vannamei* and *M. liza* in BFT system, where mullets grew 5.53g in 53-days of experiment being also fed with 1% of biomass and thus consuming the system bioflocs.

Table 5.2.b: Zootechnical performance (mean ± standard deviation) of L. vannamei and M. liza during the experiment in the different treatments: shrimp monoculture (MONO), IMTA single tank (SING) - shrimp and mullet cultured in the same tank; and IMTA multi tank (MULT) - shrimp and mullet cultured in different tanks. WWG: Weekly growth gain; FCR: Apparent feed conversion rate. Different letters in the same line represents significant differences (P<0.05) among treatments after One-way ANOVA followed Tukey's Test.

	MONO	SING	MULT
Shrimp performance			
Survival (%)	84.44±2.22	95.56±5.88	96.67±4.71
Mean final weight (g)	4.51±0.22^{a}	2.31±0.25^{b}	4.87±0.6^{a}
FCR	1.8±0.09^{a}	1.48±0.05^{b}	1.42±0.05^{b}
WWG (gweek $^{-1}$)	0.83±0.06^{a}	0.28±0.06^{b}	0.92±0.15^{a}
Final biomass (kg)	0.17±0.05^{b}	0.10±0.01^{c}	0.21±0.02^{a}
Yield (kgm^{-3})	0.77±0.02^{b}	0.44±0.02^{a}	0.52±0.03^{a}
Mullet performance	-		
Survival (%)	-	92.59±3.21	100±0
Mean final weight (g)	-	15.97±0.62^{a}	11.07±0.23^{b}
FCR	-	-	1.25±0.12
WWG (g week $^{-1}$)	-	1.79±0.15^{b}	0.57±0.06^{a}
Final biomass (kg)	-	0.27±0.01^{a}	0.20±0.04^{b}
Yield (kgm^{-3})	-	1.2±0.05^{a}	0.5±0.01^{b}
Shrimp plus Mullet			
Total final biomass (kg)	0.17±0.05^{c}	0.36±0.05^{b}	0.41±0.01^{a}
Total yield (kgm^{-3})	0.77±0.02^{c}	1.65±0.02^{a}	1.03±0.02^{b}
FCR	1.8±0.09^{b}	1.48±0.05^{a}	1.37±0.03^{a}

Experiment : Shrimp and Ulva

Significant differences for the environmental variables evaluated were observed only for pH in the first experiment and temperature for the second one, with the higher values being observed in the *U. ohnoi* and 2gL^{-1} groups, respectively (Table 5.2.c). Regarding the growth performance, *U. fasciata* exhibited a decrease in biomass, resulting in a significant lower final biomass when compared to *U. ohnoi* (Table 5.2.d). In the assessment of the two stocking densities of *U. ohnoi*, statistically significant differences were observed for final biomass and specific growth rate, in which the higher value occurred for algae cultivated in the 4gL^{-1} and 2gL^{-1}, respectively (Table 5.2d). Finally, no significant differences for the nutrient uptake rates were found (Table 5.2.e).

*Table 5.2.c: Environmental variables monitored throughout the two three-week experiments evaluating algae species (Ulva fasciata and Ulva ohnoi) and algae density in the culture of U. ohnoi when employing water from a biofloc system as fertilizer. Data presented as mean±standard deviation. *Statistically significant. ‡Welch's t-test. †Mann-Witney U-test. When symbol absent, Student's t-test. TAN: Total ammonia nitrogen. TSS: Total suspended solids.*

Variables	Seaweed species		*p*-value	Different densities (*U. ohnoi*)		*p*-value
	U. fasciata	*U. ohnoi*		2 g L^{-1}	4 g L^{-1}	
Dissolved oxygen (mgL^{-1})	6.71±0.60	6.73±0.62	0.831	5.88±0.48	5.92±0.54	0.559†
Temperature (˚C)	23.8±3.0	24.0±3.1	0.539†	30.3±1.4	29.9±1.4*	0.005†
Photon irradiance (µmol photons m^{-2} s^{-1})	58.5±44.8	92.0±90.7	0.103†	85.7±73.4	83.7±70.4	0.818†
TAN (mg L^{-1})	0.20±0.17	0.22±0.18	0.691†	0.10±0.23	0.13±0.13	0.182†
NO_2^- (mg L^{-1})	0.12±0.12	0.07±0.05	0.243‡	0.08±0.04	0.06±0.05	0.200†
NO_3^- (mg L^{-1})	3.4±1.0	2.2±1.2	0.257	7.56±1.83	6.33±1.37	0.148
PO_4^{3-} (mg L^{-1})	1.06±0.08	0.87±0.18	0.168	0.27±0.04	0.24±0.03	0.080†
pH	8.20±0.03	8.26±0.05*	0.035	8.64±0.21	8.54±1.46	0.057†
Alkalinity (mg $CaCO_3$ L^{-1})	159±9	166±18	0.329‡	125±15	127±16	0.620†
Salinity (g L^{-1})	34.3±0.5	34.4±0.3	0.267†	34.0±0.2	34.1±0.3	0.341
TSS (mg L^{-1})	-	-	-	173±46	155±38	0.194†

*Table 5.2.c: Growth performance variables assessed throughout the two three-week experiments evaluating algae species (Ulva fasciata and Ulva ohnoi) and algae density in the culture of U. ohnoi when employing water from a biofloc system as fertilizer. Data presented as mean±standard deviation. *Statistically significant. Student´s t-test used in all instances. SGR: Specific Growth Rate. †There was no growth, no biomass gain and no yield.*

Variable	Seaweed species		*p*-value	Different densities (*U. ohnoi*)		*p*-value
	U. fasciata	*U. ohnoi*		2 g L^{-1}	4 g L^{-1}	
Final biomass (g)	189.0±74.6	379.6±51.7*	0.022	301.9±24.8	378.8±53.0*	0.039
Biomass gain (g)	-†	182.5±50.3	-	181.5±24.9	137.9±53.1	0.188
SGR (%day^{-1})	-†	3.0±0.6	-	4.3±0.4	2.7±0.7*	0.006
Yield (g m^{-3} day^{-1})	-†	138.3±38.1	-	137.5±18.8	104.5±40.2	0.188

Table 5.2.d: Nutrient uptake rates assessed in a three-week experiment evaluating algae density in the culture of Ulva ohnoi when employing water from a biofloc system as fertilizer. Data presented as mean±standard deviation. Student's t-test used in all instances. TAN: Total ammonia nitrogen.

Variables	$2gL^{-1}$	$4gL^{-1}$	p-value
TAN uptake efficiency (%)	77.71±26.89	83.79±16.26	0.712
NO_3^- uptake efficiency (%)	58.40±10.97	44.64±18.81	0.253
PO_4^{3-} uptake efficiency (%)	96.78±1.64	94.54±2.10	0.145

Discussion

Experiment 1: Shrimp and mullet

All water quality parameters were all within the ideal range for cultivation of *L. vannamei* (Van-Wyk and Scarpa, 1999) and *M. liza* (Sampaio et al., 2002; Legarda et al., 2019). Concentration of TSS for shrimp culture in a BFT system should be maintained between 100 and $300mgL^{-1}$ (Gaona et al. 2017). In this study, the maximum concentrations of TSS remained below $300mgL^{-1}$ in the treatments with mullets, while when shrimp was raised in monoculture, TSS achieved $350mgL^{-1}$. A small amount of feed (1% of mullet biomass / day) offered to fish favored biofloc consumption in both treatments, especially in MULT, where fish were kept in part of the shrimp. In the SING treatment, in addition to consuming an offer action, customers compete for participation in the shrimp, or reduce the biofloc consumption by bowls in this treatment and impair the shrimp's growth performance.

Ekasari et al. (2014) observed in an integrated system composed of *L. vannamei*, *O. niloticus* and *Perna viridis*, in which the three species have consumed flakes in different manners and sizes, contributing to the reduction of TSS in the BFT system. Shpigel et al. (2016) used the mullet *M. cephalus* in an integrated culture with *Sparus aurata*, in which the mullets remained 284-days feeding only on the mud generated by *S. aurata* culture, reducing the amount of mud by 98% and obtaining survival values of 82%. The present study demonstrated that *M. liza* can consume bioflocs and can be used in IMTA with *L. vannamei* in separate tanks MULT. It was also observed by Larson and Shanks (1996) or by the consumption of microbial flakes by *M. liza* and *M. curema* demonstrating an economy of mullet consumed by the BFT system biofloc and thus acting as a filter for IMTA with *L. vannamei.*

Zootechnical performance

The ratio biomass of 2:1 shrimp and mullet to SING treatment did not affect *L. vannamei* survival, but mullet consumed a large portion of the shrimp feed, impairing the shrimp growth. Integrated culture of *L. vannamei* with mullet *M. liza* in ponds showed the same interaction between this species, impaired shrimp growth and favored mullet growth (Costa et al. 2013). This same pattern was observed by Hoang et al. (2018) in treatments with 20 and 30% *M. cephalus* mullet density in relation to shrimp density: an increase in shrimp FCR was noticed in comparison to shrimp monoculture and to cultures with lower mullet density, suggesting there was competition for food, which determined poor shrimp performance in these treatments. In an integrated culture of *Litopenaeus schmitti* and *Mugil curema*, in BFT system, in which 10:1 (50 shrimp m^{-2} and 5 fish m^{-2}) and 5:1 (50 shrimp m^{-2} and 10 fish m^{-2}) shrimp: mullet ratios were used, losses on shrimp growth were identified when compared to *L. schmitti* reared alone (Melo et al., 2016). The authors also reported competition for food and observed stress signs for *L. schmitti*, because it was observed that the shrimp were not near the wall of the tanks, or that it may characterize a stress caused by the presence of mullets.

The apparent feed conversion rate of the shrimp was significantly higher in the treatments where fish were present when compared to the monoculture treatment ($P < 0.05$) but it was within the range normally found for *L. vannamei* cultivation (Krummenauer et al., 2016 Lara et al., 2017; Legarda et al., 2019). The mullets showed an apparent feed conversion (FCR) of 1.25 ± 0.12 in the MULT treatment,

whereas in the SING treatment it was not possible to calculate the actual apparent feed conversion of the fish, as the mulls also consumed the offered ration for the shrimp. The FCR value of mullets in the MULT treatment was similar to that reported by Legarda et al. (2019) (FCR = 1.34 ± 0.12), the authors also comment that the feed conversion of mullets in a traditional system exceeds up to 4 times the value of FCR found in their study.

Shrimp yield was higher in the monoculture treatment (0.77 ± 0.02kgm^{-3}) when compared to the SING (0.44 ± 0.02kgm^{-3}) and MULT (0.52 ± 0.02kgm^{-3}) treatments. In MULT treatment the lower productivity was already expected, since the shrimp had its zootechnical performance impaired by the presence of the mull in the same tank, while in the MULT treatment, as the total volume of the system (shrimp + mullet) of 0.44m³ was considered. However, productivity decreased, if we consider the shrimp tank volume as 0.22m³, this productivity doubles, with no difference between monoculture.

Land-based IMTA are mostly closed systems, such as the one proposed in this study, which allows the control of nutrient-rich waste, thus decreasing the negative environmental impacts of aquaculture (Chopin et al., 2001; Kerrigan and Suckling, 2016). Coupling BFT system – which by itself uses minimal or no water renewal and, hence, generates low effluent discharge into the environment – to IMTA, can turn aquaculture more environmentally friendly. In this system, mullet consumed excess TSS in the BFT system, which transforms this residue into animal protein with low feed costs and still provides a marketable by-product to the farmer.

Experiment 2: Shrimp and ulva

In the first experiment, both species were subjected to similar environmental conditions, as demonstrated by the lack of statistical significance. The pH value was the exception, albeit the difference was numerically small. For the second experiment, a significant difference was observed, but only for temperature, which was again a numerically small difference. When comparing the two experiments, the rise in temperature observed in the second one was due to higher air temperatures and longer sunlight duration occurring in the months of September and October compared to June. As regards the differences in the concentrations of nutrients, they were a result of variations in the water quality of the biofloc shrimp tanks from which the water was collected. It is difficult to discuss potential differences in the growth performance of *U. ohnoi* when comparing the two experiments due to these variations and to the different stocking densities employed. Possible effects of nutrient concentrations and water temperature on its culture should merit specific studies.

In the literature, water quality values within the range observed in this study for the cultivation of *Ulva* species were reported for dissolved oxygen, temperature, pH and salinity (Khoi and Fotedar, 2011; Mantri et al., 2011; Zou, 2014; Ge et al., 2018), as well as irradiance (Fortes and Lüning, 1980; Sand-Jensen, 1988; Ruangchuay et al., 2012). The concentrations of TAN, nitrite, nitrate, and orthophosphate were also within the range found in other studies (Khoi and Fotedar, 2011; Ge et al., 2018). Alkalinity remained above 100mgL^{-1}, which is recommended to improve the availability of inorganic carbon for algae (Oca et al., 2019).

When comparing the growth of the two species, *U. ohnoi* showed significantly higher values for all variables evaluated. These results agree with previous assays performed in our laboratory in which *U. fasciata* collected from the sea did not grow well when cultured using the same methodology as described in the Material and Methods of this work for the first experiment (Unpublished data). In fact, a decrease in algae biomass was observed, similar to the case of the present study. Perhaps seaweeds growing in the lagoon were more adapted to culture conditions. For instance, growing under different environmental conditions in their natural habitats, such as stagnant water, as opposed to the wave-exposed algae collected at the beach, could have been the cause of the impaired growth. In fact, even

intraspecific morphological variation is observed for wild algae populations subjected to different environmental conditions, such as wave exposure (Bociąg et al., 2013). Different morphologies can then affect nutrient uptake rates (e.g., Raven and Taylor, 2003). We also noted that, in the experimental units, *U. ohnoi* remained closer to the water surface than *U. fasciata*. Considering that light intensity is attenuated as water depth increases, that fact may have played a role in the differing performances. Another explanation could be related to the water temperatures to which each species was adapted. It is known that this environmental variable affects the growth rate of seaweeds, with different species exhibiting different optimal temperatures for maximum growth (Mantri et al., 2011; Nakamura et al., 2020). *U. ohnoi* collected in the lagoons could have been more adapted to the warmer water temperatures occurring in the greenhouse, in contrast to *U. fasciata* collected from the sea.

In the second experiment, the final biomass was significantly higher in the stocking density of $4gL^{-1}$ of *U. ohnoi*; however, the specific growth rate was significantly higher in the lower stocking density. This could be explained either by lack of nutrients or by self-shading. However, no significant differences between treatments were observed for the initial concentration of nutrients, or the nutrient uptake rates. Alkalinity, an indicator of inorganic carbon availability, was also statistically equal among treatments. Therefore, a more likely explanation is that of self–shading caused by excessive algae biomass, which could have been caused by the type of aeration system employed, which did not promote an adequate circulation of the algae in the water column. This phenomenon of decreasing growth performance as algae stocking density increases is often observed in the literature. For instance, there was a significant decrease in the growth rate of *Gracilaria tikvahiae* when its stocking density increased from $0.g\ L^{-1}$ to $10L^{-1}$ (Kim and Yarish, 2014). In another study, the algae *Agarophyton vermiculophyllum* exhibited a significant decrease in its specific growth rate when the stocking density increased from $0.2gL^{-1}$ to $2gL^{-1}$ (Shin et al., 2020). In the case of yield, no significant differences were observed. Still, this suggests that a lower stocking density of algal biomass is to be preferred, considering that similar yields are obtained with fewer inputs.

The growth results of both experiments were altogether consistent with data reported for *Ulva* spp., namely *U. fasciata* (Mantri et al., 2011), *U. lactuca* (Khoi and Fotedar, 2011), *U. reticulata* (Msuya et al., 2006; Msuya, 2007) and *U. ohnoi* (Lawton et al., 2013).

Considering that no significant differences were observed in the nutrient uptake rates when comparing the two densities, future studies could assess if higher concentrations of biofloc effluent could produce different results, having in mind that probable higher water turbidities could compromise the growth performance and yield of the macroalgae.

Overall, the results obtained in this work add to previous results showing the feasibility of using water from biofloc rearing units as nutrient utilisation for the culture of macroalgae, in general (Pedra et al., 2017; Shin et al., 2020), and *Ulva* Spp., in particular (Ge et al., 2018).

Progress, deviations, problems & next 12M

Progress: Based on the results obtained and discussed above we consider this task to be complete by about 35%. We executed two experiments. Two more experiments and the evaluation of the prototype remain to be done.

Deviations & Problems: nothing to declare.

Outlook: next tasks are evaluating integration of shrimp and oyster, shrimp and tilapia, shrimp and mullet with different mullet feeding rates and evaluate the protocols in a pilot scale.

CST 5.3 Design, implement and carry out a profit and sustainable integrated pond-based shrimp - seaweed - oyster farming in Northeastern Brazil

Janaina Kimpara, Embrapa

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
5.3	Design, implement and carry out a profit and sustainable integrated pond-based shrimp - seaweed - oyster farming in Northeastern Brazil	T2.2, T3.2	EMBRAPA, UNESP, PRIMAR, FURG	25%	✓	M4	M4	M40	0	5	8

Introduction

Shrimp farming has suffered from the incidence of diseases such as white spot syndrome, which is caused by a virus (Bandeira et al., 2019), and the most recommended treatment for control is adequate production management (Cock et al., 2017). Among management, changes like the decrease of water renewal, treatment of farming water, and mainly the storage density decrease, which generates a low productivity and income on the farms. Integrated multitrophic aquaculture (IMTA) can be a solution to this problem. The IMTA concept describes the integration of aquatic species at different trophic levels in the same cultivation environment (Chopin et al., 2001). In this type of system, residues from the cultivation of one species serve as a source of food or fertilizers for another, imitating the natural ecological process and reducing environmental impacts through efficiency in the use of nutrients and interaction between species (Boyd et al., 2020). IMTA increases profitability, reduces financial risk due to product diversification, and can also increase the acceptability of mariculture by stakeholders (Boyd et al., 2020). The most low-trophic organisms used in marine IMTA are oysters and seaweed.

Methods

Experiment 1

The work was carried out at Primar Aquacultura, located in Tibau do Sul, Rio Grande do Norte, Brazil (6 ° 13'30.2 "S 35 ° 08'19.9" W). The farm is located in an estuarine area, around the Guaraíras lagoon, and features an environment with a high level of muddy sediment at the bottom of the ponds and low transparency in the water column. The farm's production system is organic (low density, with no artificial diet and low water renewal). Shrimp farming is the main activity of the farm, which also counts on the production of oysters in a separate system from the shrimp. This project proposes the integration of shrimp with oysters and macroalgae in an integrated system design. Until the present, three experiments have been carried out. All the licenses for the rearing were obtained.

A completely randomised experiment was carried out in a 3x4 factorial scheme, the first factor was the algae species, with three levels (*Ulva lactuca, Gracilaria caudata,* and *Hypnea pseudomusciformis*) and the second-factor four-level cultivation structures (tray, pillow, tubular net, and cables). All structures were fixed with wooden poles and positioned 10cm from the surface. Each treatment had four experimental units.

The structures were randomly distributed in a ~ 2 ha pond with white shrimp at a density of 4/m². The macroalgae were stored with a density of 5000g/m³ in each structure. The algae cultivation lasted 60 days and the algae were cleaned every two days and the temperature, salinity, pH, dissolved oxygen (DO), and the redox potential (ORP) of the culture were monitored near the seaweed structures. It was evaluated for each treatment: initial mass; final mass; and mass gain.

Results

Experiment 1

During 60 days of cultivation, the water variables did not suffer great variation. In the morning period the temperature was 29 ± 1ºC, the salinity of 36.1 ± 4.4, OD of 3 ±2mg/L, 8.4 ± 0.2, and ORP of 86 ± 25.

In the afternoon the temperature was 33 ± 1ºC, the salinity of 39.5 ± 3.4, OD of 10 ±2mg/L, pH 8.8 ± 0.2, ORP of 99 ± 26, and transparency of 33 ± 7.

The macroalgae had an apparent growth in the first 15-days of cultivation. However, there was an accumulation of particulate matter on the surface of all species, even with the cleaning of algae every two days. All treatments showed loss of biomass, except *U. lactuca*, which grew 88 ± 32g in the structure of pillows. At the end of the culture, all seaweeds presented a lot of fouling organisms and could not be fully cleaned.

Discussion

Experiment showed no adaptation of the three tested seaweeds species to the earthen pond environment. A lot of particulate material accumulate in seaweeds that did not grow and suffered from loss of biomass. Despite the biomass gain of *U. lactuca* in the pillows, they present a lot of biofouling that infeasible the biomass use. In this experiment, we noted the growth of other green filamentous macroalgae on the *Gracilaria*. These macroalgae had not been introduced into the crop and had spontaneous growth on the cultivated seaweed.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained and discussed above we consider this task to be complete by about 25%. We executed one experiment and two more experiments and the evaluation of the prototype are still to be done.

Deviations & Problems: nothing to declare.

Outlook: Next tasks are evaluating oyster and seaweed integration in a shrimp pond and teste different substrates to *Ulva* Spp. spore recruitment

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CS6

Summary of progress report for Case Study **6**

Date of report:	**24.3.2020**
Case Study name:	**Sea Urchin Roe Enhancement**
of relevane for WPs	**5, 6, 7, 9**

Abstract/Summary

In Months 0-12 reasonable progress has been made in developing the sea urchin roe enhancement facility in Stavanger, Norway. URCHINOMICS have built and are in the process of finalising the installation of the holding system that will form a key exploitable result. There have been some unforeseen complications that have been dealt with and progress is continuing, despite the impact of COVID-19 from February 2020.

Unfortunately, the market testing of the first enhanced sea urchin roe at the Brussels Seafood Expo was not possible due to the cancellation of the event but limited testing was still made by URCHINOMICS staff.

In Spain progress on the design and installation of holding systems for sea urchin roe enhancement at the Algafres facility continues after an initial trial showed that existing harvest and transport protocols were not suitable for subsequent roe enhancement of *P. lividus*. Algafres will start a second trial to test revised protocols early in the next reporting period.

There is ongoing cooperation and communication with Canadian institutes and industries developing sea urchin roe enhancement in the north of Canada.

CST 6.1 Development of roe enhancement protocols and holding systems

Responsible CS Task Leader Philip James, Nofima

CST #	Name	Reports to task(s)	Partners involved	complete d by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
1	Development of roe enhancement protocols and holding systems	T2.2, T3.2	Nofima, GMIT	12%	✓	M1	M1	M40	0	4	7

Introduction Norway

Despite a considerable amount of research into roe enhancement of a range of sea urchin species there is very little commercial scale testing of diets, protocols and holding systems. For the cool water species *Strongylocentrotus droebachiensis* there are a number of challenges regarding the harsh environment they must be harvested and transported in. For the more temperate species *Paracentrotus Lividus* there is simply not very much information on the efficacy of roe enhancement of the species. Neither species have tested the efficacy of the latest commercial feed available from URCHINOMICS, particularly in terms of market acceptance. This Tasks aims to develop harvesting, transport and live holding, roe enhancement protocols for both species. It will be split into two geographic sections: Norway and Spain.

Methods Norway

The first task will be for Nofima to define existing roe enhancement protocols for *S. droebachiensis* and distribute these to stakeholders. There are currently no existing protocols for the enhancement of *P. lividus*.

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement No 818173. This report reflects only the authors' view and that the Commission is not responsible for any use that may be made of the information it contains.

URCHINOMICS have developed a land-based tank design and this will be installed and tested at a licensed facility in Stavanger, Norway.

Results Norway

NORWAY activities in M0-12:

- The URCHINOMICS feed (previously untested on either sea urchin species) was produced in Japan and distributed in Norway in July 2019.

Holding system development:

- A site visit to was made to the proposed URCHINOMICS facility in Stavanger in September 2019 (this is one of the few commercial scale facilities in Norway with a license to hold and enhance sea urchins).
- Final design and installation by URCHINOMICS of holding tanks in flow through land-based system. The tank design was based on results of tank test conducted by Nofima Nov/Dec 2019 (Confidential Report, 2019).

Figure 6.1.a: The tipper system (based on the abalone tipper systems) installed at the Stavanger Facility.

- Results of first roe enhancement trial (See Task 6.2) in Tromsø showed a gut parasite was present in the Tromsø populations sampled, but in very small numbers.
- Bio-mitigation measures were therefore required by Mattilsynet (Norwegian Food Authority). The system in Stavanger was redesigned as a RAS system with a mechanical and chemical cleaning of all effluent water as a security measure (Jan-Mar 2020).

COVID 19 impact (February 2020 onwards):

- Limited ability for URCHINOMICS staff to access facility to rebuild RAS system.
- Collection and transport of sea urchins from Tromsø to Stavanger not possible

Discussion Norway

The development of the system in Stavanger was slower than expected due to the additional legal requirements (biomitigation) for *S. droebachiansis*. It has taken langer than expected to get the system up and running which has delayed the initial enhancement trials in the system. This has been further exacerbated by the impact of COVID 19 which has delayed rebuilds and delivery of sea urchins.

Progress, deviations, problems & next 12M Norway

Progress: Based on the results obtained to date we believe we are approximately 12% complete in the task of system installation and delivery of enhancement protocols. The transport protocols will be investigated further in future trials. The tipper tank system will be a **key exploitable result** once fully tested.

Deviations & Problems: Problems encountered include the need to rebuild system and subsequent delays due to Food safety regulations and COVID delays. We believe this has set the task back between 4-6 months.

Outlook: In the next 12M shipments of sea urchins from Tromsø will resume and enhancement trials in the new system will be conducted and market testing of product will begin.

Methods Spain

Algafres have no previous experience enhancing sea urchins and they will build a test holding system in their facility in Galicia, Spain (based on instructions from GMIT and Nofima) and test its efficacy for sea urchin enhancement. The best-known protocols, system design for roe enhancement of *S. droebachiensis* will be supplied by Nofima for *P. lividus* in Spain.

Results Spain

- The URCHINOMICS feed (previously untested on either species) was produced in Japan was distributed to Spain in July 2019.
- A stakeholder visit in Galicia (as part of a separate funded project) was conducted in July 2019 which facilitated a stakeholder meeting and discuss tank and facility design.

Holding system development

- Recommendations for system design were delivered in August 2019 and the system installed (Aug/Sep 2019)
- A site visit by Nofima/GMIT was conducted in Sep 2019 to do final installation check. Nofima and GMIT monitored the collection of sea urchins and the existing harvesting and transport protocols (currently used by local sea urchin fishermen). Subsequently, Algafres set up their first trial and conducted initial census together with GMIT and Nofima.

Figure 6.1.b: The tank system installed at the Algafres Facility in Galicia (September 2019).

- See T6.3 for initial trial set up and results.

COVID 19 impact (Feb 2020 onwards):

- Severely limited ability for Algafres staff to access facility to maintain and run trial. Limited final census conducted.

Discussion Spain

The development of the system in Galicia was completed on time. The first trial started on time but the development of COVID 19 has had a serious impact on the running of the first trial. Restricting obtaining of results in regard to the efficacy of the systems. The very high mortality data did confirm that the harvesting and transport protocols are not suitable for collection and subsequent roe enhancement of this species.

Progress, deviations, problems & next 12M Spain

Progress: Based on the results obtained to date we believe we are approximately 12% complete in the task of system installation and delivery of enhancement protocols.

Deviations & Problems: Problems encountered include the high mortality in the first trial and the COVID 19 related delays and difficulties in getting a final census conducted (this is reported further in Task 6.3)

Outlook: In the next 12M the system will be redesigned to allow much closer monitoring of individual sea urchins. The protocols for harvesting and transport will be revised and tested.

CST 6.2 Roe enhancement of Strongylocentrotus droebachiensis in Norway

Responsible CS Task Leader Philip James, Nofima

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
2	Roe enhancement of Strongylocentrotus droebachiensis in Norway	T3.2	Nofima (IRG: URCHINOMICS, Statsnail, C Flow)	12%	✓	M1	M1	M40	0	4	8

Introduction

A small-scale trial was set up and run at the Nofima facility in Tromsø utilizing the URCHINOMICS sea urchin feed manufactured in Japan. The aim was to test the new feed and to start an investigation into the impact on roe enhancement of selecting sea urchins from different areas of a fjord or sound. The trial also aimed to produce product for market testing at the Seafood Expo in Brussels in April 2020.

Methods

Sea urchins were harvested from three sources within a single sound (within 5km of each other) and held in individual compartments in the standard Nofima experimental raceways. This allows very close monitoring of individuals, in regard to feed activity, gonad development and mortalities during enhancement trials. Three separate raceways were used with 20 sea urchins from each source population in each raceway (each raceway was considered as a replicate $n = 60$). A fourth raceway was used to enhance sea urchins for the Seafood Expo.

Sea urchins were fed one pellet twice per week and the raceways were cleaned once per week. The trial was intended to run for 10 weeks (February-March). At the beginning of the trial and at the conclusion of the trial the length, wet weight (g) and gonad weight (g) were measured and the GI (%) calculated.

COVID 19 impact:

- The trial ended at 8 weeks rather than 10 weeks due to COVID restrictions at the Nofima laboratory.
- The Seafood Expo in Brussels was cancelled.
- The sample was sent to URCHINOMICS in Ålesund for taste assessment but on a very limited scale.

Results

(Data sheets will be logged with WP4 data management)

GI results were analysed with ANOVA and a post analysis Tukey-Kramer Multiple-Comparison was used to test for differences between individual treatments. There was no mortality recorded during the trial. From the 234 sea urchins sampled in the trial, 4 were found to contain a gut parasite (*Echinomermella matsi*) which has been documented from other areas of Norway previously (Stein *et al*., 1998).

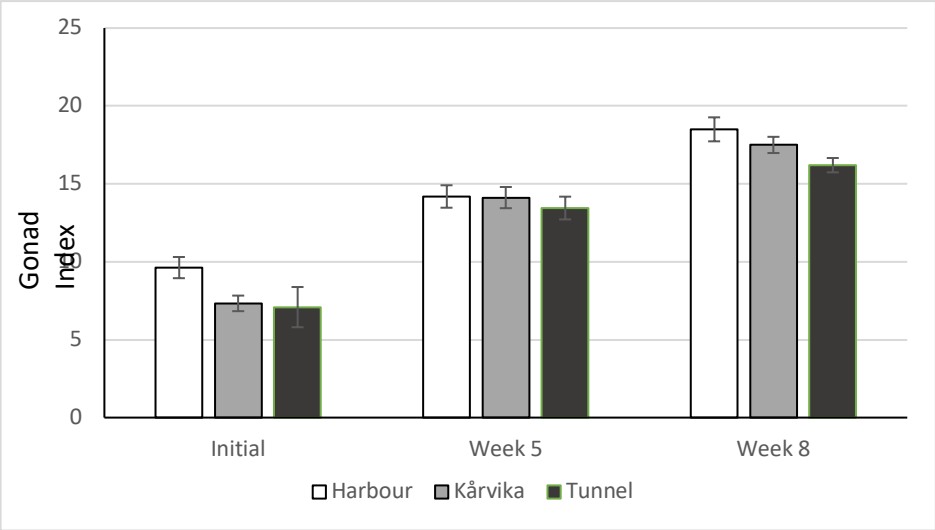

Figure 6.1.c: GI results (±SE) from sea urchins from the three source populations during the 8-week enhancement (Harbor population had a significantly larger GI than the Tunnel population at week 8, no other significant differences) .

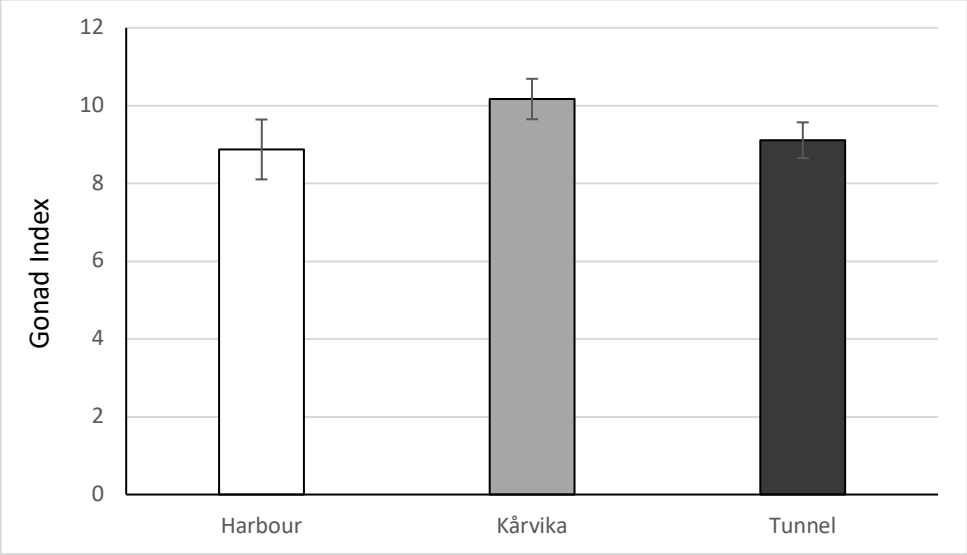

Figure 6.1.d: Increase in GI (±SE) from sea urchins from the three source populations after the 8-week enhancement (no significant differences between treatments).

Discussion

The length of the trial was shorter than expected due to the impact of COVID-19. Despite this in overall GI one of the three populations had a significantly higher GI at the conclusion of the trial. Over the 8-week enhancement period there were no significant differences in the 'increase in GI' but it appears that one of the populations was increasing at a higher rate. It is recommended the impact of source population and season on roe enhancement is investigated further. AquaVitae recommend this is included as part of the synergistic project 'InEVal' (BlueBio ERA-NET Co-fund (H2020 Project number 817992).

The trial did show that the sea urchins fed on the URCHINOMICS feed and produced an increase in roe in line with previous enhancement trial run by Nofima (James *et al*., 2015). A limited report on the taste of the sea urchins was made by URCHINOMICS after the cancellation of the Brussels Seafood Expo. The anecdotal feedback was that that overall, the taste quality was fine. It was noted that the lower yielding urchins had a slightly bitter flavor to them.

The previously undocumented occurrence of the gut nematode *E. matsi* was reported to URCHINOMICS.

Miscellaneous actions in Task 6.2

- URCHINOMICS representative attended AquaVitae kick off meeting in Tromsø in June 2019
- A case study kick off meeting was held in Tromsø in September 2019
  - AquaVitae partners present: GMIT, Nofima and CETMAR
  - Stakeholder (Industry Reference Group): URCHINOMICS, Statsnail AS
  - Discussed project and pre-trial to be undertaken in Tromsø
  - The aim of this trial is to act as a small-scale pre-trial in Tromsø whilst the system build is happening in Stavanger to test URCHINOMICS feed and the impact of source population on enhancement and to produce test product for testing at Seafood Expo in Brussels (April 2020).

Progress, deviations, problems & next 12M

Progress: Based on the results obtained to date we put the %completeness of this Task at 12%. The production of enhanced roe from *S. droebachiensis* will form a **key exploitable result** of this task. Unfortunately, due to COVID-19 the first market testing was cancelled but limited taste testing was carried out by URCHINOMICS.

Deviations & Problems: Problems encountered are the delays in converting the holding system in Stavanger to a RAS system (Task 6.1) and the impact of COVID 19 on both the initial small-scale trial in Tromsø as well as the cancellation of the Seafood Expo in Brussels. For this reason, the Task is considered 'amber' where careful monitoring is required to make sure that the Task continues in a timely manner in the coming reporting periods.

Outlook: In the following 12-months the installation at Stavanger will be completed and sea urchins supplied for enhancement trials. It is intended that product testing will be possible by M24 of the project.

CST 6.3 Roe enhancement and out of season production of Paracentrotus lividus in Spain

Responsible CS Task Leader Colin Hannon, GMIT

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
3	Roe enhancement and out of season production of Paracentrotus lividus in Spain	T3.2	GMIT, Nofima	12%	!	M1	M1	M40	0	3	0

Introduction

There has been limited research on the feasibility of enhancing *Paracentrotus lividus*. This trial was conducted as an initial trial to test the holding system installed in the Algafres facility, the URCHINOMICS feed (which has not previously been tested on this species), and the protocols for harvesting and transport currently used by fisherman in Galicia.

Methods

A draft plan was sent to Algafres in September 2019:

Draft plan for AquaVitae sea urchin roe enhancement trials in Case Study 6

Commercial partner: Algafres (Spain)

Location of trial: Algafres hatchery, A Coruña, Galicia

Research partners: Nofima (Norway), GMIT (Ireland)

Current tank set up:

Algafres have 24 shallow trays available for the sea urchin roe enhancement trial.

- Each tray is 0.47m wide, 3.18m long and 0.15m high.
- We recommend having the water level a maximum of 100mm high in each tank to restrict the
- sea urchins to a single layer
- The available bottom internal surface area of each tank is 1.5m^2
- The adults currently held at the facility had an average spine diameter of 98.6mm per sea urchin

Recommended tanks for trial:

We recommend using 16 tanks, in 4 replicates, randomly allocated the following treatments:

Treatment 1: Spray bar only – fed algae
Treatment 2: Spray bar and tipper – fed algae
Treatment 3: Spray bar only – fed pellets
Treatment 4: Spray bar and tipper – fed pellets

The sea urchin team at Algafres will decide the allocation of the treatments to the experimental tanks for the enhancement trail. These will be randomly assigned to the available tanks so that there is no experimental bias from the position of the tanks or tank effect. This means randomly selecting 4 tanks (replicates) to each treatment.

Spray bar: Consists of a simple manifold with holes drilled to create a spray curtain, which creates a laminar directional flow of water in tanks

Tipper: Consists of a container with a hinge so that as the container is filled with water it reaches a tipping point and empties into the tanks. The container then tips back into position and starts to fill again. The frequency of the tipping can be controlled by the amount of water going into the container. (see attached files showing tipper bucket systems)

Algae feed: A variety of species can be used to feed the sea urchins. The feed amounts should be controlled according to the feed rates of the sea urchins.

Pellet feed: URCHINOMICS will provide 50kg of pellets feed. The feed rate will be based on a rate of 0.5% wet weight/day but will change according to the dietary needs of the sea urchins.

All tanks will be fed twice per week per treatment.

Cleaning

Cleaning should be done twice per week, once by just picking out uneaten pellets and one where the tank is flushed fully.

Sea urchins

Sea urchins will be collected from local populations. Either Colin or Phil will be in A Coruna to assist. The collections and transport protocols are shown in the attached Nofima report.

It is crucial that the sea urchins are not stressed by rough handling, exposure to wind and exposure to high or low temperatures during the transport. This could result in long-term mortalities during the experiment.

- We recommend 100 sea urchins per tank
- Total of 1650 sea urchins (an additional 50 sea urchins for the initial sample)

NOTE: the density in the tanks can be less if this is too many urchins to collect

Water supply

All tanks should be supplied with constant ambient, sand filtered seawater throughout the trial. No air supply will be required.

- Water dissolved oxygen should be measured twice per week
- Water temperature should be measured daily

Timeline for initial trial:

September-October

- Set up holding tanks with flow through water supply

December/January

- Fish approximately 170kg urchins
- Transfer sea urchins alive to holding system at Algafres
- Do an initial sample on 30 sea urchins to assess % GI

January-March

- Roe enhancement period of 8-10 weeks
- Final assessment and market assessment of sea urchin quality

April

- Report back to Algafres

A site visit and harvesting and sea urchin harvesting occurred on 23rd Jan 2020. The first census conducted with Nofima and GMIT staff present on 24th September and the trial began on 27th September 2019.

COVID 19 impact:

- AquaVitae partners GMIT and Nofima were unable to return to the facility in A Coruna to undertake the mid or final census.
- Algafres staff access to facility becomes severely restricted as a result of COVID 19.
- Following on from Trial one there was very restricted access to the facility between March 2020 and September 2020

Results

The mortality results from the roe enhancement trial starting on 27th January 2020 show that 78% of the total mortality occurred in the first two weeks of the trial (Figure 6.3.a). It is common for harvesting and/or transport mortality in sea urchins to occur over a two-week period after the harvesting event (James *et al*., 2015).

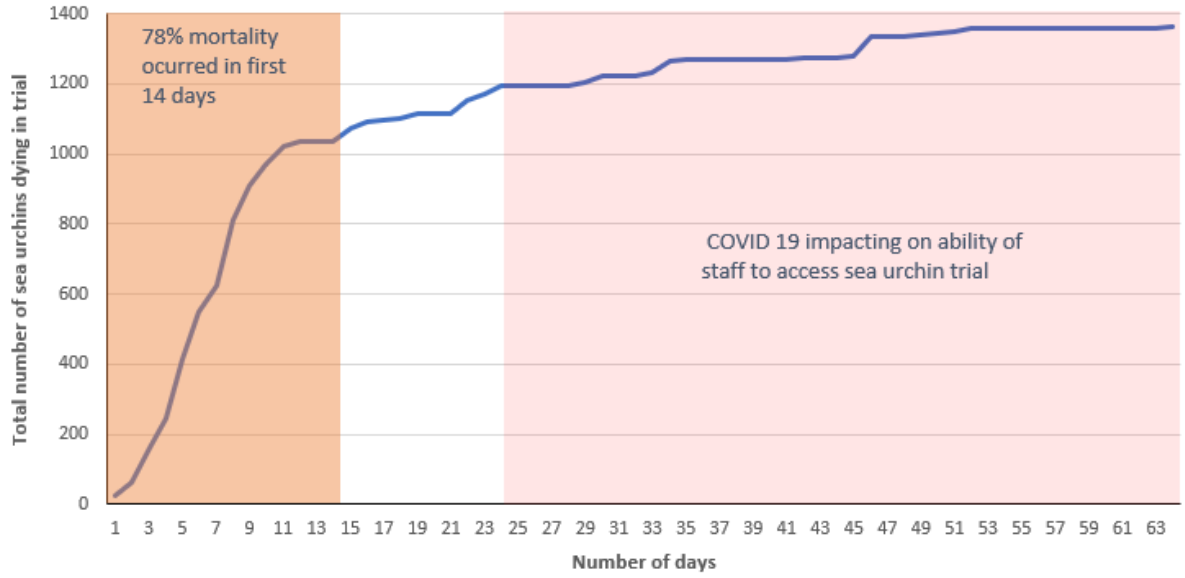

Figure 6.3.a: Cumulative mortality in the first trial at the Algafres facility from January 2020 – March 2020.

Unfortunately, the impact of COVID-19 began shortly after the start of the trial (Figure 6.3.a) and it was very difficult for the staff at Algafres to access the facility for a mid-point census but they did manage to do a late final census on the experiment. Figures 6.3.b and 6.3.c show the Final Gi results of the trial.

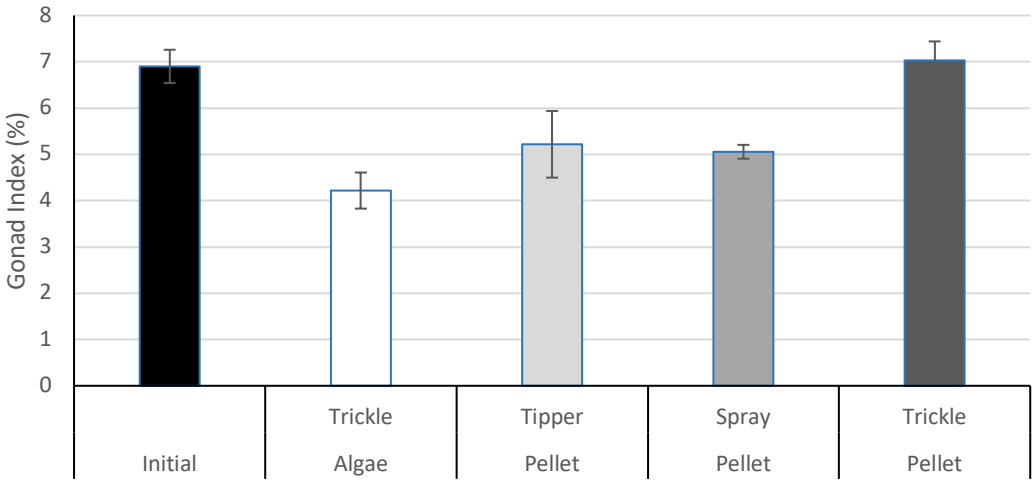

Figure 6.3.b: The initial and final GI (±SE) of the sea urchins held in the Algafres facility from January 2020 – March 2020. The results show the final GI for sea urchins held in different tank treatments.

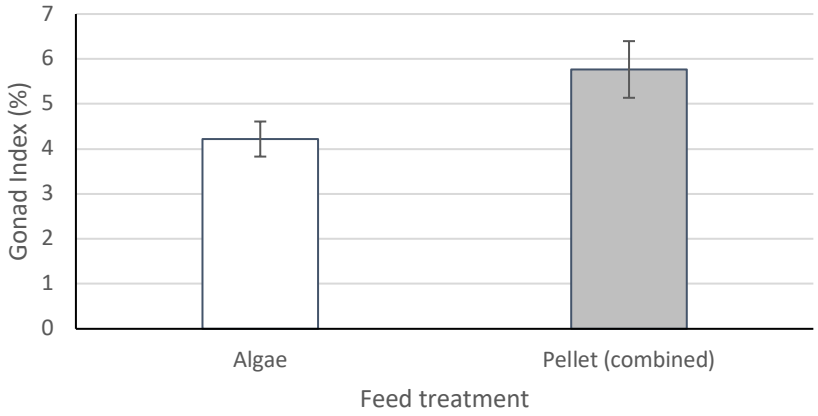

Figure 6.3.c: The final GI (±SE) of the sea urchins held in the Algafres facility from January 2020 – March 2020. The results show the final GI for sea urchins fed macroalage or manufactured feed (pellet).

In addition to the very high initial mortality the initial stress of harvesting and transport to the facility had an extended and detrimental effect on the subsequent enhancement of the sea urchins (Figure 6.3.b). The sea urchins held in the tanks with a simple trickle inlet has significantly higher GI than all other tank treatments but there was no difference between the GI of sea urchins in this treatment at the conclusion of the experiment and the GI of the sea urchins at the initial census. The sea urchins fed on macroalgae had a smaller GI at the conclusion of the experiment than those fed on the manufactured URCHINOMICS feed (Figure 6.3.c).

Discussion

The very high mortality in the first two weeks of the trial showed that the method of harvesting and transport techniques used to traditionally fish sea urchins in the area are not suitable for harvesting this species of sea urchin for subsequent enhancement. Observations during the harvesting and transport showed that the techniques used were not unusually rough (when considering the techniques used for other species of sea urchins), but it was noted that improvements could and should be made in the transport system, particularly to reduce the impact of wind on the sea urchins.

These results indicate that *P. lividus* is particularly susceptible to either harvesting and/or transport stress.

Due to the impact of the initial harvesting stress, it is difficult to make any meaningful conclusions regarding the efficacy of the water inlet system (spray bars/tippers/trickle) or feed type (manufactured URCHINOMICS feed/macroalgae) that the trial intended to test.

After extensive discussions with Algafres a new experiment design was produced to test a variety of harvest and transport methods prior to enhancement. A new holding system was designed to allow Algafres to monitor the results from individual sea urchins instead of from groups. This system has been extensively used by Nofima in the past on *S. droebachiensis* and is their standard experimental tank set up. The design was modified to adjust to the size of the species and the raceways available at Algafres. The refit of the facility will be undertaken in the following reporting period.

Miscellaneous actions in Task 6.2

- Kick off meeting held in Galicia on 21 September 2019
- AquaVitae partners present: GMIT, Nofima and CETMAR
- Stakeholder (Industry Reference Group): Algafres
- Discussed project and first trial to be undertaken in Galicia
- CST 6.3 Initial trial set up: The aim of this initial trial is to test start up protocols and tank systems in Alagfres Facility.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained to date we calculate the % completeness of the CST to be 12%.

The results showed there are major changes required to the harvesting and transport techniques used for sea urchins to be enhanced. This species also appeared to show a clear reluctance to start feeding in the enhancement system but it was uncertain if this was due to the initial stress they were exposed too or whether this was a behavioural characteristic of the species. This must also be considered in future trials.

The **key exploitable result** from this task will be the production of enhanced sea urchin roe from *P. lividus*. Limited progress has been made at this point.

Deviations & Problems: Problems encountered include the impact of harvesting and transport on *P. lividus* and subsequent mortality and reluctance of the species to feed. Potential solutions include a review of harvesting and transport protocols and rebuilding the holding system to be able to test the impact of handling prior to enhancement and the feeding of individual sea urchins during enhancement.

For this reason, the Task is reported as 'amber' where careful monitoring is required to make sure that the Task continues in a timely manner in the coming reporting periods.

Outlook: In the following 12Ms we will produce a plan for testing whether harvesting and transport techniques play a role in high mortality in first trial. A redesign of the holding systems will be implemented to allow testing and further trials will be conducted as soon as COVID-19 restrictions allow.

CST 6.4 Support commercial production of enhanced sea urchin roe in Canada

Responsible CS Task Leader Philip James, Nofima

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
4	Support commercial production of enhanced sea urchin roe in Canada	T2.2	Nofima, GMIT	10%	✓	M1	M1	M48	0	4	0

Introduction

CS task 6.4 aims to support the commercial development and implementation of land-based sea urchin roe enhancement on the East coast of Canada. The AquaVitae project partners planned to create synergies with the research that is underway in Canada and wanted to form a larger international multi-stakeholder group including representatives from Spain, Norway, Ireland and Canada (and South Africa if possible) focused on the commercial development of sea urchin roe enhancement and markets on an international level.

Methods

- not applicable -

Results

- Consultation with Canadian stakeholder (Joint meeting with InEVal held on 29.1.2020)
- Held multiple discussions with the Nature Conservancy in USA regarding sea urchin roe enhancement and harvesting techniques being developed in Norway.

Discussion

COVID 19 impact:

- The inability to travel has made this task difficult.
- The participation of Nofima in the Canadian Aquaculture Conference was cancelled.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained to date we calculate the Task to be 10% complete as ongoing discussions and participation in meetings has taken place, but no physical meetings have been held.

Deviations & Problems: Problems encountered are primarily the result of COVID-19 and the limited activities that have taken place.

Relevant results from both Canada and Norway will continue to be discussed in the following reporting period. The task is considered 'amber' where careful monitoring is required to make sure that the Task continues in a timely manner in the coming reporting periods.

Reference CS6

James, P., J, Siikavuopio, S.I., Mortensen, A., 2015. Sea urchin aquaculture in Norway. In. Echinoderm Aquaculture. Edited by Brown., N., and Eddy, E., John Wiley & Sons Inc, Hoboken, New Jersey. Pp 147-173.

Stien, A., Leinaas, H.P., Halvorsen, O. and Christie, H., 1998. Population dynamics of the *Echinomermella matsi* (Nematoda) - *Strongylocentrotus droebachiensis* (Echinoida) system: effects on host fecundity. MARINE ECOLOGY PROGRESS SERIES, Vol. 163: 193-201.

CS7

Summary of progress report for Case Study 7

Date of report:	**31.03.2020**
Case Study name:	**Sea Cucumber Aquaculture**
of relevane for WPs	**1, 2, 3, 5**

Abstract/Summary

The sea cucumber aquaculture case study (CS7) kicked off in AquaVitae in June 2019 with some fundamental changes – these were related specifically to the nations and partners involved. Where the initial plan will have been to only work in Brazil with sea cucumber, South African partners showed renewed interest in sea cucumber aquaculture and were rapidly recruited to join CS7 activities. On the other hand, activities of FURG Brazil did not receive specific CS7 funding, and successful efforts were made at the kick-off meeting to establish a suitable UNESP / EMBRAPA Student led set of experiments in Brazil.

The methodological development of tasks 7.1 and 7.2 was agreed with South African and Brazilian partners. In the case of Brazil, a set of Skype meetings was held with Janaina Kimpara (EMBRAPA) and Karina Ribeiro (EMBRAPA/Universidad Federal Vicosa) along with email correspondence. The species selection was rapid as only one detritivore species is available in the Rio Grande do Norte (RN) coastal region – Holothuria Grisea. An analysis of the existing literature showed it is a species of commencing interest in Brazil (Task 7.1). The methodological development was completed with EMBRAPA and the commercial partner Ostramar and wild collected juveniles were integrated into an existing IMTA structure with Oysters in February 2020 following the agreed protocols and with measurement of weight and survival (Task 7.2).

In the case of South Africa, after active discussion with the partners Ms. Daphne Taylor of Wild Coast Abalone and Prof. Clifford Louis Wiltshire Jones of Rhodes University, among others, a basic protocol was developed for the workplan. In November 2019 a kick-off meeting was held in South Africa to meet prospective University academics and graduate students (inclusion of Fort Hare as IRG) was organised by WiCoAb in Morgan Bay South Africa. Literature analysis by WiCoAb and AWI revealed the only sea cucumber of viable interest (size and presumed deposit-feeding) was the species *Neostichopus Grammaticus* (Task 7.1). Discussions with local divers commercially active for WiCoAb revealed *N. Grammaticus* as the only sea cucumber visibly present on the coast and also provided information on potential survey sites (Task 7.1). The kick-off meeting was used to visit WiCoAb, agree tanks designs and assess abalone waste as a sea cucumber diet. Methods were finalised for tank experiments with RhU and Fort Hare Academics, tanks construction was completed and reported as follows (Task 7.2). Methods for GSI analysis in collected animals were established with RhU (Task 7.3) and the first SCUBA diver surveys were undertaken in South Africa allowing the first data collection for gut content analysis and for gonad somatic indexing in February and March 2021 respectively.

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement No 818173. This report reflects only the authors' view and that the Commission is not responsible for any use that may be made of the information it contains.

CST 7.1 Species Identification and selection

Responsible CS Task Leader Matthew Slater, AWI

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
7.1	Species Identification and selections	1.1., 1.2, 1.4; 2.1. 3.1, 3.2.	AWI, RhU, UNESP, EMBRAPA, WCA	75%	✓	M2	M4	M13	M13	n.a.	n.a.

Introduction

In order to meet the goals of CS7, it was required in M1-12 to confirm research consortia and organise kick-offs/coordination meeting in both Brazil and South Africa. At these meetings (and in preceding interactions by phone and email) it was essential to identify the species of interest in both nations (by locality). Prior to the kick-off meetings it was also important AWI provide methodological suggestions to allow academics to develop and confirm the methods which could/can be applied under existing conditions: an Oyster Farm Site in Brazil and an Abalone Farm in South Africa.

At kick-off meetings at both sites, the sites were to be surveyed and final designs established to commence work in all tasks. This was possible in South Africa due to the rapid organisation of the kick-off meeting. In Brazil, the decision to delay the meeting until shortly before the proposed consortium meeting caused cancellation. Following the kick-off meetings, methodological development was continued with academics and graduate students at Fort Hare and RhU and animal collections and surveying were able to commence in February 2020.

Methods

7.1.1/3/5 Review of species Brazil and South Africa

Requests for data and interviews were sent to all partners and associates to CS7. This involved requests for information by email and non-structured telephone interviews related to:

- Fished and non-fished sea cucumbers species in the locality if known.
- Local knowledge (divers, fishers and aquaculture workers/practitioners) knowledge of fished and non-fished sea cucumbers species in the locality if known.
- Web of science key word searches by nation for relevant literature.
- Ecosia and GoDuckGo searches by nation for relevant literature.
- Compilation of proposed species
- Discussion of proposed species with CS partners and selection of most relevant.

7.1.2/4 Field surveys and site surveys

In South Africa, commercial divers employed by WiCoAb were tasked with conducting transects at three sites to count by area and collect chosen sea cucumber species:

Dive/snorkel transects carried out ca. 100 m2 50 m collecting all animals 1 m L:R and provided for weighing and gut content analysis.

The following was performed:

- Dissection of all animals on collection.
- Removal of full digestive tract.

Parameters to be recorded per animal:

Total Length (CM) Width (CM) Weight (G) Drained weight gutted weight Gut length Gut weight Gonad weight Sex GSI Gut index

Storage of gut with gut content for future analysis

Parameters to be determined gut content:

Grain size analysis Total Organic Matter C/N ratio.

Results

Literature searches and reviews were completed for Brazil and South Africa. Please refer to the reference section for a detailed list of available literature.

All tasks South Africa

Species selection and site surveys / collection revealed the density and suitability of the following species:

Neostichopus grammaticus (Clark) a benthic detritivorous stichopodid found on the WIO coast of South Africa (Thandar, 1987; Figure 7.1.a). This species is the only commonly occurring stichopodid commonly occurring on the South African WIO coast within the study region.

Local abalone diver and fisher feedback indicated patchy areas of presence in the coastal region of the species.

Sites were surveyed by commercial divers near the WiCoAb site and also near East London. *Neostichopus grammaticus* was collected and identified on 10th February at a site north of WiCoAb with the coordinates 32°45'02.4"S+28°16'43.5"E within the East London Closed Area - Nyara River Mouth to Great Kei River Mouth (Table 7.1.a).

Table 7.1.a: Weight and gut data from February sampling of N. grammaticus.

	DATE = 10th February 2020									
No	Total Length (CM)	Width (CM)	Weight (G)	Drained weight	gutted weight	Gut length	Gut weight	Gonad weight	sex	Gut index
1	12	4,2	58	37	17	30	18	N/A	N/A	31,03448
2	9,5	4,6	62	26	14,5	25	6,5	1,3	F	10,48387
3	11,2	5,5	83,6	57,8	23	41,5	24,8	1,1	F	29,66507
4	10	4	53,4	34,5	15,5	31	15,6	0,7	F	29,21348
5	8,5	3,5	31	19,6	9,1	25	6,2	0,4	F	20
6	11	4	32	25	10,6	31	7	0,5	M	21,875
7	9,5	4	36,5	27	11,3	28	8,7	0,3	M	23,83562
8	12,5	3,8	45,5	31,8	14,5	32	10,5	0,8	F	23,07692
9	13	3,2	27	17,2	9,5	31,5	2,1	0,25	M	7,777778
10	10	2,5	27,5	23,7	8	33,5	9	0,6	M	32,72727
11	9,2	2,5	25,3	19,6	9	30	5,5	0,25	M	21,73913
12	9,5	3	20	14	8,45	37	3,3	0,3	M	16,5
13	8,5	3,5	25,55	19,6	9,7	30	7,65	0,29	M	29,94129
14	8	3,6	30,9	24,69	10,2	37,5	9,69	0,5	F	31,35922
15	8,5	3,5	25	19,31	7,67	30	6	N/A	F	24
16	9,5	3	21,4	19,5	9,6	30	5	N/A	N/A	23,36449
17	9,5	3,5	22,6	18	9,3	23	7,7	0,2	N/A	34,0708
18	8	3	19,9	16,5	8	33	6,6	0,05	F	33,16583
19	6,2	2,7	15,2	12	5,2	30	4,1	0,3		26,97368
20	10	3,1	24,2	18	10	23,5	3,7	0,5		15,28926
21	8,5	2,8	18,1	14	6,9	27	5,25	0,3		29,00552
22	7	3,5	17,8	15	7,2	38	5,4	0,3		30,33708
23	6	2,5	9	8,1	4,27	24,5	3	0,19		33,33333
24	5,5	2,5	9,2	8	4,3	23	2,5	N/A		27,17391
25	7,5	3	23	20,7	9,5	39	8	1	M	34,78261
26	5,5	2,5	14,6	13,4	6,5	36	5,2	N/A		35,61644
27	5,7	3	14	11,83	6,2	30	5,23	0,4	M	37,35714
28	5,8	2,5	11,9	10,6	4,8	30	4,57	N/A		38,40336

Figure 7.1.a: Wild-collected N. grammaticus ready for transfer to laboratory sites.

All tasks Brazil

The species selection for Brazil (Rio Grande do Norte) was completed after discussions with EMBRAPA, UNESP and Ostramar. The species selected was *Holothuria grisea*, which is fished in the area and appears to associate with the oyster farm sites (Ponte et al. 2019). Site selection and collections were carried out and the piloting of an experimental IMTA system agreed, however all activities ceased due to the pandemic from April 2020 onwards.

Discussion

Overall, the results of the 1-12 Month activities in CS7.1 are encouraging given the severe access limitations created by the COVID-19 situation in all associated nations. The presence of *H. grisea* and *N. grammaticus* within the bounds of both farm types indicates suitability to IMTA and feeding ecology likely to accept biowastes from aquaculture. The diverse methods to be applied in South Africa and their piloting in the first few months indicate that the methods are robust and that sites and animals are readily available to obtain acceptable data. Brazil efforts must be revisited when the situation normalises in Rio Grande do Norte.

Progress, deviations, problems & next 12M

Progress: Based on the methods developed so far there should be a suitable level of data available in 12 month's time to begin publication and make clear statements about *N. grammaticus* natural diet and site selection. This task is considered ca. 75% completed as gut samples and date have been obtained in multiple sampling events, an application of suitable laboratory methods will ensure a full dataset is available to complete the task.

Deviations & Problems: no major deviations but limitations in literature availability (very few publications and few candidate species). Pandemic development beginning to limit SA activities and heavily impacting BR activities

Outlook: Data collection (gut content) for 7.1 and animal collection for GSI.

CST 7.2 Controlled feeding experiments

Responsible CS Task Leader Matthew Slater, AWI

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
7.2	Controlled feeding experiments	1.1., 2.1. 3.1, 3.2.	RhU, UNESP, EMBRAPA, AWI	10%	✓	M14	M7	M19	M36	n.a.	n.a.

Introduction

Task 7.2 revolves around understanding the sea cucumbers feeding ecology and conducting controlled feeding experiments to exactly determine their feeding ecology, including feeding on IMTA wastes. The kick-off meeting was imperative to developing the methods for this task and prior to the kick-off meetings it was also important AWI provide methodological suggestions to allow academics to develop and confirm the methods which could/can be applied under existing conditions: an Oyster Farm Site in Brazil and an Abalone Farm in South Africa. The first year involves method development and understanding the way sea cucumbers react to an IMTA diet and collecting data on consumption rates to establish feeding protocols.

Methods

7.2.1 Site Setup

On-site visits were conducted to WiCoAb and a discussion held with WiCoAb manager on tank availability. The site on farm was selected and available small tanks for controlled feeding experiments agreed. Methods were agreed for controlled feeding experiment between AWI, Fort Hare and WiCoAb. Construction of tank systems agreed.

7.2.2 Feeding experiment

Methodology agreed in multiple online meetings with AWI, Fort Hare please see also results section.

Results

Experimental design feeding experiment

Sixteen flow-through tanks (LXWXH: 40.5 x 25.5 x 27.5 cm) are to be used for the experiment (Figure 7.2.a). Each treatment will be randomly distributed across the tanks. The experiment will be made of four treatments, and four replicates. The animals are to be stocked at a density of 5 animals per tank, resulting in a stocking density of 46 animals/m^2.

The flow tanks are to be fitted with an airline, and the tank will have a replacement of 6% new water per minute, the total tank volume will be replaced every 17 minutes. The water temperature will be measured and dissolved oxygen measured daily using Oxy-Guard Handy Polaris probe at 9:00 am. Water pH will be measured with a pH metre; Ammonia, nitrate and nitrite are to be measured once weekly with a Palin- test photometer 7100.

Experimental feeds

Four experimental feeds are to be used for the trial. They are abalone waste, sand, commercially produced pellets (Abfeed ES26 PRIME) and fermented *Ulva rigida* mixed with sand (Table 7.2.a).

Abalone waste will be sourced from Wild coast abalone Pty, hand-picked to remove large chunks of pellets and the slurry will be then passed through a 5µm mesh sieve to reduce the water content. After which it will be stored in -18°C deep freeze; sand will be collected from the top surface (2mm) of the natural environment of the sea cucumbers (control) and kept at -20°C; for the production of fermented *Ulva rigida*. *Ulva rigida* will be taken from WiCoAb farm, water will be added to it, then it will be

mashed with a hand blender. Wet sand/sediment from the natural environment of the sea cucumbers will be added to the slurry. The ratio of *Ulva rigida* to sand will be 960g:700g. The mixture will be left to ferment for two weeks. After which the mixture will be sieved with a 5µm mesh to reduce the water content.

10g (d/w) of pellets and 10g (w/w) of each wet feed (abalone waste, *Ulva rigida* and sand) are to be given to the animals daily. These feeds are to be given to the sea cucumbers daily to mimic daily deposition of waste from an abalone tank. Feeds are to be allowed to defrost for two hours before feeding.

The airflow and seawater supply will be interrupted before the introduction of the feeds. The defrost feed will be added as a slurry and left for 30-mins to settle, thereafter, the airflow and seawater supply are to be restored. Tanks are to be cleaned once weekly. Any dead animals are to be recorded and discarded and are to be not replaced in the experiment.

Table 7.2.a: Preliminary TOM analysis of proposed diets for controlled feeding experiment.

Feed	Total organic matter (TOM%)
Abalone waste	70.17±0.73
Ulva rigida	10.67±0.33
Pellet	96.3±0.33
Sand	5.43±0.73

Sample collection

To estimate the feeding rate of *N. grammatus*, left over feed and faeces are to be collected from each tank daily for eight days. It will be easy to identify and cleanly collect the fresh faeces (which is a sausage shape) this will be siphoned each morning by 9 am and oven-dried at 60°C for 48-hours.

Diet and sediment assays

Three samples of abalone waste diet and fermented Ulva rigida diet are to be stored at -18°C. Freeze-dried samples are to be analysed for lipid according to AOAC standard method, protein (Block digestion method after Kjeldahl), ash (direct ashing, muffle furnace at 550°C for four hours) and carbohydrate (calculated by difference).

The total organic matter (TOM) will be determined for all diets according to by Byers et al. (1978). Samples are to be oven-dried at 60°C for 48 hours and weighed. Oven-dried samples are to be then placed in a furnace at 500° C for 6 hours for combustion to be complete after which samples are to be weighed. The Percentage total organic matter will be calculated by sample weight loss after combustion.

Data calculation

Specific growth rate (SGR), ingestion rate (IR); faecal production rate (FPR) and apparent assimilation efficiency (AAE) are to be calculated as per Yuan et al. (2006).

SGR (% d−1)= 100*(LN (Wf) – LN (Wi))/t

IR (g ind−1 d−1) = C/n/t

FPR (g ind−1 d−1) = (Wfa/n)/t

AAE (%) = 100*(IR−FPR)/IR

Where C= Dry weight of food consumed in a tank (dry weight of food given - dry weight of uneaten food)

n= number of sea cucumbers in the tank

t= time in days

Where Wf is the weight (g) of each sea cucumber at the end of the experiment;

Wi= the initial weight (g) of each sea cucumber at the beginning of the experiment;

Ct, the dry weight (g) of the food consumed during the whole experiment in each tank;

Wfa, the dry weight (g) of the faeces of the sea cucumbers in each tank;

n= number of sea cucumbers in each tank and t is the time in days.

Statistical analyses

At the beginning of the experiment, the animals' mean weight will be compared to ensure there will be no bias in the sizes of the animal in the various treatments. All Data are to be tested for homogeneity (Levene) and normality of variances (Shapiro-Wilk). A one-way Anova will be used to test for IR, FPR, and AAE. Where a significant difference will be detected, Tukey's test will be used for pairwise comparisons.

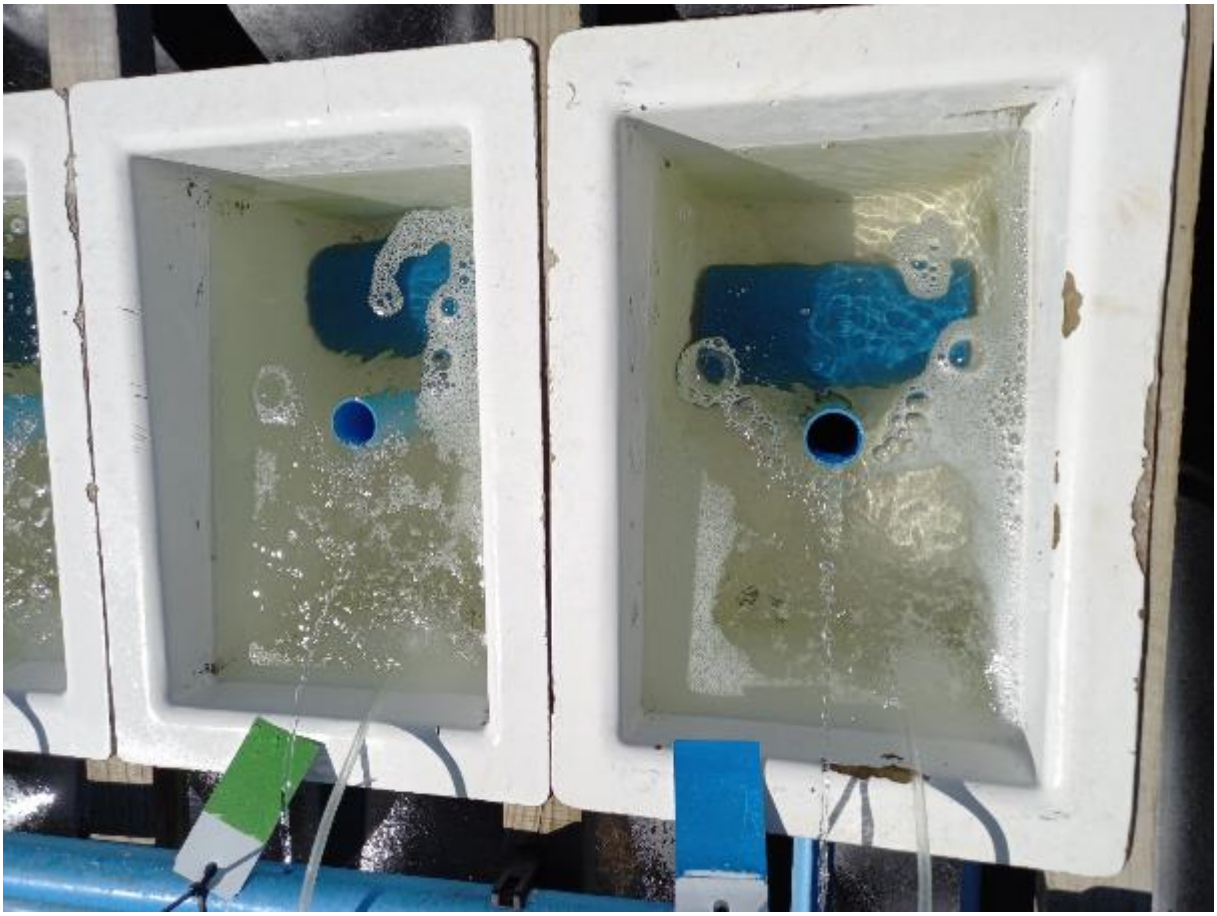

Figure 7.2.a: Designated holding tanks at laboratory site for controlled feeding experiments. Note PVC shelter provision (TBC) in blue.

Discussion

This task is advancing acceptably with the strong support of WiCoAb and Fort Hare. The species seems very well suited to the proposed feeding experiments and the facilities are being constructed at the time for reporting. Understanding of the feeding ecology is likely to be expanded with the data collected thus far and insights into potential IMTA diets will be obtained.

Progress, deviations, problems & next 12M

Progress: This task is on schedule in South Africa and it is expected that controlled feeding experiments will begin in the next few months. Methods are complete and all system requirements identified. It is estimated that this constitutes 10% completion as there are major works (system build, animal collection and preparation) along with major potential risks (lack of acceptance of diets etc.), which must still be completed/overcome.

Deviations & Problems: none, unless site access is further limited at WiCoAb.

Outlook: Data collection – experimental completion feeding experiment.

CST 7.3 Basic hatchery methods

Responsible CS Task Leader Matthew Slater, AWI

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
7.3	Basic hatchery methods	T1.2, T1.4, T3.2	AWI, RhU, UNESP, EMBRAPA	5%	✓	M20	M9	M43	M44	4	7-9

Introduction

In this task we evaluate the suitability of standardised hatchery protocols for the production of larvae and juveniles at laboratory scale and determine basic growth rates for early juveniles. This requires an understanding of the reproductive cycle of the species and application of a series of spawning, larval rearing, settlement and on-growing methods which are established in the literature but require adaptation / selection based on the reproductive ecology of the given sea cucumber species. This task aims to test and adapt these methods and suggest a protocol for hatchery production of the species based on laboratory work.

Methods

Gonad somatic index methods and collection and observation methods agreed with RhU in combination with diver survey activities in 7.1 please see also results section.

A multispecies hatchery design/build was proposed by the director of WiCoAb and agreed by all participants.

Results

Task 7.3 methodology is linked to the animal collections at sites outlined at 7.1.

Experimental design reproduction cycle/gonad development

July 2020 to December 2020 At the determined sampling site North of WiCoAb with the coordinates 32°45'02.4"S+28°16'43.5"E within the East London Closed Area - Nyara River Mouth to Great Kei River conduct monthly samples obtaining at least 10 wild-collected sea cucumbers:

- Farmed sea cucumbers @ 18˚C
- Wild sea cucumbers

Parameters to be recorded per animal:

- Gonad Index
- Samples for gonad histology
- Staging of maturity of gonad

Methods were piloted during February and April sampling and initial GSI data recorded (Table 7.3.a).

Table 7.3.a: Preliminary GSI data from piloting of task 7.3 methods March/ April 2020.

N. grammatus gonad descriptions: April 2020 - April 2021						
Wild and farmed sea cucumbers are dissected monthly to highlight differences caused by tank conditioning.						
Date	Body weight (w/out pcf & gonads)grams	Sex	Gonad weight (pat dry) grams	GI = (Gw/Dw) x100	Gonad color	Stage Stage I – Indeterminate; Stage II – growing; Stage III – Mature; Stage IV – partly spawned and stage V – spent
23/03/2020						
W1	8,28	F	0,06	0,72	Grey-yellow	I
W2	9,38	M	0,12	1,28	Whitish yellow	I
W3	7,7	F	0,11	1,43	Grey-yellow	I
W4	8,35	Indeterminate	0,09	1,08	Grey-yellow	I
F1	21,08	M	2,13	10,10	white-yellow	III
F2	10,1	F	0,5	4,95	transparent-grey	II
F3	9,02	F	0,58	6,43	transparent-grey	III
F4	12,69	F	0,16	1,26	transparent-grey	II

Discussion

The basic data collection has commenced for this task and will continue reliably. The remaining sub-tasks are higher risk than the GSI data collection and require hatchery facilities which are planned but not yet there.

Progress, deviations, problems & next 12M

Progress: This task is on schedule in South Africa. GSI data will take 12-months, but the capacity for collection and upcoming spawning work is present. This task is only 5% complete as a full year of monthly samples must be collected. Currently we have only one preliminary dataset for a month and we have established methods.

Deviations & Problems: Development of the remainder of the tasks in SA are highly dependent on hatchery facilities which currently do not exist.

Outlook: data collection – experimental completion GSI data, completion of spawning experiments (next 6-12 months).

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CS8

Summary of progress report for Case Study 8

Date of report:	**6.4.2020**
Case Study name:	**Improving seed availability and grow-out of native and non-native oysters**
of relevane for WPs	**1, 2, (3), (6), 9**

Abstract/Summary

For the first 12-months of the AquaVitae, oyster production as part of CS8 has identified and overcome some of the technical challenges seen by producers across the Atlantic. A work plan has been developed to facilitate implementation of all activities and knowledge transfer of methods within the CS8 group, and a template for bi-monthly progress monitoring has been established. Activities have started on all task in accordance to the work plan and several activities have made significant progress. In particular the hatchery and juvenile production tasks (8.1 and 8.2) has achieved great progress. For hatchery processes in Brazil, local microalgal strains have been identified, cultured and analysed for macronutrients, and a system for improvements in water quality has been designed. A protocol for spatting pond production has also been developed and evaluated using one season of data from Ireland. An industry lead activity related to low-tech nursery systems was implemented as a pilot trial, and the sea-based wild settlement activities have also started and significant collaboration with stakeholders has been achieved. Additionally, a first prototype for an algorithm for automated species identification has been developed. Despite the onset of the Covid-19 pandemic, CS8 has seen great progress.

CST 8.1 Hatchery production of native oysters & techniques for local production of tetra- & triploid selectively breed Pacific oysters

Responsible CS Task Leader Colin Hannon, Galway Mayo Institute of Technology

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
8.1	Hatchery production of native oysters & techniques for local production of tetra- & triploid selectively breed Pacific oysters	T1.2, T1.3, T1.4, T1.5	EmBraPa, PrAq, IVL, GMIT, CPS	0-5%	!	M1	M3	M46	0	3-5	5-7

Introduction

T8.1 aims to increase availability of spat of native and underutilised oyster species in Brazil and Scandinavia by developing hatchery production of native oysters & techniques for local production of tetra- & triploid selectively breed Pacific oysters. The task includes six specific sub-tasks as described in the CS8 workplan (annex 3 of D1.1) and reflected by the six subsections under each heading:

1. **Larval diet**
2. **Water quality**
3. **Conditioning**
4. **Production of triploid Pacific oysters**
5. **Nursery systems**
6. **Spatting pond production**

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement No 818173. This report reflects only the authors' view and that the Commission is not responsible for any use that may be made of the information it contains.

To address the different needs identified in different parts of the Atlantic related to development of new hatchery protocols for native oyster species (reported in the CS8 work plan), different approaches have been adopted in Brazil and Europe.

The ambition of T8.1 for M1-12 was to plan and initiate all sub-tasks, both by making a detailed work plan describing the methods and timeframes of the work, and by beginning data collection in most of the activities. The work plan for CS8 is reported in D1.1, and according to plan, activities have been initiated in all sub-tasks. The activities performed are described in detail below.

One of the major bottlenecks in the production of *C. gasar* in Brazil is access to seed. Hatchery protocols for the species is under development, but many challenges still persist, e.g. low larval survival. From related species, aspects such as conditioning of broodstock, nutritional quality of broodstock and larvae food and water quality are known to affect hatchery success. To increase survival of larva in the hatchery system, three activities were identified as relevant;

1) development of a larval diet using native microalgae instead of the conventional microalgae used in the hatchery,

2) improvements in water quality through water recirculation and treatment system to enhance quality of the estuarine water used for larviculture tanks at the Primar hatchery.

3) the need for a protocol for conditioning of broodstock, and

One product and two protocols were identified as the potential outcome of the work:

CSTP8.1.1 A new diet regime for *C. gasar*

CSTP8.1.2 A new conditioning protocol for *C. gasar*

CSTP8.1.3 A new protocol for water improvements in small-scale oyster hatcheries using estuarine water

Larval diet

The conventional diet offered to larvae of *C. gasar* are microalgae widely known and used in hatcheries for various marine species such as shrimp, fish, and more specifically Pacific oysters. The conventional microalgae and the origin of Primar strains are listed below:

Chaletoceros calcitrans / UFSC

Chaetoceros muelleri / UFSC

Nannochloropsis sp / USP

Isochrysis galbana / UFSC and UFPR

Pavlova lutheri / UFSC and UFPR

Rhodomonas salina / UFRJ

Tetraselmis sp / UFSC

Thalassiosira sp / Aquatec RN

Navicula sp / Aquatec RN

Many of the above microalgae are too large to be offered in the first larval stage for *C. gasar* and enter the diet only when the oysters reach the seed stage, above the size of 200 to 300µm. A possibility for

the first larval phase is a diet with microalgae, preferably native, in the size below 5 µm that could be introduced right at the beginning of the culture, when the larvae are 35 µm in size. Diatoms and flagellates, equivalent to the microalgae that make up the *C. gasar* natural diet, would need to be collected, identified and produced in Primar's hatchery for the new proposed diet.

Water quality

Another major issue to be developed is the improvement of water quality for the larvae tanks. Any change in the water supply to the larviculture tanks causes stress on the larvae and consequently mortality. The current system at the Primar hatchery is based on flow through with estuarine water treated in a series of steps to enhance water quality. One reservoir containing the treated water lasts for 5-7 days of use in the hatchery after which the hatchery system must be switched to the other reservoir which has then been refilled. This infers a disturbance of the hatchery system which results in low stability and predictability to the operation. The larvae of *C. gasar* has been observed to be very sensitive to the disturbance associated to switching reservoirs and often high mortalities are observed associated to changes in the water system. Therefore, a solution where the water quality is as uniform and regular as possible is needed, and could provide a substantial improvement in the survival of the larvae of *C. gasar*.

Conditioning

In regions of temperate or subtropical climate, seasonal variations in water temperature occurs. In Brazilian tropical estuarine regions, the temperature remains relatively constant throughout the year and the reproductive cycle of oysters is more influenced by variations in salinity caused by the occurrence of dry and rainy periods. In the subtropical climate of southern Brazil, hatcheries collect oysters at the end of winter, when the animals are at rest and subject them to waters with higher temperatures to initiate gametogenesis. Since oysters in tropical water regions reproduce throughout the year and do not enter the resting phase, traditional oyster maturation and conditioning methods using temperature variations are not suitable for Brazilian tropical waters. Previously conducted studies have shown that there are salinity ranges in which *C. gasar* produces, but does not release gametes, indicating that the manipulation of salinity in hatcheries may be a viable alternative for maturation and conditioning of this species.

Production of triploid Pacific oysters

Ploidy manipulation of oysters has several advantages for the culture of species outside of the natural geographical distributions thus reducing the risk of creating an non-native invasive species which has been seen around Europe. Triploid production of oysters has created a market year round for oysters where they are consistent and do not produce genetic material and putting this energy directly into growth. Producing triploid oyster via genetic means is a complex task which can be done however the methodology is under licenced use.

Nursery systems

One bottleneck for culture of the native oyster *O. edulis* in Scandinavia is low seed survival when hatchery reared seed are transferred from the hatchery to sea-based systems for grow-out. The Scandinavian oyster industry is small as seed supply from the local hatchery has, for many years, been erratic. There is consequently no possibility for the industry to invest in more large-scale nursery systems such as Floating Upwelling Systems (FLUPSYs), and alternatives to enhance survival of *O. edulis* is therefore requested by the industry. During consultations with IRG members after AV was funded, low tech nursery systems were raised as a barrier in need of exploration. IVL therefore agreed to extend their activities to include an IRG lead activity related to development of low-tech nursery systems for *O. edulis* seed. The activity was initiated as planned M1, and one protocol was identified as the potential outcome of the work:

CSTP8.1.4 A new protocol for enhanced survival of flat oyster seed using small-scale, low-tech nursery systems.

Spatting pond production

One of the most valuable bivalve species in Europe is the European flat oyster, *O. edulis*, and the interest in aquaculture of the species is high. Commercial culture of the species is, however, hampered by access to spat as neither sea-based collection nor hatchery production have been able to provide reliable sources of seed. Consequently, there is a need to develop alternative methods to produce oyster spat to support the development of oyster industry. One method that is receiving increasing attention internationally is pond based production of oyster spat, i.e. spatting pond production. This technique is used by some companies around Europe, but the possibility of transfer of the technique to new areas is limited as there are no protocols available and knowledge exist as industry know-how only. The purpose of this sub-task is therefore to develop, evaluate and refine a protocol for reliable spatting pond production of flat oyster seed. The work to develop a protocol for spatting pond production of *O. edulis* as a means to achieve a robust juvenile production method for the *O. edulis* industry in an economically viable time frame was initiated as planned M1, and one protocol was identified as the potential outcome of the work:

CSTP8.1.5 A new production protocol for flat oyster spatting pond production

Methods

Larval diet

The task to develop a larval diet adapted to the requirements for the native *C. gasar* in Brazil was initiated as planned M3. Water samples with native microalgae were collected from the culture ponds at the Primar hatchery and in the Canguaretama estuary, in a municipality close to the Primar facilities. 12 different microalgae (Figure 8.1.a) were identified to species or genus, isolated in partnership with the Federal University of Paraiba. The first cultivation protocol for native microalgae was developed and evaluated at experimental scale. Successful cultures were transferred to intermediary and mass culture (Figure 8.1.b). In the first evaluation five microalgae strains (all <5µm) were used. The same procedure as with the normal microalgae was used for the large-scale cultures. Protocols were maintained to keep records of the process and cell counts which will be compare to microalgae species commonly used in oyster hatchery production. The information will be presented in the best practice guide produced in WP1.

Figure 8.1.a. 12 microalgae isolated from the Primar channel and Canguareta estuary.

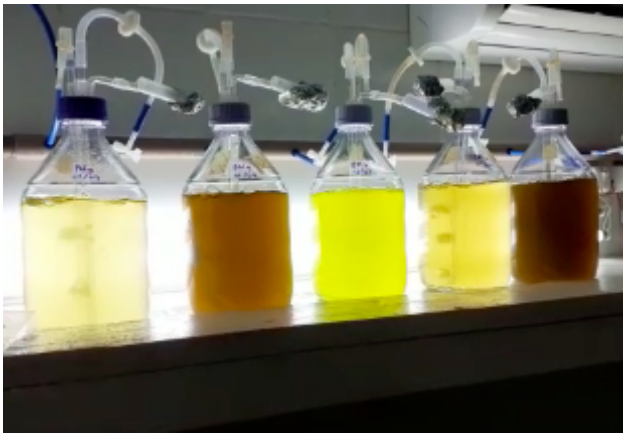

Figure 8.1.b: Vials with native microalgae collected from the culture ponds at the Primar hatchery and at Canguareta estuary and the subsequent large-scale culture trials with selected species.

After the large-scale cultures were mature, 10L with concentrated biomass of each cultured microalgae strain was shipped to the Federal University of Paraiba for analysis of nutritional value (macronutrients - fat, protein, carbohydrates) (Figure 8.1.c), fatty acid composition and genetic species identification. Due to Covid-19, the lab closed and the samples were frozen for later analysis. Only the macronutrient analysis was completed at this point.

Cepa	Proteínas hidrossolúveis (%)	Carboidratos totais (%)	Lipídeos totais (%)	Total (%)
P-M5BG	19,89	15,61	7,31	42,81
P-M9BG	16,74	9,71	10,74	37,19
P-M11BG	14,38	11,98	8,65	35,01
P-M5P	4,66	3,71	5,86	14,23
P-M7P	15,47	12,00	9,22	36,69
P-M9P	21,72	13,51	14,45	49,68
P-M16P	2,10	4,21	6,56	12,87

Figure 8.1.c: Examples of macronutrient analysis of the native microalgae cultured at the Primar hatchery.

Water quality

The task to evaluate the effect of water quality on hatchery production of *C. gasar* was initiated as planned M6. The current hatchery structure (based on a flow through system) will be modified according to the principles of a semi-RAS system and the improvements in water quality will be evaluated by comparing hatchery success before and after system modification. Between M6-12, the bottlenecks of early oyster spat production at the Primar hatchery was described, literature was reviewed and adjustment options for hatchery water quality improvements were identified and verified using the expertise of external and AV partners. The first draft for water quality improvements was produced (Figure 8.1.d).

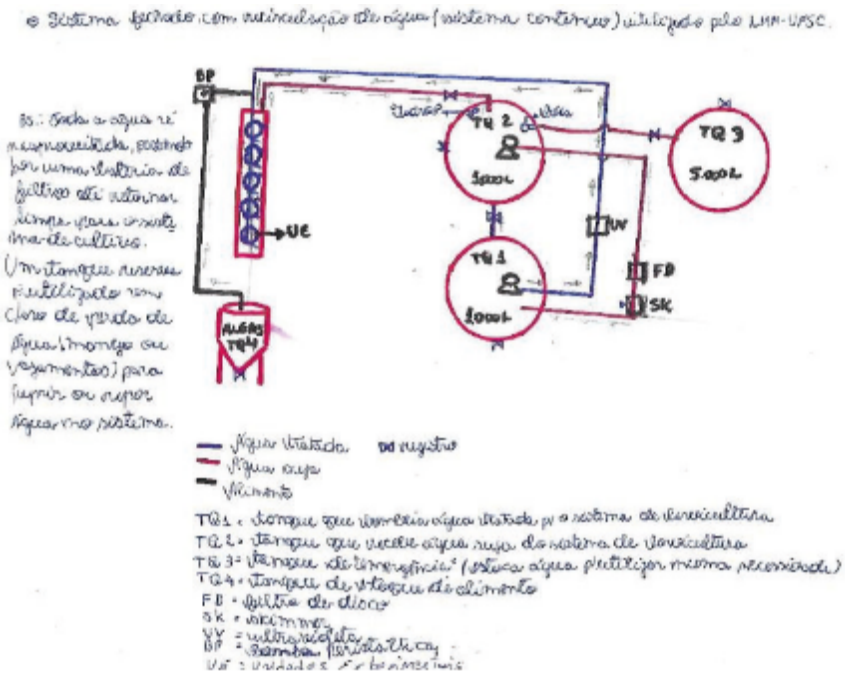

Figure 8.1.d: The first draft of the system for improvement of water quality at the Primar hatchery in Brazil.

Conditioning

In a previous project run by UFSC and Embrapa, a first prototype of a protocol for maturation and conditioning was developed. During the AquaVitae project this protocol will be validated and improved in a new hatchery facility that will be installed at Embrapa. A company was contracted (not within the AV project) to develop and install the small-scale hatchery in April/May (M11/M12). The installation was delayed due to Covid-19 as the lab was closed.

Production of triploid Pacific oysters

To address the need for local production of triploid Pacific oyster spat using tetraploid broodstock, a practical guide for initiation of this kind of activities will be included in the best practises report in WP1.

Nursery systems

During a series of meeting with IRG members, different alternatives for small scale nursery systems were discussed. Possible solutions were identified from systems used by small scale oyster farmers around the Atlantic observed by IVL in previous projects, through tech-transfer from north American partners and through reviews of published and grey literature. 4-5 different main types of systems were identified, ranging from land- based flow through and small-scale FLUPSY systems to sea-based cage and floating systems. Based on this, the IRG partners prioritized one system, a floating, surface based structure. Benefits perceived with this system was that the surface based systems would enable the seed to have access to the highest food availability while also being exposed to the highest temperatures (hence increasing growth rates) and to tumbling by waves that may enhance the shape of the oysters. A first prototype (Figure 8.1.e) was developed by members in the IRG.

Figure 8.1.e: The nursery system developed by members of the IRG (left) and protocol development and training of IRG member during initiation of the pilot trial (right).

In the beginning of July 2019, 22 500 *O. edulis* seed of 8-12mm and 35,000 seed 5-8mm were bought by the IRG partners from a local hatchery for a pilot trial. The seed were transported to the Kristineberg research facility where the average weight (g) of the seed was measured using bulk sampling (i.e. total mass for a known number of seed divided by the number of seed). The active IRG partners and IVL jointly developed a protocol for monitoring seed survival and growth based on a sub-sampling regime including bulk weightings on a monthly basis (Figure 8.1.d). The seed in each size class were divided into 8 groups of equal weight and were distributed between 4 wooden frames (0.7 x 0.5 m) covered by 1.4 mm nylon mesh and 4 plastic seed cages normally used as nursery systems. The systems were transferred to the sea where they were placed at two shellfish production sites (4 cages and 4 wooden frames per site, 2 of each type containing the small size seed and 2 of each type containing the larger seed). The seed cages were hung at approximately 2 m depth while the wooden system floated on the surface. The systems were checked on a bi-weekly basis during the trial and the wooden frames were flipped from one side to the other at every check to reduce fulling issues.

Spatting pond production

The first protocol prototype was developed by IVL based on literature and results from a previously performed project[31] (Figure 8.1.f). The protocol was translated to English and reviewed by IRG partners experienced in spatting pond technology before being passed on to AV partners in Ireland (GMIT and Cartron Point Shellfish).

31

https://www.researchgate.net/publication/332144621_Produktion_av_ostronyngel_Ostrea_edulis_i_havsbaserade_tankar_Biologisk_och_teknisk_forstudie

Part of Report C 380 (2019). Hatching of *Ostrea edulis* in sea-based ponds – a biological and technical pilot study. Chapter 6.

6. Pond production protocol

In the following chapter, a protocol is described for how sea-based pond cultivation of flat oyster (*Ostrea edulis*) spat may be achieved. Note that this protocol is based on information collected during study visits and literature studies only, which is why the protocol needs to be evaluated and possibly adjusted by practical experience gained from future cultivation trials. The protocol is divided into different tasks, whose temporal implementation is illustrated in Figure 23.

Figure 23. Time allocation of different activities in the culture protocol.

6.1 Preparations

November-May:

- The ponds are drained and kept empty.
- Mussel shells for later monitoring of spat settlement is laid out for cleaning (aim for 10 shells per pond and per week, sampling every week from mid-June to August -> about 500 shells for 4

Figure 8.1.f: Illustration of the first prototype of the spatting pond protocol developed by IVL as a basis for the spatting pond sub-task.

Cartron Point Shellfish Ltd, New Quay, Co. Clare, Ireland began the production of juvenile *O. edulis* in their 1 Million litre spatting ponds (Figure 8.1.g) in the summer 2019 after the kick-off of the AquaVitae project. The preparation of the spatting ponds took place in May 2019 for inclusion of brood stock *O. edulis*. The ponds were thoroughly cleaned, brushed and washed down. The ponds were then rinsed with seawater and finally flushed out prior to filling again.

Four spatting ponds were filled with seawater filtered through a 4mm mesh sock at the inflow pipe of each pond thus retaining medium and large sized potential predators (mostly crab and inshore intertidal fish species), between the 7th and 14th of June 2019. Ambient seawater temperature on filling was 13.1°C on 12th June 2019 for pond two whereas it had reached 15°C, 17°C, and 16°C for filling ponds 7,8 and 9 respectively by the 14th of June. Seawater was pumped using a submersible pump on incoming tides to the required depth (approximately ¾ of the full volume) after which the pumping was stopped and water quality was monitored. In all ponds the water quality from the first filling was found to be within the limits of salinity and pH required. On the 24th of June all ponds had reached a temperature of 19°C or above exceeding the minimum temperature suitable for larval development in the ponds.

Figure 8.1.g: Cartron Point Shellfish, New Quay, Co. Clare, on the South shore of Galway Bay Ireland. The spatting ponds can be clearly seen in the figure along with the hatchery building (Image courtesy of Iarfhlaith Connellan, 2019).

Broodstock were collected from three different sources (Tralee bay, Co. Kerry, Clarenbridge & Redbank, Co. Galway) and conditioning of Broodstock was allowed to take place on each of the source beds until deemed suitable for spawning in the spatting ponds. Tralee bay oysters were monitored from 15th of May and when 25% were either white or black sick the relevant stocks were transferred to the ponds at New Quay.

The stock held at Eaninish channel in Aughinish Bay, these oysters were displaying 25-30% white or black sick by the 5th of June and were deployed immediately into the spatting ponds. Clarinbridge oysters were obtained from dredging on the 19th of June and showed 20% with larvae within the mantle cavity. Salinity and pH of each pond was monitored every 3-4 days, and phytoplankton and oyster larvae counts were carried out daily throughout the spatting season.

Larval monitoring indicated that by the end of August larval releases had reduced to a trickle or had ceased entirely. A programme of seawater flow-through was therefore initiated and maintained throughout September and October. This consisted of 10 to 15 % of the water in each pond being replaced every other day throughout the period. This ensured that all ponds were reduced in temperature initially by 1-2 degrees per day until all ponds had reached ambient seawater temperature.

Two settlement surfaces were tested for efficiency of settlement of *O. edulis* in each of the 4 spatting ponds.

- Mussel shells
- Crushed shell particles of *M. gigas*

The Mussel shell substrate was a waste stream product from an industrial mussel processing plant and had been weathered outside for minimum of 24-months before use which resulted in up to 80% of the periostracum had slewed off. This gave the shell a distinct advantage in order to maintain adhesion of oyster spat post settlement and transport.

Eight tonnes, at 5kgs per bag, of mussel shell was packed into 10 mm mesh oyster bags. The oyster bags were placed around the periphery of each pond awaiting deployment as soon as larval settlement had been ascertained by visual means.

The oyster bags containing the mussel shell were suspended using two cords per bag, on the sloped surface of the side walls of each pond. Bags were suspended in positions 10cm from the bottom to a height 10 cm below the working seawater surface of each pond.

Weathered Pacific oyster shell was crushed to a particle size ranging from 4 mm to 30mm with a hammersmith mill. This fraction of the *M. gigas* shell was placed in oyster bags, with 5kg/bag, and deployed around each of the spatting ponds. In the same method as the mussel shell cultch but at a ratio of one oyster shell bag to three mussel shell bags the entire cultch volume was introduced to the ponds when oyster larval development was observed to have reached settlement stage. This varied from pond to pond in both timing and intensity throughout the settlement period.

Data from the settlement on the two cultch substrates will evaluated at transportation to sea stage. For the purpose of the settlement data, Unglazed pottery plates were hung at a depth of 1-metre in each pond. Each plate was retrieved and replaced with a clean dry plate (daily for the season) and the plate removed from the pond was examined under a dissecting microscope.

Results

Larval diet

No results yet, see also Methods on “Larval diet”.

Water quality

No results yet, see also Methods on “Water quality”.

Conditioning

No results yet, see also Methods on “Conditioning”.

Production of triploid Pacific oysters

No results yet, see also Methods on “Production of triploid Pacific oysters”.

Nursery systems

Due to an unexpected increase in work load during the summer months, the IRG failed to monitor the progress of the oyster seed as planned. The systems were, however, checked regularly, the seed were sorted by size on a monthly basis, and qualitative data on performance was obtained. After four weeks the floating system was affected by fouling of green macroalgae inside the system which caused smothering of the oyster seed. Similar fouling was not observed in the cage systems that hung at approximately 2m depth. The fouling made it hard to size sort the seed and many were lost during cleaning of the system. After approximately 6 weeks the seed were sorted by the IRG partners and seed from the small size class that had not grown over the past six weeks and consequently remained in the size range of 5-8 mm were discarded as sub-sampling indicated a high level of mortality for this group. In October, all seed previously kept in the nursery system were transferred to suspended grow-out oyster baskets, again without further quantifications of survival and growth. During the summer, the system with oyster seed in cages disappeared from the farm and could not be found despite extensive search. This made comparisons of the success of the pilot system at this site impossible. All oyster in the remaining system (previous from the pilot nursery system) at that site were quantified by IVL in December. Survival at that time quantified as number of oysters remaining compared to number of oyster seed at the start of the trial was approximately 58%. At site two, survival of oysters originally

placed in the pilot nursery system quantified in a similar way as for the first site was approximately 46%. The corresponding number for oysters continuously maintained in the cage systems was 8%. Due to the lack of replication of this system the significance of this is hard to evaluate.

Spatting pond production

Phytoplankton samples were taken throughout the summer to monitor food availability for oyster larvae and analysis indicated a predominance of unicellular micro-flagellates throughout the spatting season. The density of these predominant flagellates varied from 40-60 cells/µL to a peak of 300 cells/µL in pond seven for a period of one week.

Larval releases began in Pond two and continued throughout June. Larval density never exceeded 12 larvae/litre throughout the season (Figure 8.1.h and 8.1.i) and larvae seldom developed beyond 200 µm until mid-July. Settlement counts of larvae on a plate could vary from 1-2 spat to several thousand in any one 24-hour period (Figure 8.1.j and 8.1.k). Plate counts of settled larvae included settlement obtained on both sides of each plate.

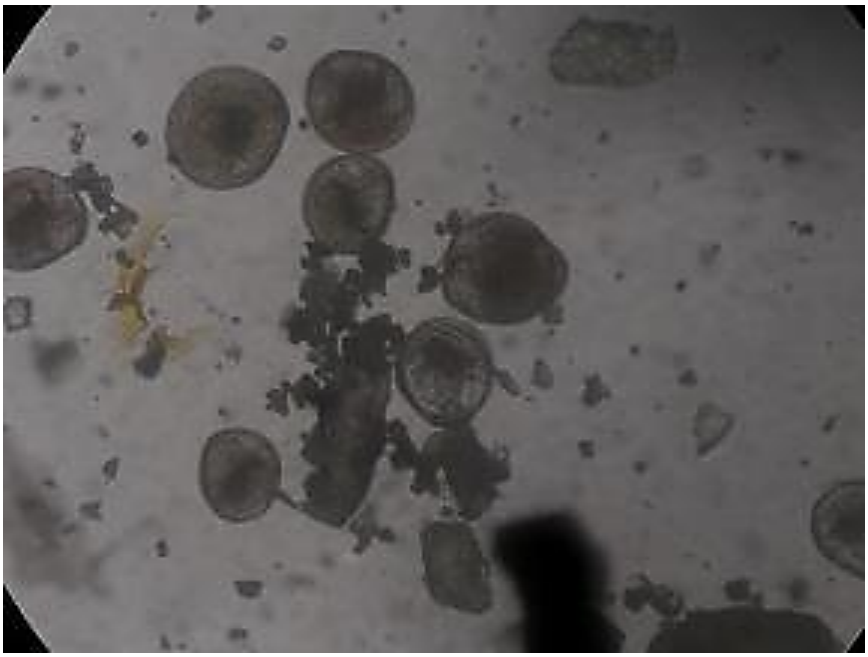

Figure 8.1.h: Plankton sample containing O. edulis larvae (Image courtesy of Iarfhlaith Connellan, 2019).

In Pond seven larval releases reached 45 larvae/litre showing 12% at settlement stage by the 30th of June 2019. The peak of larval numbers in pond seven was reached on the 10th of July (189 larvae/litre) of which 60 larvae/litre were at the settlement stage.

Larval releases in Pond eight began gradually in mid- June and reached a peak of 108 larvae/litre on the 30th of June 2019. This peak consisted of 30% larvae 250 microns in shell length and were deemed competent and ready to settle.

Larval densities in the ponds varied over time and peaked between Jun 30th and July 10th as illustrated in figure 8.1.i, this trend was observed across all ponds. One pond only showed slow release of larvae throughout the summer and never peaked. Larvae 250 µm in shell length were deemed competent and ready to settle.

In general, the percentage of competent larvae (i.e. ready to settle) at peak spawning was between 12 and 30%.

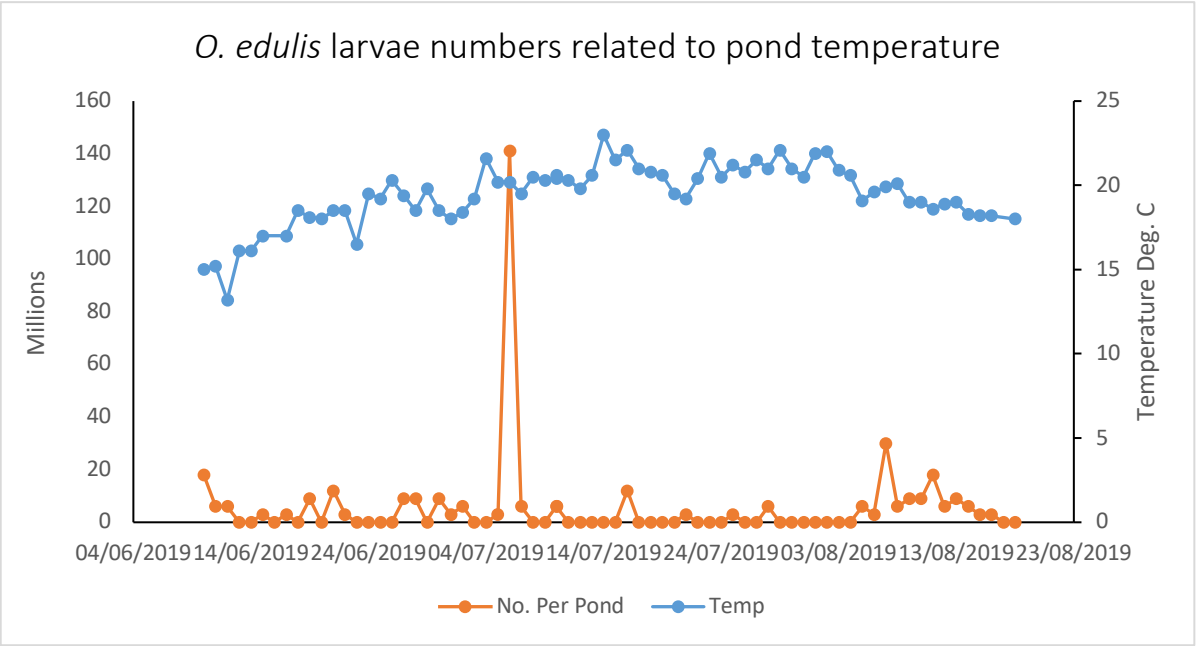

Figure 8.1.i: initial O. edulis larvae numbers for pond No. 2 for the 2019 season.

Figure 8.1.j: Unglazed plates used for assessing daily settlement numbers. Settled O. edulis post larvae can be seen on the plate (Image courtesy of Colin Hannon, 2019).

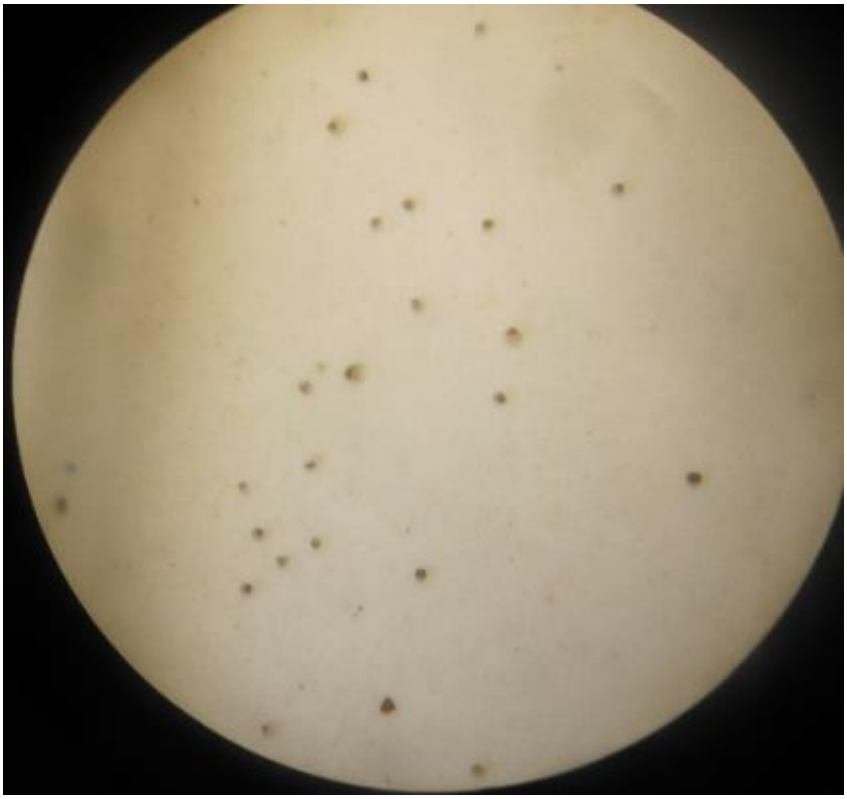

Figure 8.1.k: Settled individual O. edulis as seen on the settlement plates under a dissection microscope (Image courtesy of Iarfhlaith Connellan, 2019).

Discussion

Larval diet

The first results from culture trials and macronutrient analysis look very promising. Based on the culture trials, all five species tested so far are suitable for large scale production in hatcheries, and their macronutrient composition is suitable for oyster larvae. The coming fatty acid composition analysis will provide further insight into what microalgae species may be most suitable for culture of *C. gasar* larvae.

Water quality

The implementation of a semi-closed RAS system at the Primar hatchery was identified to be the key to a substantial improvement in the rearing environment of *C. gasar* larvae. The best option for the hatchery is a combination of the current continuous supply system and a new semi-closed RAS that concurrently supply the hatchery with water for the cultivation of seeds and larvae, respectively (the semi-closed RAS system being used for the more delicate and vulnerable larval stages). With the support of external partners, linked to the Federal University of Santa Catarina and the Federal University of Parana, the first studies will be planned during M13-18 and a budget plan for acquisition and assembly of the semi RAS systems must be developed. The system will be funded by the Primas hatchery, but experimental work is included in the AV project to explore effects of the system modifications on larval survival.

Conditioning

Not available

Production of triploid Pacific oysters

Not available

Nursery systems

Although the protocol for monitoring of system performance was agreed with the IRG partners at the initiation of the trial, it turned out to be too labour intensive for the farmers to maintain. For future trials monitoring should either be performed by the research partner or ambitions for data collection lowered and the protocol simplified. Moreover, qualitative observations of the pilot system highlighted possible drawbacks (such as green macroalgae fouling inside of the pilot system) and adjustment needs of the system. One option discussed for next season was to deploy the system in more exposed locations and increase flipping frequency. Overall the IRG partners were enthusiastic about developing the system further.

Spatting pond production

The production of *O. edulis* juveniles for was successful for the 2019 season. The methodology presented not only validates a process for implementation at other spatting pond facilities but also boosts the utilisation of this technology. Further data exploration and analysis from the 2019 season is needed. Cartron Point shellfish plan to contribute their 2020 season data also as a compassion once completed and analysis can be carried out on data from two consecutive years of production. Spatting pond settlement has the capacity to produce Ostrea edulis in a very economical and timely manner when compared to conventional hatchery production. It is possible that the greatest weakness of hatchery produced of native European oyster spat is the narrow genetic base forced on producers by the selection of Broodstock, seldom more than 6-8 individual females contributing to any one production batch. This results in a very homogenous genetic origin of all hatchery produced oysters with low genetic diversity. This can easily result in inadvertent selection for adverse genetic characteristics and inbreeding in a stock.

Spatting pond production requires broodstock of more than 1000 oysters for each pond providing an annual genetic potential contribution from 10,000 to 12,000 individual oysters. It is also possible in a spatting pond production run to provide a mixed genetic contribution from a variety of sources of broodstock. Further investigation is required to identify the intricacies between ponds and groups of oysters to gain a better understanding of the complex environment in a spatting pond.

A technical report will be produced based on all data from this trial which will contribute towards best practice methodologies for WP1.

Progress, deviations, problems & next 12M

Larval diet

Progress: Native microalgae has been sampled, isolated and grown as planned. The transfer of cultivation technology between Larbim UFPB and Primar was successfully carried out. Biomass of native microalgae for nutritional analysis was produced and sent for analysis. Macronutrient analysis of the cultured microalgae was completed. Progress in the task has been slow between M9 and 12 due to Covid-19. Progress on the overall task is between 0-5%

Deviations & Problems: Due to closure of the lab at the University of Paraiba due to Covid-19, the cultured microalgae had to be frozen and stored for later analysis of fatty acid composition. The task will experience a delay and it remains to be seen if the stored samples can be used once the lab reopens. The identification of microalgae by molecular analysis also depends on the reopening of

laboratories on behalf of Covid-19. The expected outcome of those tasks remain unchanged at this time.

Outlook: Fatty acid composition analysis of the produced samples and selection of microalgae to be used in feeding trials with *C. gasar* larvae will be performed in the months to come. The prototype protocols for microalgae culture will be tested again before next seasons larvae production (estimated to start M20).

Water quality

Progress: Progress was according to plan until M9 and then slowed down between M9 and 12 due to Covid-19. Progress on this task is between 0-5%.

Deviations & Problems: Slow progress during M9-12 due to Covid-19 as industry partners experienced a need to focus more on their core activities in contrast to research activities.

Outlook: Adjustment of the hatchery for hatchery trials during M12-18.

Conditioning

Progress: Progress was according to plan related to the protocol development. The lab construction was delayed by Covid-19. Progress on this task is between 0-5%.

Deviations & Problems: Due to Covid-19, the lab at Embrapa was closed and the hatchery system could not be installed. This inferred a delay in the task until the lab can reopen.

Outlook: Installation of the hatchery system at the Embrapa lab as soon as possible.

Production of triploid Pacific oysters

Progress: The activity was planned to be initiated M18 and consequently there was no progress during M1-12.

Deviations & Problems: None at this point

Outlook: None until M18

Nursery systems

Progress: A prototype low-tech nursery system was developed and tested during summer 2019. Qualitative data was obtained and adjustment needs for development of a second prototype was identified. Progress on this task is between 0-5%.

Deviations & Problems: The data collection by the IRG partners was limited. The planned continuation (M12) was cancelled due to the members in the IRG having to focus on their core activities instead of innovations in their production systems due to Covid-19.

Outlook: A new trial was planned for summer 2020 (M12-18), but this was cancelled due to time constraints for industry partners.

Spatting pond production

Progress: Data on productivity and larvae settlement was collected throughout the summer 2019 and data analysis will take place during fall and winter 2019. The task is proceeding as planned. Progress on this task is between 0-5%.

Deviations & Problems: None at this point.

Outlook: The evaluation data will be used to refine the protocol, after which a second run of evaluation, including more industry partners, will take place.

CST 8.2: Evaluation of alternative techniques and development of new protocols for collection of wild settled oyster spat targeting native oyster species in Scandinavia and Brazil

Responsible CS Task Leader Åsa Strand, IVL Swedish Environmental Research Institute

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
8.2	Evaluation of alternative techniques and development of new protocols for collection of wild settled oyster spat targeting native oyster species in Scandinavia and Brazil.	T1.2, T1.3, T1.5, T3.2	IVL, GMIT, EmBraPa	0-5%	✓	M1	M3	M46	0	4-5	7

Introduction

T8.2 aims to increase availability of spat of native and underutilised oyster species in Brazil and Scandinavia by evaluating alternative techniques and develop new protocols for collection of wild settled oyster spat targeting native oyster species in Scandinavia and Brazil. The task includes three specific sub-tasks, each containing one activity, i.e.

1. evaluation of **substrate** specific settlement preferences of native species,
2. development of a **protocol** for targeted capture of native oyster species over Pacific oysters (non-native species in both Brazil and Scandinavia), and
3. development of an algorithm for **automated spat identification,** as described in the CS8 workplan (annex 3 of D1.1).

The target species in AV in Sweden and in Brazil are *O. edulis* and *C. gasar*, respectively. Until a few years ago production of *C. gasar* was based on harvest of wild seed from wild oyster beds, and producers collected oysters about 5-6cm in the estuarine environment and kept them in grow-out structures until they reached about 8-10cm. Recently, pressure on natural stocks has increased and this practice has become unviable. Since there are few established hatcheries and the techniques and technologies for producing *C. gasar* in the laboratory are not yet well defined, the development of methodologies for collecting seeds in the natural environment is important for supplying producers with seed. Also, in Sweden, the oyster industry relies on the successful settlement and collection of natural occurring oyster larvae as hatchery production of the native oyster is erratic. However, sea-based seed production in these areas will result in multi-species seed production, often including unwanted species such as the invasive Pacific oyster (e.g. in Sweden) which due to its status is not allowed for culture, or the slow growing *Crassostrea rhizophorae* (in Brazil).

Therefore, the aim of T8.2 is to explore how the capture of native species can be enhanced and optimized in relation to non-target species. Despite protocol development it is unlikely that the target species will constitute the only species on the collectors, and in many cases the individuals of different species are difficult to tell apart, especially as seed. Therefore, we also aim to develop a tool for automated sorting of oyster seed by species to facilitate the process of extracting the target species from seed collected using sea/estuarine based collectors. One product and one protocol were identified as the potential outcome of the work:

CSTP8.2.1 A new protocol for sea based native oyster spat production

CSTP8.2.2 A new software for automatic identification of oyster species

The ambition of T8.2 for M1-12 was to plan and initiate all activities, both by making a detailed work plan describing the methods and timeframes of the work, and by beginning data collection in all of the sub-tasks. The work plan for CS8 is reported in D1.1, and activities have been initiated in all sub-tasks in accordance with the work plan. The activities performed are described in detail below.

Substrate test

For efficient capture of oyster seed using sea-based seed collectors, a suitable substrate that attract the oysters must be used. The collectors must also be easy to handle for the farmers, cost-efficient and durable as they will be used repeatedly. Different bivalves may display different substrate preferences, yet little is known about the preferences of the target species in Sweden and Brazil. To evaluate substrate specific settlement preferences of native species in Brazil and Scandinavia relative to non-native oyster species, different substrates will therefore be deployed during the main recruitment period of the native species.

Protocol development

Different species may also display different preferences in terms of depth preferences, influence of proximity to wild populations of the target species and they may occur in varying abundances in different geographical regions. In Sweden, the native oyster has been observed to settle during the same time period as the non-native Pacific oyster and capture of the native species can consequently not be separated from the non-native species by timing of collector deployment. There are, however, indications that the Pacific oyster may be more prone to settle on substrates close to the sea surface while the native oyster has a broader depth interval for settlement. Moreover, being a gregarious species, oyster larvae are attracted to conspecifics, indicating that the presence of adult oysters may enhance seed capture. Similar patterns have been observed for Pacific oyster seed on collectors placed on or away from native oyster beds, but has not yet been documented for the native oyster. Moreover, there are strong indications by anecdotal information provided by IRG members that seed capture success in general, and for native oysters in particular, vary significantly between geographical areas. By targeting specific areas for seed collection, fouling of unwanted species such as Pacific oyster may consequently be reduced, yet this pattern must be verified before any recommendations on seed production can be produced. To optimize capture of target species in Scandinavia relative to non-native oyster species, factors affecting species distribution and settlement preferences will be explored. Similarly, in Ireland, very little is known about the settlement preferences of the native oyster. CS8 has therefore developed a synergistic collaboration with the Marine Institute in Ireland and Comharchumann Sliogéisc Chonamara Teo. (Connemara shellfish Co-op Ltd) in Cill Ciaran, Co. Galway. The Co-op wants to better utilise their 140K hectare shellfish aquaculture licensed area starting with the sea-based collection of wild *O. edulis* spat collection. The AV project and the Marine Institute are at this point collaborating on this task to explore similar aspects as targeted in Sweden.

Automated spat identification

Oysters are healthy and sustainably produced foods with great economic value. One of the most valuable bivalve molluscs in Sweden is the flat oyster, *O. edulis*. However, domestic production of oysters fails to meet the demand on the local market at the same time as the Swedish aquaculture industry has difficulties expanding due to limited availability of oyster spat. Traditionally in Sweden, spat for aquaculture have been collected with the help of sea-based collectors, but since the introduction and establishment of the Pacific oyster (*M. gigas*) in 2006, the possibility of collecting spat with this field-based technology has drastically decreased as both flat oyster and Pacific oysters attach to the collectors. Since aquaculture of Pacific oysters is not allowed in Sweden due to that the species is classified as an invasive species, the collected oyster spat must be sorted by species and all Pacific oysters must be destroyed, which is neither practical nor economically feasible for the industry today. The overall aim of this activity is therefore to contribute to an increase in Swedish oyster cultivation by facilitating access to flat oyster spat by the growers by development of an automated way of sorting oyster seed.

Methods

Substrate test

Brazil

In Brazil, a trial with four different collector types traditionally used in Brazilian oyster aquaculture (plastic bottles, plastic bottles covered with lime, bottles with shell powder and sanded bottles Figure 8.2.a) was performed before the initiation of AV. Oysters were sampled from the substrates and were sent for species identification using genetic analysis. In the experiment, some treatments were found to be unsuccessful, e.g. lime on bottles (due to lime falling of the substrate) and shell powder on bottles (too expensive), while others, e.g. sanded bottles resulted in good seed capture. The AV activities build on the results from that trial. There may, however, be more efficient methods for seed collection, as indicated by the use of coupelles in the French oyster industry. The best substrates from the study (sanded bottles) will therefore be complemented with other substrate types, e.g. perforated and non-perforated coupelles and ZapCo spat collectors. To evaluate the role of structure for seed capture, settlement on PVC pipes treated in different ways (sanded, with structure, "plain") will also be evaluated. The substrates were ordered during the first months of AV but were delayed due to Covid-19. Approaching M12, PVC strips for construction of collector types were received and four types of collectors were constructed (sanded PET bottle, smooth PVC, sanded PVC and factory-textured PVC).

Figure 8.2.a: Examples of collector types evaluated during the initial trial. The best substrate in that trial will be used as a reference compared to new types of collectors.

Sweden

In Sweden, oyster farmers are currently using coupelle-racks from France for sea-based seed production. However, the structures are big and heavy, especially at retrieval when they are full of oyster seed and fouling, and the farmers are therefore eager to explore alternatives to this technique. A range of suitable materials have been identified by a combination of tech-transfer between AV partners, industry know-how, review of scientific and grey literature and own ideas. Six different substrates (lamella collector, thick plastic sheet, thin plastic sheet, plastic bottles with and without

lime and a structured plastic sheet) were selected after consultation with members in the IRG and materials were ordered and prepared for deployment. Two replicates of each substrate type will be deployed at three different sites in mid-July (M13), the main swarming period for *O. edulis* in Sweden. Limed coupelles will be used as controls. For each substrate the area was quantified.

Protocol development

Sweden

A stakeholder meeting attended by 13 industry representatives was hosted by IVL in January 2020 (M7, Figure 8.2.b) to discuss industry needs and participation in research activities.

Figure 8.2.b: Participants at the workshop hosted by IVL M7 to initiate the protocol development for targeted capture of native oyster seed using sea-based collectors.

All industry partners agreed to join the study to develop a protocol for native oyster seed collection using sea-based collectors. 22 different sites distributed along the Swedish west coast were selected (Figure 8.2.c), and the farmers reported depth conditions and access to existing infrastructure, as well as proximity to native oyster beds adjacent to the culture sites, to IVL.

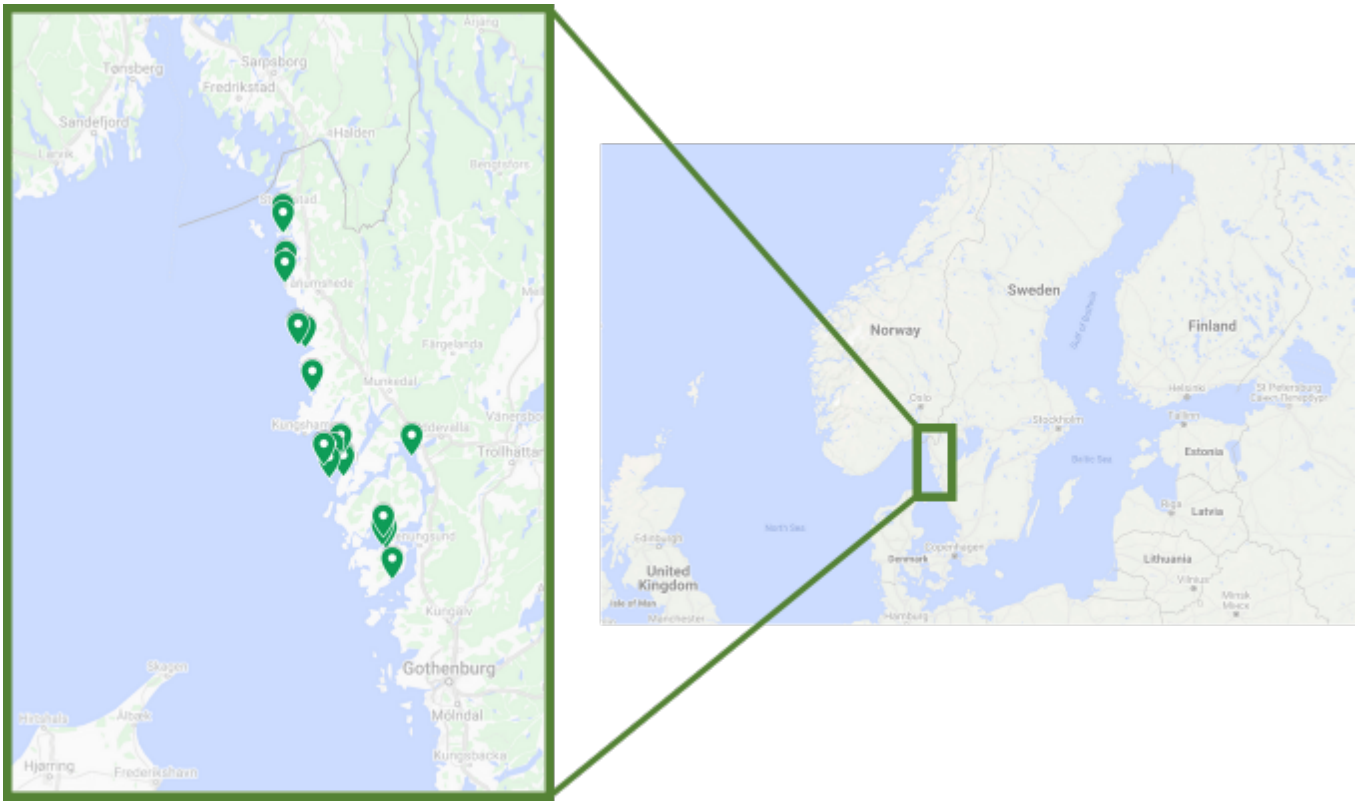

Figure 8.2.c: Illustration of site location where collectors are to be deployed during the protocol development study to evaluate aspects affecting native oyster settlement.

Based on depth conditions at the sites and access to infrastructure, different designs of the systems to be deployed were needed. For sites with existing infrastructure (e.g. longline systems, rafts or other structures), lines with collectors were planned to be attached to the existing structure. For sites with no existing infrastructure, collector ropes were anchored by a weight and supported by a surface based buoy. At each site, five limed collector discs (coupelles) were placed at 1m depth. Depending on the dept of the site, an additional five discs were also placed at 5, 10 and 15 m depth, respectively, if possible as illustrated in figure 8.2.d. An application for a deployment permit for the 22 sites was submitted to the County administrative board, and was granted, before deployment of the structures.

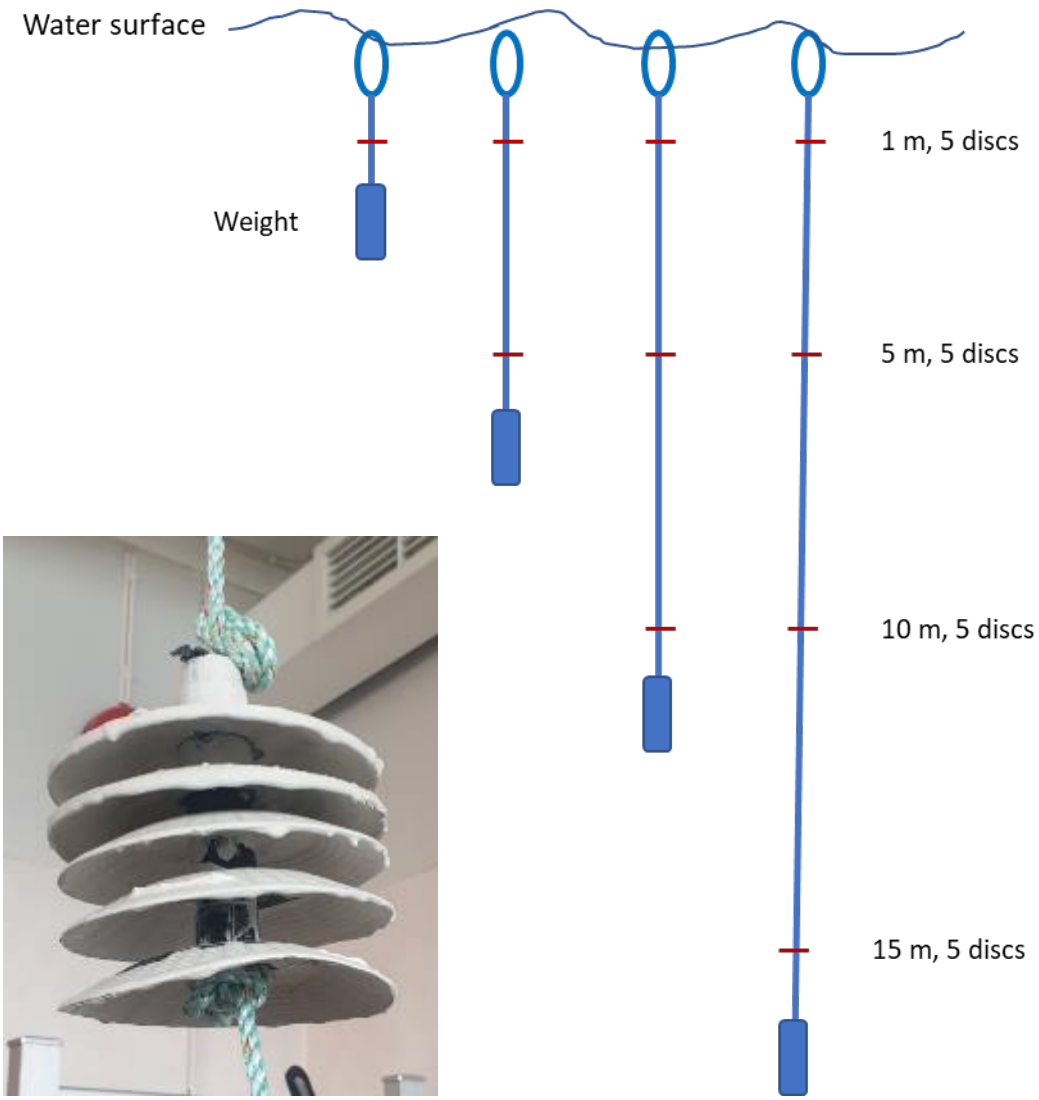

Figure 8.2.d: Illustration of the collector study setup at different sites with different depths, including a picture of what the collector stacks (coupelles) used placed at each depth look like.

Ireland

During M1-12 of AV, several meetings were attended to explore the level of interactions and the scope of activities suitable for these stakeholders and a number of activities were agreed upon. These were:

Mapping of wild beds of native oysters (for site selection of collector deployment)

Performance of industry scale field trials including collector racks

The aspects included in the collector study in Sweden were discussed, ant it was decided that similar aspects should be evaluated also in Ireland, e.g. geographical differences in settlement success, importance of proximity to broodstock oysters, importance of deployment mood of collectors (suspended or bottom based) and evaluation of depth dependent patterns in settlement of the species.

Site selection was carried in out in May 2020 (M12) for initial validation of oyster settlement on mussel and Pacific oyster shell at Cill Ciaran Bay, Co. Galway, Ireland (Figure 8.2.e). The planned large-scale deployment of longlines, collectors and cultch was delayed due to logistics. However, the Co-Op was able to deploy large quantities of cultch at one surveyed location.

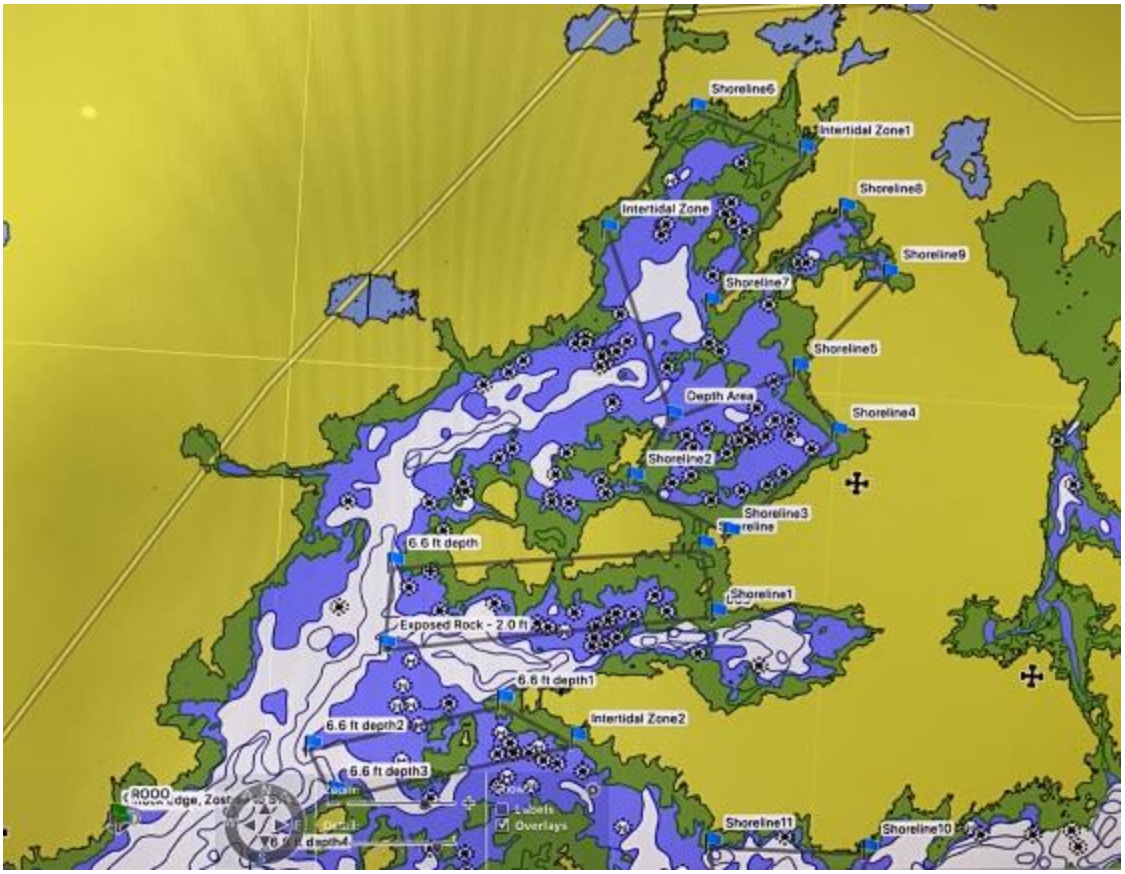

Figure 8.2.e: Surveyed areas of Cill Ciaran Bay, Co. Galway, Ireland.

Automated spat identification

The development of an analysis algorithm for oyster seed identification is a continuation of a previous project run by IVL. In that project, oyster seed was sources from an oyster culture project where sea-based collectors were used to capture oyster seed at five sites along the Swedish west coast in 2018. The collectors were harvested in November 2018 and the seed were sorted manually by species into the categories “flat oysters”, “Pacific oysters” and “unsure” (i.e. individuals for which species could not be determined). The seed were size sorted and the oysters in the categories “flat oysters” and “Pacific oysters” were photographed in February 2019, and then placed for grow out in a suspended culture system at the marine research station at Kristineberg, Sweden. The oysters were approximately 4-5 cm at that time. The pictures were used to produce a first prototype of an algorithm (see below) that can classify images of oysters as either Pacific oysters or flat oysters through image processing and machine learning (artificial intelligence - AI systems).

The AI software uses supervised learning methods. This means that a lot of images of oysters along with corresponding labels of what species they contain is required as training material for the algorithm. It learns from examples, and the more available examples to learn from the better the algorithm. The supervised learning method used was a Convolutional Neural Network (CNN) which is known to be good at classifying images. The specific CNN architecture used was based upon the previously successful network VGG16, known to have performed good in different image classification challenges. Some adjustments had to be made to the network architecture since it initially were created to classify images of 1 000 different classes while this project only required two – Pacific oysters and native oysters. In this project, the pictures were of oysters and the fact their species identification. Over 1 000 images of oysters were used to improve the algorithm's performance. The images were divided into 80% training data and 20% validation data. Training data was used to train

the algorithm and the performance of the algorithm was evaluated based on validation data not used during the training procedure.

After the initiation of AquaVitae, the project was expanded to include also unsure oysters. In October 2019 (M5), 200 oysters from the “flat oysters” and the “unsure” categories were photo documented. Individuals of 4-5 cm were selected to ensure a similar size range as during the previous sampling. The “Unsure” oysters were analysed using qPCR (quantitative Polymerase Chain Reaction) and species-specific primers to determine species. Pictures with a large number of oysters not neatly organised as in the previous pictures were produced to simulate a more realistic sorting situation on a conveyer belt. The same procedure as described above was used to process the images.

Results

Substrate test

No results are available in M12.

Protocol development

No results were obtained during M1-12.

Automated spat identification

No results are available in M12.

Discussion

Substrate test

Not available.

Protocol development

Not available.

Automated spat identification

Not available

Progress, deviations, problems & next 12M

Substrate test

Progress: Progress in Sweden is as anticipated and in accordance to the workplan. Substrates have been identified and prepared for initiation of field trials. In Brazil the activity has experienced significant delays. Progress on this task is between 0-5%.

Deviations & Problems: The activities in Brazil has experienced significant delays due to Covid-19. Equipment has not been delivered and field work has not been possible due to travel restrictions.

Outlook: The field trial in Sweden will be initiated M13 according to plan. Activities in Brazil will be initiated as soon as travel restrictions are released.

Protocol development

Progress: Progress in Sweden is as anticipated and in accordance to the plan. A protocol for the field trial was developed and equipment for the experiment was prepared to be deployed. Progress on this task is between 0-5%.

Deviations and Problems: No problems experienced in Sweden with this sub-task. The Irish collaboration has had their initial plans scaled back due to logistic problems and will aim to increase activities in the 2021 season. Moreover, Covid-19 has reduced interactions in Ireland due to local restrictions, however it may be possible to deploy some material under strict supervision.

Outlook: The field trial in Sweden will be initiated M13 according to plan. Cultch will be deployed and settlement monitored during M13-18 in Ireland.

Automated spat identification

Progress: Progress in Sweden is as anticipated and in accordance to the plan. Pictures of oysters of both species and of unsure individuals have been collected and the unsure individuals have been sent for genetic identification of species. Progress on this task is between 0-5%.

Deviations and Problems: None at this point

Outlook: Once the genetic data is received, the algorithm will be trained on unsure individuals and its performance will be evaluated.

CST 8.3: Developing new, and adjusting existing, culture techniques/grow out systems to native oyster species and local culture conditions

Responsible CS Task Leader Jefferson Legat, Embrapa

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
8.3	Developing new, and adjusting existing, culture techniques/grow out systems to native oyster species and local culture conditions.	T2.2	IVL, BoHu, EmBraPa + IRG members	0-5%	!	M6	M4	M46	0	3-5	5-7

Introduction

T8.3 aims to improve economic sustainability of oyster culture in Brazil and Scandinavia by developing new, and adjusting existing, culture techniques/grow out systems to native oyster species and local culture conditions. T8.3 includes three sub-tasks each containing one activity as described in the CS8 workplan (D1.1). Sub-tasks in T8.3 include

1. **Evaluation and adaptation of existing techniques.** This task aims to innovate and adapt existing growing methods and technology and evaluate the current status of technology for the on-growing of oyster species across the Atlantic area
2. **Development of new culture techniques** and implementation of newly developed systems in the working environment.
3. **Fouling treatments.** As CS8 covers the whole value chain of production the quality of the end product is also taken into consideration by development of protocols to eliminate fouling species on the shells of market ready oysters. Three protocols were identified as the potential outcome of the work:

CSTP8.3.1 Recommendations of oyster grow-out systems for Scandinavia and Brazil including adaptation of existing techniques and newly developed systems.

CSTP8.3.2 New culture system for oysters

CSTP8.3.3 A new protocol for heat treatment of fouling on oysters will be developed

The ambition of T8.3 for M1-12 was to plan and initiate all activities, both by making a detailed work plan describing the methods and timeframes of the work, and by beginning data collection in all of the

sub-tasks. The work plan for CS8 is reported in D1.1, and activities have been initiated in all sub-tasks in accordance with the work plan. The activities performed are described in detail below.

Evaluation and adaptation of existing techniques

The cultivation of *C. gasar* along the Brazilian coast is based on the French system of fixed structures in shallow areas subject to greater tidal variations, practiced in the State of São Paulo and in the north and northeast regions. This system has shown satisfactory results in the South and Southeast regions. However, recent studies have indicated that the adoption of this type of system in the north-eastern region can affect the growth and survival of oysters exposed to high temperatures during periods of low tide. For this reason, is necessary to evaluate and adapt *C. gasar* grow-out systems to different regions of the country.

Similarly, in Sweden, the main culture system used for oysters is the longline system with suspended oyster cages. This system is traditionally used for mussel aquaculture which is an activity that has been performed for many years in Sweden while oyster aquaculture is an emerging activity. Consequently, the systems are not adapted to the requirements of oyster farming, and both oysters and the infrastructure are exposed to significant amounts of fouling during the summer months. The systems are often also placed in deeper water (approximately 10-15 m), hence restricting the sites available for culture, and also cause opposition and reduced social licence due to visual pollution in an area that is heavily used for tourism. The potential of alternative systems better suited for oysters in this area must therefore be explored.

Development new culture techniques

In coastal areas there is a constant conflict of interest between different maritime activities, e.g. tourists, shipping, fishing and aquaculture, and aquaculture is often considered as “visual pollution” since a lot of the structure is visible from the surface. This visual pollution can also lead to a more difficult time obtaining a permit. A way to get around this issue is to use submerged culture systems, where the structures are not visible from the surface. There are some submerged systems available on the market, but most of them are static. Static systems may work for species that doesn’t require a lot of maintenance, and in tidal areas where regular desiccation reduces fouling, e.g. during low tides when the culture systems are exposed to air. Sweden is, however, a microtidal area, and fouling pressure, especially during summer, is very high. The main production area for bivalves is also highly attractive for tourism and recreational activities, and consequently the conflicts between aquaculture and these activities are many. The objective for this sub-task is therefore to develop a prototype for a dynamic system for submerged oyster aquaculture with the potential for automated fouling management, and to test it in field-conditions on the west coast of Sweden.

Fouling treatments

A global challenge for oyster growers is that fouling by marine organisms reduces the survival and market values of the produced oysters (Figure 8.3.b). The type of fouling varies with geographical region, type of environment and depth. Soft bodied fouling organisms such as macroalgae and tunicates are more easily handled compared to calcifying organisms such as barnacles and tube worms, which are notoriously difficult to remove. Current methods for dealing with biofouling on oysters is physical removal (by hand or machine) which is both time consuming and labour intensive. The use of chemicals and biocides are avoided in general within aquaculture due to food safety standards as well as having deleterious effects to the environment. Consequently, alternative methods for treatment of fouling during the culture cycle must be developed. In the Swedish mussel industry, activities have been initiated to develop systems for on farm heat treatment of fouling on blue mussels. As oysters in Sweden are often cultured using blue mussel longline systems (although with oyster baskets instead of mussel lines), there is a high probability that the same system, or with minor modifications, can be

used to treat fouling also on oysters. The effect of heat treatments is, however, affected by season, and treatment temperatures and exposure times are likely to differ between mussels and oysters due to differences in size and shell characteristics. Protocols for heat treatment of oysters must therefore be developed.

Figure 8.3.b: Oyster with fouling by calcifying worms (Pomatoceros triqueter).

Methods

Evaluation and adaptation of existing techniques

Brazil

Six types of grow-out systems for *C. gasar* will be evaluated (Figure 8.3.c):

- Fixed PVC table
- Fixed rope table
- Floating PVC table
- Galvanized steel floating table
- BST basket system
- Pillows fixed on rope according to the traditional method of the producers of Sergipe.

Figure 8.3.c: Examples of grow-out systems to be evaluated in Brazil.

In each type of system, four experimental units (EU) containing 1,000 oyster seeds/EU will be placed. The height of 30 oysters from each EU will be measured monthly, totalling 120 oysters measured per system. In the early stages, survival will be calculated by counting a volumetric sample in each EU. The oysters will be placed in a 200ml container and dead and live oysters will be counted. According to the growth of oysters, the EU will be replaced by EU with a larger mesh and the number of oysters will be reduced to 500 individuals per EU. From that stage on, all oysters will be counted for evaluation of survival.

Sweden

As for the sub-task related to protocol development for sea-based seed collection in T8.2, this sub-task was initiated through the stakeholder meeting previously described (Figure 8.2.b). During the meeting, information about different culture systems used for oyster aquaculture around the Atlantic were presented to the farmers. The information was based on IVL staff experiences from previous projects and observations from conferences and study visits, reviews of scientific and grey literature and knowledge exchange with AV partners. The same partners that decided to work on seed collection also decided to join the grow-out trial. A protocol for data collection was developed jointly and was adapted to industry partners skillset, access to equipment (scales and more) and willingness to invest time.

Sampling will take place every second month during the experiment except for during winter (November-Marsh) when oysters should not be handled due to low air temperatures. 500 oysters will be placed in each system, and bulk sampling will be used to record average wet weight and mortality of the oysters. Length will be measured by IVL every 6-months.

All partners were offered a choice of three systems (Figure 8.3.d), and the suitability of different systems was discussed with each farmer and selection was adapted to site specific conditions (e.g. depth, substrate, exposure and more). An application for a deployment permit for the 13 sites was submitted to the County administrative board and was granted. Native oyster seed were ordered from the local hatchery to be delivered during August 2020 (M15).

Figure 8.3.d: Examples of culture systems offered to the farmers for the grow-out trial of native oysters in Sweden.

Development new culture techniques

A literature study was done to look at different designs of submerged systems globally. Most systems identified were static and relied on tidal movements or required manual cleaning. The basic concept of a bottom bound cage system was selected as a base and adapted to allow for integration into a future automatic cleaning system. The new system consists of a cylinder-shaped oyster cage made of metal. The cage includes 5 compartments and has a hatch to make sure that the oysters are easy to access. With this cylinder shape, the cages can be rotated to clean them from fouling. The cages can also be linked to one another, which makes this system usable both for small-scale farmers and at a larger scale. The design was sent to a contractor in China that produced 40 cages.

Fouling treatments

Literature was reviewed for establishment of a baseline in terms of suitable temperatures and exposure times of oysters and the development of a protocol for lab trials was initiated.

Results

Evaluation and adaptation of existing techniques

No results are available in M12.

Development new culture techniques

No results are available in M12.

Fouling treatments

No results are available in M12.

Discussion

Evaluation and adaptation of existing techniques

Not available

Development new culture techniques

Not available

Fouling treatments

Not available

Progress, deviations, problems & next 12M

Evaluation and adaptation of existing techniques

Progress: Progress in Sweden is as anticipated and in accordance to the plan. Culture technology to be tested has been identified and oyster seed for initiation of field trials have been ordered. In Brazil the activity has experienced significant delays. Progress on this task is between 0-5%.

Deviations and Problems: Due to restrictions caused by Covid-19, non-essential services remained closed for much of the period M8-12 in Brazil, making it difficult to purchase materials. In addition, restrictive travel measures were imposed, restraining access to cultivated areas.

Outlook: Oyster seed and culture equipment will be distributed to the industry partners M15 and growth trials will be initiated. Activities in Brazil will be initiated as soon as travel restrictions are released.

Development new culture techniques

Progress: A new system for oyster aquaculture in microtidal areas was designed and ordered from China. Progress on this task is between 0-5%.

Deviations and Problems: Progress was according to plan until M8 when manufacturing and transports from China was stopped due to Covid-19.

Outlook: Growth trials will be initiated as soon as the new systems is delivered.

Fouling treatments

Progress: The task has started included setting up of the experiment. Progress on this task is between 0-5%.

Deviations and Problems: Progress was according to plan.

Outlook: During M13-18 the protocol for heat treatments will be finalised and evaluated in a pilot trial.

Summary of progress report for Case Study 9

Date of report:	**31.3.2020**
Case Study name:	**Mussels**
of relevane for WPs	**1 and 2 and potentially 3 (task 4)**

Abstract/Summary

CS9 have progressed more or less as planned within M1-M12 with only minor delays. In Task 9.1 a method for larval production and settlement was developed and this method was transferred to DTU staff at the CS9 workshop between DTU, GMIT and CPS. In Task 9.2. the produced mussel seeded lines (Task 9.1) were prepared at CSP to be transferred to sea. The mussel seed on the collectors grew well, however due to Covid-19 restrictions in Ireland the deployment of seeded lines was delayed. The design and deployment of the submergible tubes has progressed as planned (Task 9.3). One of the submergible tubes have been deployed at the DTU mussel farm and seems to work fine, whereas the approval of license for deployment of the platform is still pending. The laboratory experiments with heat-exposed as anti-fouling treatment have been delayed covid-19 and is therefore postponed to M13-M18 (Task 9.4).

CST 9.1 Hatchery protocol for blue mussel seed production

Task Leader Pernille Nielsen, DTU

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
9.1	Hatchery protocol for blue mussel seed production	T1.4, T1.5	DTU, GMIT, CPS	25%	✓	M3	M5	M48	0	4	8

Introduction

A blue mussel seed hatchery protocol will be developed in task 9.1. within M1-M12, CPS and GMIT will test, run and modify the initial protocol developed by CSP in CPS's hatchery. Furthermore, a training programme for DTU staff at the CPS hatchery will also take place to ensure the knowledge transfer from CPS to DTU. DTU will modify the hatchery protocol to fit the DTU hatchery setup and prepare to initiate the hatchery work, which is planned to be carried out in M13-17. The development of a robust transferable protocol will enable continuity of seed supply in failed natural wild spawning and settlement events and also the supply of blue mussel seed in offshore environments.

Methods

CPS staff collected adult blue mussels from the North shore of Galway bay from October 2019 every two weeks to assess fecundity. On the 9th December 2019 collected mussels were ready for conditioning and moved them to their broodstock holding system to feed and monitor fecundity.

Settlement racks were made up from nylon string and wrapped around plastic frames (Fig 9.1.a). the frames were used for the settlement of mussel larvae in down-wellers once larvae are competent.

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement No 818173. This report reflects only the authors' view and that the Commission is not responsible for any use that may be made of the information it contains.

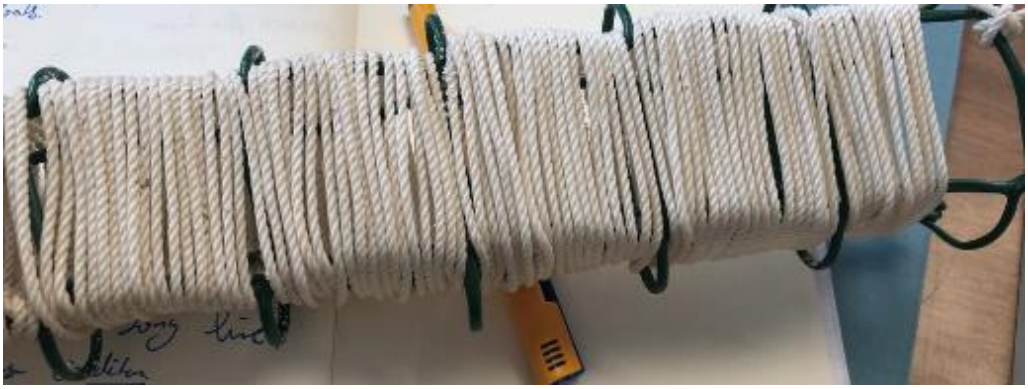

Figure 9.1.a 50m of 2.5mm nylon string on a plastic collector.

Fecundity was assessed visually weekly and were deemed ready to begin spawning at the end of January 2020. Spawning of male and female mussels was induced by temperature shock on the 25th of January 2020.

Conditioning

50-60 adult mussels were collected from the wild (these can also be collected from mussel farm lines). If collecting from the wild medium sized animals of 35-45mm in shell length, if selecting line grown mussels select the largest animals.

Spread the mussels over the bottom surface of the conditioning unit to avoid the mussels clumping together which will decrease the feeding rate. Mussels were maintained at 18°C with a flow rate of 500L/day. The broodstock mussels were fed a ration of 40:60 ratio of *Isochrysis* Spp. and *Chaetoceros* Spp. at an optimum of 120 cells/μL as a maximum. To maintain conditioning the cells per litre must not go below 60 cells/μL.

The period of time for conditioning depends on the animals collected but we held the broodstock for 4-6 weeks and fecundity (ripeness) was assessed weekly.

Spawning & Fertilisation

Two buckets are required at two different temperatures of filtered sea water at 10°C and 25°C, these will be used for the temperature shock. Two further buckets at 22°C will be needed and labelled males and females to separate the broodstock, once the mussels start to spawn they will be placed in their respective bucket by sex.

Once all the buckets are set up, the first set of mussels (n=10) can be taken from the conditioning unit at 18°C and placed in the 10°C bucket (5L of water) where they will remain for 20 minutes. After 20 minutes they are then moved and placed in the 25°C bucket for a further 20 minutes. If no spawning, repeat the process every 20 minutes.

Spawning was successful males and females are divided into their separate labelled buckets which contains 5L of 22°C water. Males release sperm which is white (Fig. 9.1.b), and they are usually the first to spawn. The females release eggs (brown/orange colour) which sink to the bottom (Fig. 9.1.c).

Fertilisation

When the female mussels stop or slow down the release of eggs they are removed from the bucket. The eggs are sieved and rinsed through a 40μm sieve and put into a clean bucket at 22°C with 5L of

clean seawater. At this stage it is important to get an accurate count of the eggs. 50ml of well mixed sperm is added to the bucket of eggs and the gently mixed.

Sample the eggs every 15-minutes to assess fertilisation. At this stage we need to see five sperm per egg, any less than this 50ml of sperm needs to be added again, and wait 15-minutes before sampling again. If there are 5 sperm visible per egg in the sample after mixing every 15-minutes, after one hour indication of successful fertilisation should be visible. Indications of successful fertilisation are polar body expulsion or cellular division.

Once fertilisation is confirmed the eggs are rinsed once more in a 40μm sieve and the eggs were transferred to a 5000L larval bin at 22°C, D-larvae (Fig. 9.1d.) will be visible after 24 hours and an optimum stocking density of 8 larvae/ml was achieved.

After 48 hours after hatching, the water in the larval bin needs to be changed and the larvae need to be sieved without removing the bottom 2.5cm of the larval bin. The larvae can now be replaced into the bin at 20°C and feeding can begin. Full water exchange was carried out ever 48 hours. The larvae are graded by sieves at the water exchanges. For the first 10-days a 30μm sieve then 60μm and finally 100μm.

Larval feeding

The mussel larvae receive a mixed live algal diet of for week one of 75:25 ratio of *Isochrysis* Spp. and *Chaetoceros* Spp. at 60 cell/μL. Week two the larvae received a 50:50 of the same species at 60 cell/μL. the final stage before settlement the larvae receive a ration of 25:75 *Tetra selmis* and *Skeletonema* Spp. at 60 cell/μL. The larval period prior to settlement works out at 20 days at 20°C.

Settlement

Settlement assessment at the final larval stage is assess visually of the larvae when 15-20% foot activity is seen in the cohort of graded competent larvae.

The down-weller sieves (150μm) are set up prior to competent larvae been introduced. The collector frames and string are placed in the down-wellers 24-hours before settlement at 20°C. Larvae are transferred from the larval bins to the down-wellers at 250K larvae per sieve. Larvae were feed to hunger which resulted at 30 cells/μL. Within a couple days settled larvae were visibly attached to the collectors (Fig. 9.1.e). The temperature in the down-wellers was gradually reduced in preparation of transferring the collectors to sea.

Results

The collection and identification of fecund wild broodstock, spawning, larval rearing and settlement has been documented by CPS and GMIT for the winter 2019/2020 was successful and a method for larval production and settlement was developed. This method was transferred to DTU staff during the settlement phase at the CS9 workshop between DTU, GMIT and CPS.

Data analysis from the larval period and initial settlement is currently ongoing by GMIT and CPS.

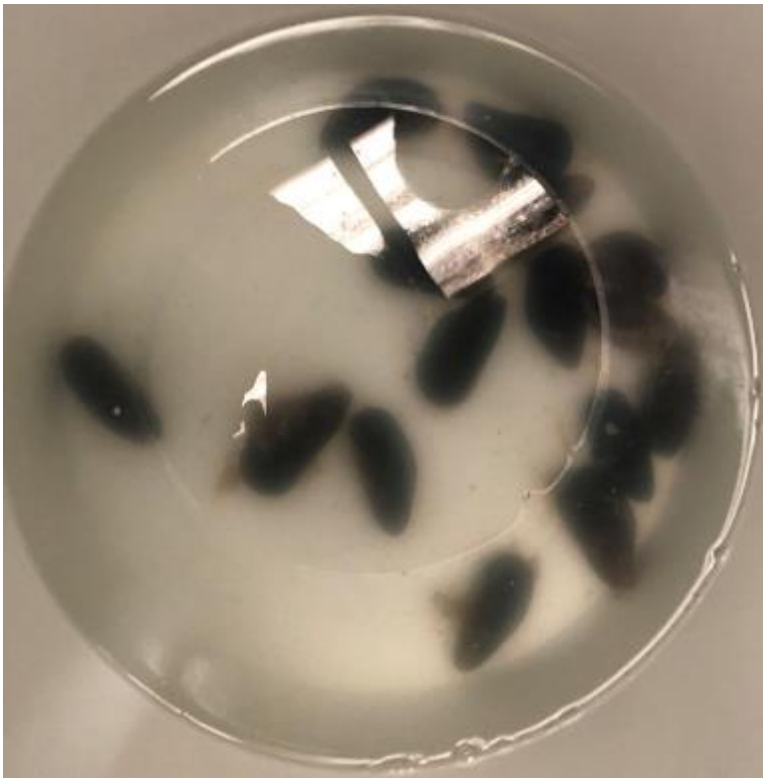

Figure 9.1.b Male blue mussels releasing sperm. The water can be seen to be clouded with sperm (Photo CPS/GMIT)..

Figure 9.1.c, Female blue mussels begin to release eggs, the eggs are the brown lines on the middle right of the photo (Photo CPS/GMIT).

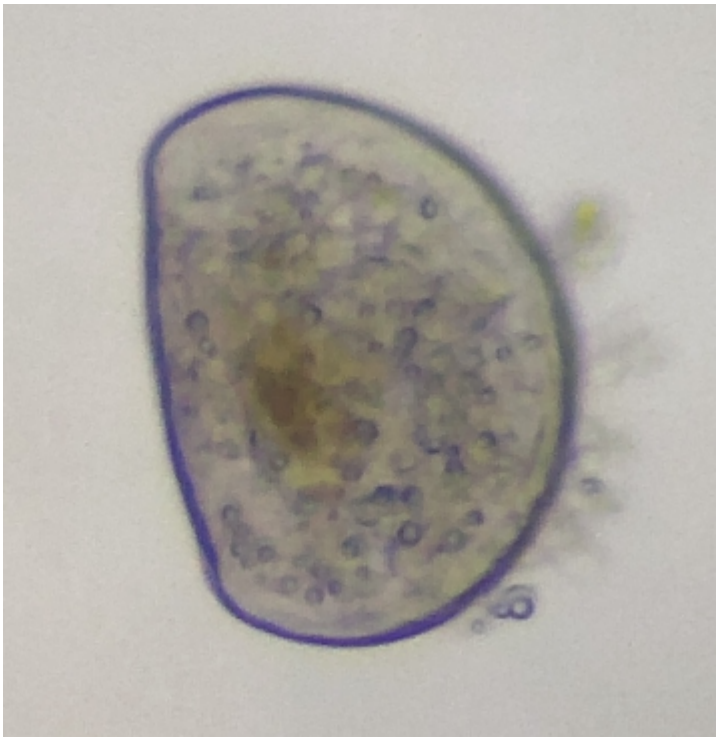

Figure 9.1.d, Blue Mussel D-larvae (Photo CPS/GMIT).

Figure 9.1.e, Settled blue mussel larvae can be seen attached to the collector string (Photo CPS/GMIT).

Discussion

Task 9.1 for CS9 is well developed and a robust hatchery production method was developed with all partners.

The practical components of the tasks in the hatchery were completed in March 2020 for Ireland. However due to nation Covid-19 restrictions it was not possible to continue the monitoring of the seeded collectors during the nursery stage.

A Successful training workshop was held at the CPS hatchery at the end of February 2020 and beginning of March 2020. The workshop included hands on practical work and understanding of the holding of broodstock, conditioning, spawning and larval rearing of blue mussels. During the workshop swimming larvae were transferred for settlement on the collectors which was completed by all CS participants.

Data analysis and exploration of results is currently on going by partners.

Progress, deviations, problems & next 12M

Progress: We assume a 25% completeness for this task. The hatchery practical part of the task in Ireland has been completed as has been the technology transfer of the method to DTU. Report and data analysis are currently ongoing.

Deviations & Problems: The work planned for M13-M17 at the DTU hatchery has been delayed because of COVID-19. The work has been postponed to M16-M20.

Outlook: DTU will initiate the delayed work with testing the modified protocol in M16-M20.

CST 9.2 *In*-situ seed growth

Task Leader Pernille Nielsen, DTU

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
9.2	In-situ seed growth	T1.4, T1.5	DTU, GMIT, CPS	5%	✓	M6	M8	M48	0	4	8

Introduction

A successful production of hatchery produced mussel seeds are not only relaying on the survival rate and production numbers of seeds produced in the hatchery but also on a successful transfer of the seeds from the hatchery to the field. Task 9.1 has developed a transferable hatchery production method which will be implemented and adapted by DTU.

Seeded collector string is maintained in a down-welling system and fed mixed live algal diets prior to the transfer on to droppers at sea. The juvenile mussel seed will as they grow transfer full onto the dropper where at grading the remaining collector line can be removed before redeploying the mussel line.

Methods

Cartron Point Shellfish has previously documented that the transfer of mussel can be done but using similar techniques as for macroalgae. The procedure is that the mussel larvae settle on thin “seed lines” (Figure 9.1.a), which is then wrapped around continuous droppers, which will provide sufficient growth-material for the mussels to attach to, when the grow, and thereby reduce the loss of mussels by self-thinning. Mussel growth will be monitored in relation to e.g. biomass, shell length, numbers/m during the growth season.

Results

T9.1 developed seeded line with blue mussel seed which was awaiting transfer to CSP growing line at sea. T9.2 was well developed and under way at CPS and GMIT when national restrictions due to Covid-19 came into place. GMIT staff were unable to monitor or visit CPS facilities.

CPS will maintain mussel seed.

Discussion

On-growing systems at sea in Galway bay were prepared in early March 2020 at CPS in preparation of the transfer of seeded lines to sea. The mussel seed on the collectors grew well, however Covid-19 restrictions where put in place in Ireland in the middle of March 2020 which delayed the deployment of seeded lines.

Progress, deviations, problems & next 12M

Progress: We assume 5% completeness for this task. Seeded line of blue mussel seed was successfully maintained and on-grown at CPS land-based nursery unit but the majority of the work is yet to come.

Deviations & Problems: Due to the COVID-19 restrictions the mussel seeds produced at the hatchery at CPS have not yet been transferred to grow-out and is currently still in the nursery. The mussel seeds will be lost if they are not transferred to grow-out very soon.

Outlook: If the CPS produced seeds are transferred to grow-out the biomass monitoring will be initiated and continued during M13-M24. Furthermore, DTU reared mussel seeds will be transferred to grow-out and monitored in M20-M24.

CST 9.3 Modification of novel production systems for mussel producing in exposed areas

Task Leader Pernille Nielsen, DTU

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
9.3	Test of modification of novel production systems for mussel production in high-energy areas	T2.2	DTU	10%	✓	M11	M11	M48	0	2	6-7

Introduction

DTU will identify problems with mussel farming in exposed areas in Denmark, which will include meetings with Musholm A/S and Kerteminde Muslinger IVS from the IRG. Based on inputs form the two mussel farmers, two different mussel productions systems will be tested at two different locations in Denmark. One modified tube and net system that can be submerged and a classical “Spanish” raft made of concrete and steel. In M1-M12 the focus will be on preparation, planning and modification of novel production systems to be ready to deploy the system in the coming mussel production season starting at the end M12.

Methods

DTU will design a submerged tube and net system by modification of the existing system designed and used at Musholm A/S. To be able to submerge the tube, three inner tubes will be installed in the main tube. Furthermore, DTU will engage and advice in the discussions of moorings and deployment of the platform at Kerteminde Muslinger IVS as well as apply for a license for the platform at the authorities.

Results

Two submergible tube systems of each 50m have been designed and produced. In M10, one of the tubes was equipped with a 17.5 cm mesh net, then towed out to DTU’s mussel farm and moored to

two screw anchors (Fig. 9.1.a), whereas the other tube was towed from DTU in Nykøbing Mors, Denmark to Musholm.

Figure 9.3.a: Submergible tube deployed at the DTU mussel farm. The tube is kept elevated at outlet side

The approval for the licence for deployment of the platform is still pending in M12. Furthermore, due to COVID-19, installation of the platform was not possible in M11 as planned.

Discussion

The design and deployment of the submergible tubes has progressed as planned and seems to work fine based on the very limited time for observation after the deployment at the DTU farm.

Progress, deviations, problems & next 12M

Progress: We assume a 10% completeness for this task. The design and deployment of the submergible tubes has progressed as planned, whereas the deployment of the platform is delayed due to COVID-19 and approval of the licence is also still pending.

Deviations & Problems: The delay of the deployment of the platform has the consequence that no biomass monitoring of mussel growth can be initiated until the next spring larvae settlement (M23-M25). However, the performance of the platform after deployment will still be possible.

Outlook: The second submergible tube is planned to be deployed at Musholm in M13 with subsequently biomass monitoring for both tubes (DTU farm and Musholm farm). Furthermore, deployment of the platform has been planned for M16.

CST 9.4 Heat-based anti-fouling method

Task Leader Åsa Strand, IVL

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
9.4	Heat-based anti-fouling methods	T2.2	IVL, BoHU	0%		M8	M8	M46	0	3	6

Introduction

A field-based non-toxic antifouling heat-based method for calcifying worms (*Pomatoceros triqueter*) on blue mussels will be developed in Task 9.4. In M1-M12 preparation, design of laboratory studies and laboratory experiments will be carried out to develop a protocol for heat-based anti-fouling treatment.

Methods

To identify the optimal temperature and exposure time for antifouling treatments of blue mussels. Trails testing six different temperatures between 30 to 55°C with 5°C interval and all with an exposure time of 45 seconds will be carried out. The mortalities of the worms and the survival rate of the mussels were calculated. The aim for the protocol is that >90% of the worms will die and <10% of the mussels will die of the treatment.

Results

No results have been obtained, since the COVID-19 delayed the laboratory experiments. The design of the laboratory experiments is ready as well as the design of the field study of fouling organisms, which will be initiated in M13.

Discussion

N/A

Progress, deviations, problems & next 12M

Progress: We assume a 0% completeness for this task. Task 9.4 has been delayed due to COVID-19. However, a contingency plan is being prepared to ensure that the laboratory trails can be initiated within the next 6 months. However, if this is not possible, major changes might be needed for Task 9.4.

Deviations & Problems: The delays of the laboratory trails due to COVID-19 has postponed the work to M13-M18 and might need modification of the experimental design.

Outlook: The postponed laboratory trails are planned for M13 along with a field study of the time occurrence of fouling organisms. This knowledge will be used together with the industry build antifouling head-based equipment that can be used on-site at the mussel site, which is planned to be initiated in M20-M24.

Summary of progress report for Case Study **10**

Date of report:	**06.04.2020**
Case Study name:	**Freshwater fish production**
of relevane for WPs	**1, 2**

Abstract/Summary

Within the period from M1 to M12, CS10 progressed satisfactorily especially considering the overall difficulties faced by task leaders on their different activities. CST10.1 ("Optimisation of captive reproduction of Pirarucu *A. gigas"*) started; problems were faced with the delay on having earth ponds (infrastructure) for testing reproduction protocols on pairing fish at EMBRAPA Fisheries and Aquaculture (originally scheduled for M6-M2). These earth ponds will be ready for 2021 (M18-M24) when trials will be set-up. On the other hand, a pilot experiment has been conducted at EMBRAPA Fisheries and Aquaculture aiming at the collection of gametes after hormonal stimulation. This test involved inducing two males and two females with different hormonal therapies. A key output from this pilot study was the demonstration of the feasibility in the collection of milt in the species, a subject which should be further studied along AquaVitae lifetime. The feasibility of egg collection could not be demonstrated, and potential causes are discussed. Also within CST10.1, another activity involved a visit of Embrapa at one stakeholder (Hidrobios, Palmas-TO, Brazil), with the aim of identify the sex of *Arapaima* broodstocks, paving the way for future trials on hormonal induction at this place. Regarding CST10.2 ("Large scale production of triploid tambaqui *C. macropomum*"), Embrapa's original planning of developing a protocol for triploid induction using temperature shocks we changed (Oct/2019) to apply a pressure shock instead. This was made possible after the borrowing of Nofima's hydraulic pressure vessel shipped from Tromsø (Norway) to Palmas-TO (Brazil) and a physical meeting with the execution of a trial at Embrapa facilities at Dec/2019 (CS10 kick-off meeting) involving EMBRAPA and Nofima's researchers. In this occasion, a protocol development plan was discussed and started being implemented. At the same time, a manual pressure vessel from UFSC (AquaVitae partner) was borrowed and shipped to Embrapa Occidental Amazon (Manaus-AM), with the aim of having two facilities to run the proposed trials along AquaVitae lifetime. A partnership has been established between Embrapa (Manaus-AM) and Fiocruz Institute (Manaus-AM) aiming to have larvae samples analysed for triploidy induction (flow cytometry) at their laboratory. A trial aiming to determine the time post-fertilisation for pressure application has also been conducted. Work on the flow cytometry protocol to be applied at Fiocruz (Manaus-AM) has been done, but Covid-19 caused the closure of laboratories and experimental sites both at Palmas and Manaus from April/2019 onwards. Finally, CST10.3 ("Characterisation of the intermuscular bones development of tambaqui *C. macropomum"*) has started, aiming to generate important information on the development of intermuscular bones (IB) in Tambaqui. So far, three families of tambaqui have been produced at Embrapa and whole fish samples collected from 75 - 121 days-post-fertilisation (DPF). Following, one pilot test clearing samples with pancreatin enzyme and staining bones with alizarin dye has been done aiming to describe intermuscular bones. As for other CS tasks, closure of laboratories and facilities due to Covid-19 forbade advancing with data collection and fish clearing procedures from April 2020 onwards.

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CST 10.1 Optimisation of captive reproduction of Pirarucu (*A. gigas*)

Responsible: Lucas Simon Torati, EMBRAPA Fisheries and Aquaculture (Palmas-TO)

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
10.1	Optimization of captive reproduction of Pirarucu (A. gigas)	T1.2, T2.2	EMBRAPA, UNESP	10%	✓	M1	M4	M48	NA	3	5

Introduction

CST 10.1 has the overall aim to optimise protocols used for captive reproduction of *A. gigas*. The current protocol relies on the natural reproduction of couples separated in earthponds, where spawning is expected to occur naturally during the rainy season (Nov to May), though this method is inconsistent and inefficient. Aiming to improve this protocol, this task will test hormonal and/or environmental manipulations to try to stimulate natural reproduction and try to develop novel protocols to enable the collection of gametes, evaluating the possibility of doing artificial fertilisation in the species. Research on Arapaima reproduction strongly relies on the availability of large number of earth ponds and broodstocks, which are expensive and difficult to be used for research purposes. CST 10.1 relies on limited amount of earth ponds (potentially 12) at Embrapa Fisheries and Aquaculture. So, in the first 12-months of AquaVitae, efforts were initially turned into find and develop three different stakeholders in North Brazil for potential collaboration on the research to be developed along AquaVitae lifetime. Also, the feasibility of collecting gametes in the species is very questionable and so far has not been demonstrated. Since this would promote a step change in studies with the reproduction of the species, a pilot study has been organised and developed (Dez/2019) during CST 10.2 kick off meeting (Palmas-TO). Work has then been done in one of the three stakeholders (Hidrobios, Palmas-TO), where their broodstocks had their sex identified by Embrapa using cannulation method. On this occasion, couples were also paired in earth ponds, and this paved the way for future tests on the induction of reproduction using GnRHa implants at this stakeholder.

Methods

A pilot experiment has been conducted at Embrapa Fisheries and Aquaculture on Dec/2019. This trial aimed to evaluate the possibility of gamete collection in the species after application of varied hormonal therapies at considerably “high doses”. Table I summarise weight of animals and hormonal treatments applied. To do so, two adult females and two adult males were selected from earth ponds. Females had their gonad maturation evaluated after collection of an ovarian biopsy following Torati et al (2019)[32]. Both females had their leading cohort oocytes at the final maturation stage. Blood samples were collected before and after injections for future steroid analyses. After application of second dose, animals were conditioned at two circular 15,000L tanks aiming to be observed for possible egg release (Figure10.1.a). It was given a period of 300 degree-hours from second hormonal application, and then tanks had their water level lowered, and gamete collection was attempted after: 1) stripping females contained over a wet mattress and 2) emptying the urinary bladder of males with a cannula, and striping males and for milt collection using dry 1ml syringes (Figure10.1.a).

[32] Torati, L. S., A. F. Lima, L. N. Ganeco-Kirschnik, and H. Migaud. 2019. Endoscopy and Cannulation as Non-Invasive Tools to Identify Sex and Monitor Reproductive Development in *Arapaima gigas*. Copeia 107:287-296.

Table 10.1.a: Details of the hormonal therapy pilot study on Arapaima gigas at Dec/2019. Details on fish Passive Integrated (PIT) ID Number, sex, weight, the hormonal therapy applied with dose and application schedule. CPE – Carp Pituitary Extract; PGF – Prostaglandin F; GnRHa – Gonadotrophin Release Hormone analogue; DOM - Domperidone.

Fish ID	Sex	Weight (Kg)	Therapy	Dose	Schedule
006225	F	22.8	CPE +PGF	5 $mg.kg^{-1}$ + 0.1$mg.kg^{-1}$	10%+90% (12h)
107787	M	22.2	CPE	3 $mg.kg^{-1}$	100% (single)
563329	M	18.5	GnRHa	100$\mu g.kg^{-1}$	100% (single)
563364	F	22.1	GnRHa + DOM	200$\mu g.kg^{-1}$ + 3.8 $\mu g.kg^{-1}$	20%+80% (12h)

Figure 10.1.a On the left, an Arapaima gigas broodstock on a circular tank prior to attempt of gamete collection. Water level is low to facilitate fish handling. On the right, procedure of milt collection after fish being contained on a wet mattress.

Also, a technical visit has been made at one stakeholder (Hidrobios, Palmas-TO) by Feb/2020, where a total of 15 adult broodstocks had their sex identified using cannulation method and couples separated into earth ponds for future use in the project (Figure10.1.b). All fish were measured for its total length (TL), weighted and an ovarian biopsy taken for sex identification. When cannulation could not be performed (coelomatic cavity could not assessed), fish were regarded as male according to Torati et al (2019)[33].

Figure 10.1.b. On the left, the capture of Arapaima gigas broodstock broodstocks in earth ponds using a net. On the right, the procedure of cannulation for sex identification in the broodstock for pairing couples in earth ponds and increase reproduction probability.

Results

The hormonal therapies tested in the females did not cause evident ovulation and/or release of eggs on the circular tanks where broodstocks were contained. On the other hand, we were able to collect milt from the Arapaima, confirmed by the microscope identification of spermatozoa (Figure 10.1.c).

[33] Torati, L. S., A. F. Lima, L. N. Ganeco-Kirschnik, and H. Migaud. 2019. Endoscopy and Cannulation as Non-Invasive Tools to Identify Sex and Monitor Reproductive Development in *Arapaima gigas*. Copeia 107:287-296.

From each male, volumes from 1.3 to 3 ml were collected. The collected milt was a viscous translucent liquid. Observed spermatozoa were already activated when observed, indicating a possible urine contamination. After moving the four fish back to their original earth ponds, three of them (two females and one male) died two days post hormonal injections.

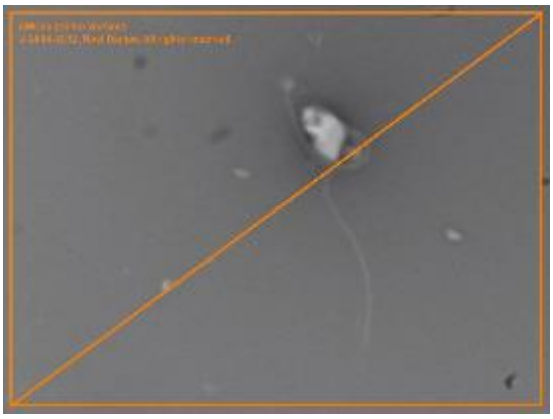

Figure 10.1.c. Arapaima gigas spermatozoa observed under a microscope.

In Table 10.1.b we summarize the biological data collected at Hidrobios stakeholder (Palmas-TO), where we were able to pair six couples in different earth ponds. These are now set up for future tests and monitoring of reproduction along AquaVitae.

Table 10.1.b. Details of the sex identification work on Arapaima gigas from Hidrobios farm at Feb/2020. Details on fish Passive Integrated (PIT) ID number, weight, total length, sex after cannulation, fish original and destined ponds after sex identification.

Fish PIT Tag	Weight (kg)	Total Lenght (cm)	Sex after cannulation	Pond of Orign	Destined to pond
11401236	54,4	1,84	F	27	16
00387047	51,2	1,71	M	27	16
00769405	24,5	1,41	F	2	6
00031998	48,9	1,74	F	2	7
00770456	52,8	1,73	M	2	7
90005410	54,1	1,77	M	1	2
00770457	32,4	1,58	M	1	6
00757570	46,9	1,8	F	1	2
00769406	42,3	1,75	M	1	9
00769443	60,8	1,86	M	1	8
90005471	17	1,37	M	1	Tucunaré
11328191	20,6	1,34	M	1	Tucunaré
00769403	43,3	1,72	F	5	9
00770459	24,8	1,56	F	5	Tucunaré
00769401	29,2	1,36	M	5	Tucunaré

Discussion

The main goal of our pilot study was to provide insight into the feasibility of collecting gametes in *A. gigas*. A first discussion among Embrapa and UNESP researches was made during CS10 kick off meeting, when the more likely hormonal therapy to apply on a pilot study. It was taken into account the high risks of losing such valuable fish, but attempts to collect gametes in the species had never

been done using such hormones. Although these therapies resulted in high mortality, some insights were gained and should guide our actions in the experiments to come in AquaVitae lifetime. First, a good sign was the indication that collection of milt can become feasible in the species. Effort should be directed to this as a potential Key Exploitable Result (CSTP10.1.2.). Future trials from M13 to M24 should aim at providing basic information after physiological analysis of the collected milt. For this, Computer Assisted Sperm Analysis (CASA), ATP concentration and osmolality aspects of milt should be investigated. Second, results obtained so far indicate that acute applications of hormone therapies are risky to broodstocks when compared to the application of slow-release delivery systems, already used for the species[34]. Therefore, efforts should be made towards the in-house making of such implants for tests on experiments aiming to stimulate natural reproductions in couples paired in earth ponds. These implants should be tested together with environmental manipulations on water depth as predicted in the original work plan. Acute injections should be avoided in future experiments with the species. Finally, an important step has been given with the broodstock screening (weight, TL, sex) at a key stakeholder from CS10. It is expected that once we become able to make GnRHa implants, application/testing on this stakeholder be feasible.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained so far, 10% of the work has been done towards the achievement of results from CS10. 1: "Optimization of captive reproduction of Pirarucu (*A. gigas*)". CST10.1 advanced well with the involvement of three stakeholders in the project activities, also with the execution of a pilot test using different hormonal therapies in an attempt to stimulate ovulation/spermiation. Results indicated the feasibility of milt collection in the species and future actions are now turned into this (CSTP10.1.2.).

Deviations & Problems: The feasibility of egg collection remains uncertain (CSTP10.1.3) and will require experiments to monitor oogenesis in females along the experiments to come. Experiments initially programmed to occur at Embrapa relied on the finalization of an infrastructure (12 earth ponds) forecasted for 08/2019, but given issues on the execution of this infrastructure project, these earth ponds could not be available in time to allow the execution of experiments as they were programmed in CS10 work plan. Therefore, we more than ever relied on the infrastructure from stakeholders. Turning our efforts to them, an initial work was conducted at Hidrobios farm (Palmas-TO), where an action was made to Identify the sex of 15 broodstocks (cannulation method) and now five couples are separated in different earth ponds, able for use in future experiments (CSTP10.1.1.). This task was then impacted by the start of Covid-19 pandemic from April/2020 onwards, when Embrapa's laboratories were closed and travelling banned.

Outlook: In the coming months (M13-M24), the planning's are to perform the sex identification at another stakeholder (Fazenda Água Limpa, Aliança do Tocantins-TO), also to revise the available protocols for the in-house fabrication of Ethylene-vinyl acetate (Evac) implants to be used in the coming experiments to start at 2021.

[34] Torati, L. S., J. Taylor, P. E. C. Mesquita, and H. Migaud. 2020. GnRHa implants and size pairing effects on plasma and cephalic secretion sex steroids in *Arapaima gigas*. General and Comparative Endocrinology:113614.

CST 10.2 Large scale production of triploid tambaqui (*C. macropomum*)

Responsible: Fernanda Loureiro de Almeida O´Sullivan, EMBRAPA Occidental Amazon (Manaus-AM)

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
10.2	Large scale production of triploid tambaqui (C. macropomum)	T1.2, T2.2	EMBRAPA, NOFIMA	5%	✓	M1	M4	M48	NA	3	7

Introduction

CST 10.2 has the overall aim to identify an optimal protocol for the large-scale production of triploid sterile tambaqui *C. macropomum*, and further evaluate this protocol under farm conditions and at different climate zones of Brazil. During the first 12-months of AquaVitae project, this task advanced with the initial identification of stakeholders at three different states (Amazonas, Tocantins and Rondônia). The original plan of using temperature shocks was changed to apply a pressure shock onto the fertilized oocytes since it has been discussed pressure shocks are less prone to cause deformities on the treated progenies. For the development of a triploidy protocol using pressure shocks, there are three key parameters that will be optimised. These are: 1) time post-fertilization for when to apply the pressure (Experiment I); 2) pressure intensity (Experiment II) and 3) the duration for pressure application (Experiment III). These experiments are dependent from each other, and should result in an optimal protocol to be developed along AquaVitae lifetime. During the first year of the project, meetings have been made and equipment (pressure vessels) put in place to enable experiments at two different Embrapa units (Palmas-TO and Manaus-AM). Also, a partnership has been established between Embrapa (Manaus-AM) and Fiocruz Institute (Manaus-AM) aiming to have larvae samples analysed for triploidy induction (flow cytometry) at their laboratory along the project.

Methods

At Embrapa Fisheries and Aquaculture, experiments will rely on a hydraulic pressure vessel (TRC Hydraulics, TRC-APV 6.0), whereas at Embrapa Occidental Amazon a manual pressure vessel (Aquatic Ecosystems Inc) is set for experiments (Figure 10.1.a). These two equipment have different internal volumes and reach required pressures at different times. Therefore, for having the application of pressure soon after fertilization (90 seconds post fertilisation), only the TRC Hydraulic equipment can be used, limiting functionality of the manual pressure vessel for Experiment I.

Figure 10.2.a. The hydraulic TRC pressure vessel (left picture, Palmas-TO) and the manual Tuckers pressure Vessel (right picture, Manaus-AM) installed for the development CS10.2. activities on triploidy of tambaqui Colossoma macropomum.

The first Experiment testing the time post-fertilization for when to apply the pressure was made twice during the reproductive season from Dec/2019 and Mar/2020 at Palmas-TO using the hydraulic pressure vessel (Figure 10.1.a.). The set-up of this trial is detailed in Table 10.2.a. This trial consists of six treatments, which are times post fertilization of eggs: 90. 120, 150, 180, 210 and 300 seconds post fertilisation. For this trial, eggs and milt of tambaqui were obtained after stimulation of ovulation/spermiation with conventional reproduction protocol for the species[35]. Each treatment was run in triplicate, and for which 12.5ml of eggs from one female were fertilised with 120µl of milt from a pool of three males. A fixed pressure of 8000psi was applied in the eggs for a fixed 2-minute-time, after which the fertilized eggs were individually incubated in 21 incubators (200L), including a control group for which no pressure was applied. After 6-hours from incubation, fertilisation rates were calculated after counting the viable eggs in triplicate for each incubator. After 12-hours from fertilisation, a problem arose with the RAS, and the fertilised eggs remained without water flow for an uncertain period of time. This caused their egg membrane to stick to each other turning it difficult to accurately estimate the fertilisation rates. After 24-hours post fertilisation, larvae have been photographed and frozen for being shipped to Manaus-AM for flow cytometry analysis to identify percentage of triploidy induction on the treatments.

Table 10.2.a. Experimental set-up to test different post fertilization times for pressure application in fertilized eggs of tambaqui Colososma macropomum.

	Fertilization time (s)	Temp (°C)	Time to start the pressure (s)	Time to reach the pressure (s)	Reach the required pressure (s)	Degree sec to reach pressure (°C.s)	Pressure (psi)	Induction time (s)	End of pressure treatment (s)	Treatment (Time post fertilization) (s)
1	T=0	28	T=52	38	T=90	2520	8000	120	T=210	90
2	T=0	28	T=82	38	T=120	3360	8000	120	T=240	120
3	T=0	28	T=112	38	T=150	4200	8000	120	T=270	150
4	T=0	28	T=142	38	T=180	5040	8000	120	T=300	180
5	T=0	28	T=172	38	T=210	5880	8000	120	T=330	210
6	T=0	28	T=262	38	T=300	8400	8000	120	T=420	300
7	CONTROL (no pressure)									

[35] Woynárovich, A., and R. V. Anrooy. 2019. Field guide to the culture of tambaqui (*Colossoma macropomum*, Cuvier, 1816). Food And Agriculture Organization Of The United Nations.

Figure 10.2.b. On the left, a picture of larvae from treatment 120 seconds post fertilisation collected and fixed frozen for flow cytometry analysis. On the right, the flow cytometer equipment used on the process of protocol development for larvae of tambaqui Colossoma macropomum at FioCruz institute (Manaus-AM).

Results

So far, experiment I has been attempted twice, with results limited to the fertilisation rates (Table 10.2.b). Samples have been collected for flow cytometry but these analyses have not been performed yet. Regarding flow cytometry analysis at Fiocruz Institute (Manaus-AM), preliminary tests have been made with different concentrations of Propidium Iodide. Technical problems were found with the detection of high peaks on the equipment, and the use of a specific DNA filter was required.

Table 10.2.b. Results from the fertilisation rates obtained for Experiment I, which tested different post fertilisation times (90, 120, 150, 180, 210) for the application of a pressure of tambaqui Colossoma macropomum.

Treatment	% Fertilization
Control a	99
Control b	93
Control c	94
T90 a	85
T90 b	95
T90 c	97
T120 a	96
T120 b	91
T120 c	98
T150 a	91
T150 b	98
T150 c	96
T180 a	97
T180 b	89
T180 c	91
T210 a	98
T210 b	91
T210 c	99

Discussion

So far one of the three experiments required for the development of a protocol for triploid production in tambaqui have been executed, with problems faced for maintaining the progenies further. This would have enabled detect possible eventual problems frequently occasioned by a third set of chromosome (i.e. deformities). It has to be acknowledged the enormous effort made on this task to enable the original change from temperature to pressure chock for this protocol development. This required bringing along very heavy equipment either from Norway to Brazil and from Santa Catarina to Amazonas State in Brazil. In terms of logistics, things are in place for execution of trials. Another important fact is the current partnership that has been established between Embrapa and Fiocruz, which will enable analysis of larvae with flow cytometry, essential for this task development. Important to be observed is the short window (November to March) of tambaqui reproduction, which limits execution of trials, and the dependency trials I, II and III have from each other.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained so far, 5% of the work has been done towards the achievement of CS10.2.: "Large scale production of triploid tambaqui (*C. macropomum*)". In a nutshell, CS10.2 advanced well with the establishment of three different stakeholders in three states (Amazonas, Tocantins and Rondônia), with the transportation of pressure vessels among AquaVitae partners and with Fiocruz Institute (Manaus-AM) for flow cytometry analysis.

Deviations & Problems: Experiment I has been partially executed with samples remaining to be analysed for flow cytometry. Initial issues Embrapa had to receive first payment (which required an amendment to the GA), flow cytometry protocol method could not be started earlier (on diploid samples). Problems were also faced with the beginning of Covid-19 from April/2020 onwards, when laboratories from Embrapa and Fiocruz were closed or limited forbidding staff to advance with flow cytometry analyses.

Outlook: In the coming months (M13-M24), the planning is to run experiment I again at Palmas-TO, and await for Fiocruz (Manaus-AM) reopen for partners. At the moment, Fiocruz is heavily being demanded by Covid-19 testing.

CST 10.3 Characterization of the intermuscular bones development of tambaqui (*C. macropomum*)

Responsible: Eduardo Sousa Varela, EMBRAPA Fisheries and Aquaculture (Palmas-TO)

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
10.3	Characterization of the intermuscular bones development of tambaqui (C. macropomum)	T2.2	EMBRAPA	10%	✓	M1	M1	M48	NA	2	5

Introduction

This task aims to characterize the development of the intermuscular bones (IBs) in the tambaqui, *C. macropomum*, with the ultimate goal of generating predictive models to identify the IB type, number and length variation. During the first 12-months of AquaVitae project, this task advanced fairly well with the captive production of three different families of tambaqui which provided important samples for the method implementation and initial morphological analyses. So far, this task is at the stage of processing data (images) from these samples, and has the expectations to include more tambaqui families (genetic variability) and developmental stages in the analyses along AquaVitae lifetime.

Methods

Three different families of *C. macropomum* were produced at Embrapa after stimulation of oocyte maturation, ovulation/spermiation using conventional protocol using Carp Pituitary Extract (CPE), thus enabling artificial fertilisation and larvae propagation. Larvae of the different families were individually reared in 200L incubators and fed with Artemia nauplii seven times a day until 14-days post hatching (DPH). After 14DPH, fingerlings were fed six times a day with powder feed and transition to extruded feed 0.8-1.0mm (45% crude protein) and 1.3-1.5mm (45% crude protein) four times a day, when fingerlings were transferred to different 15m^2 earth ponds composed by an open flow system. Samples were collected (n=30 per family) at 75, 80, 86, 90, 95, 100, 104, 109, 114, 121 days-post fertilisation (DPF) and sacrificed with a lethal dose of Benzocaine (220mg.L-1), then fixed in formalin 4% for diaphanisation and X-ray analyses. In total, 148 individuals we collected. Diaphanisation analyses were made at Embrapa laboratories following protocol from Yao, W. et al. (2015)[36]. The same individuals were also examined using a portable veterinary X-Ray equipment (RX 110/100 Ecoray) at the Catholic University of Tocantins (FACTO, Palmas-TO) after partnership with Embrapa. Images from cleared and scanned individuals were individually analysed visually aiming to identify and categorize the different IBs at the different developmental stages. Also, for IB analysis, tambaqui body has been divided into different body areas (Trunk over Axis, Tail Hindquarters Shaft, and Tail Hindquarters under Shaft) areas aiming to understand the impact different IB types may have in the different tambaqui cuts for the industry after model development. These body areas were based on different similar works done on other finfish species[37][38].

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[36] Yao, W., Lv, Y., Gong, X., Wu, J., & Bao, B. (2015). Different ossification patterns of intermuscular bones in fish with different swimming modes. Biol Open, 4(12), 1727–1732. https://doi.org/10.1242/bio.012856.

[37] Guo, H. H., Zheng, G. D., Wu, C. Bin, Jiang, X. Y., & Zou, S. M. (2018). Comparative analysis of the growth performance and intermuscular bone traits in F1hybrids of black bream (Megalobrama terminalis) (♀) × topmouth culter (Culter alburnus) (♂). Aquaculture, 492(March), 15–23. https://doi.org/10.1016/j.aquaculture.2018.03.037

[38] Cao, D. C., Kuang, Y. Y., Zheng, X. H., Tong, G. X., Li, C. T., & Sun, X. W. (2015). Comparative analysis of intermuscular bones in three strains of common carp. Journal of Applied Ichthyology, 31(1), 32–36. https://doi.org/10.1111/jai.12483

Results

Table 10.3.a. summarises the morphometric preliminary results from IBs collected and measured so far. Overall, preliminary image analyses of the three different families indicated that IB ossification starts around 75 DPH with fingerlings measuring approximately 3.0cm in total length (TL). Also, the stage of ossification first appears in the medium loin then bi-directionally toward the head and tail at approximately 86 DPH. It has been observed the complete IB appearance between 90 and 95 DPH with fingerlings measuring approximately 3.8cm and 5.8cm in SL in most samples analysed. Also, the mean number of IBs observed at 100 DPH decreased suddenly to zero in all samples and families. Further analysis should be addressed to test other clearing methods or experimental designs with various calcium levels. After 109 DPH the IB turned back to appear and increase as growth in SL. There was no difference observed between the number of IBs comparing individual body sides in all DPHs. Compared to the tail body portion, the fish central dorsal part presented a higher number and more varied IB types (Figure 10.3.a). At least 80 DPH in the three families, it was possible to identify six different IB types according to standard IB classification (Wang et al., 20144). These were types "I", "丿" , "卜" "丬" , "one-end-multifork", "two-end-multifork", either epineural and epipleural origin.

Table 10.3.a. Intermuscular Bone (IB) variations in found in Colossoma macropomum at different Days-Post-Hatching (DPH) in individuals from three different families.SL = Standard mean.

DPH	N	IB mean	IB_SD	Min	Max	SE	CI	SL (mm)	SD
75	15	0	0	0	0	0	0	38.093	5.718
80	15	15.933	14.767	0	40	4.846	10.393	35.801	8.641
86	14	34.000	4.624	21	40	1.236	2.670	38.004	6.194
90	15	35.267	2.052	32	40	0.530	1.136	49.254	8.779
95	15	35.667	1.877	33	39	0.485	1.040	58.709	5.301
100	15	0.000	0.000	0	0	0.000	0.000	62.088	14.148
104	15	6.067	9.816	0	32	2.534	5.436	62.831	14.739
109	15	21.267	16.202	0	37	4.183	8.972	77.905	14.312
114	14	28.214	10.245	5	39	2.738	5.915	83.267	14.147
121	15	22.800	11.441	0	38	2.954	6.336	81.905	16.349

The Figure 10.3.a. summarises measurements made on the different body areas (Standard Length, Head Length, Trunk over Axis, Tail Hindquarters Shaft, and Tail Hindquarters under Shaft) along the development of tambaqui.

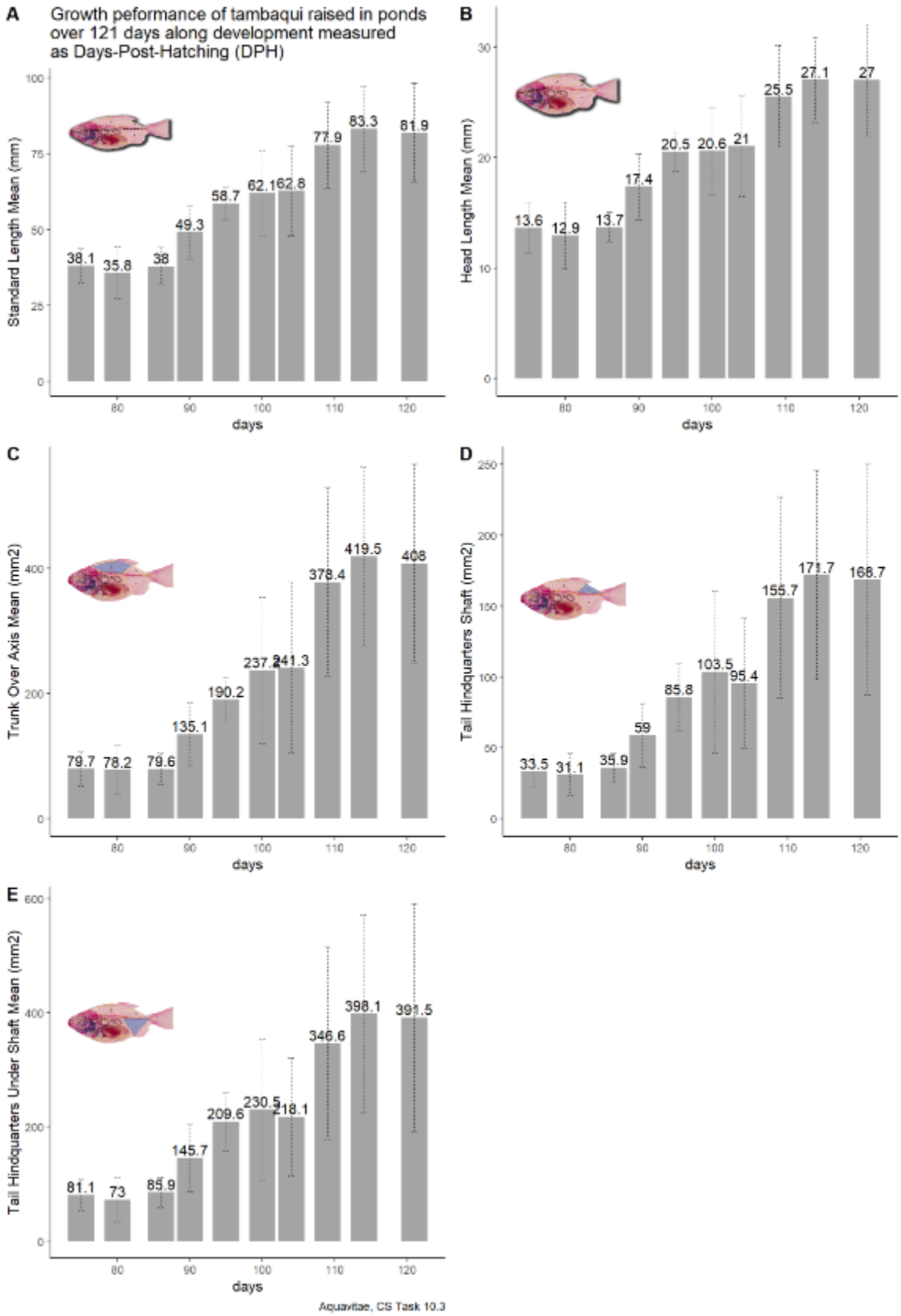

Figure 10.3.a: Different body measurements made on Colossoma macropomum individuals along development measured as Days-Post-Hatching (DPH). A. Standard Length. B Head Length. C. Trunk over Axis. D. Tail Hindquarters Shaft. E. Tail Hindquarters under Shaft.

Discussion

Although preliminary, results obtained so far have demonstrated the feasibility of diaphanization method to produce reliable results on IB morphology in tambaqui. If compared to X-ray scanning, only ossification stages could be observed from 75 DPH onwards. Given diaphanization is more time consuming and expensive than X-Ray imaging, the former method should be applied only to investigate individuals before 95 DPH in in future analyses, and X-Ray should be preferred from 240 DPH onwards.

Overall, results showed little morphological variability within the three different families in early stages, indicating that it is possible count IB phenotypes in precocious periods and should be used for future analyses aiming to generate a robust predictive model to identify the IB type, number and length variation as previous classification models such as Kernel Methods for Pattern Analysis (Mery et al., 2010)[39]. This said, CST 10.3 is in track of its main goal, considerably effort has been made so far and preliminary data gives insight into its potential to generate impact with future breeding programs/genomic editing tools.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained so far, 10% of the work has been done towards the achievement of CSTP10.3.1: "Predictive models to identify the type, number and length variation of intermuscular bones in tambaqui, *C. macropomum*". In a nutshell, CST10.3 advanced well with three families of tambaqui being produced and partially analysed. Pilot tests with clearing-staining (diaphanization) has been conducted and individuals were analysed with the method. An important partnership with the Catholic University of Tocantins (FACTO, Palmas-TO) has also been established with Embrapa and an X-Ray equipment has been made available for use in AquaVitae project. This is especially important to reduce the overall costs of sample analyses.

Deviations & Problems: Some issues were faced with the delay on the building of a new indoor RAS infrastructure at Embrapa, that would have enabled the production of a higher number of families for analysis for this task. Problems were also faced with the start of Covid-19 from April/2020 onwards, when Embrapa's laboratories were closed or limited forbidding staff to advance with image analyses and fish clearing procedures.

Outlook: In the coming months (M13-M24), the planning is to generate more tambaqui families and then increase sample variability (genetic diversity) and turn data modelling statistically more robust.

39 Mery, D., Lillo, I., Loebel, H., Riffo, V., Soto, A., Cipriano, A., & Aguilera, J. M. (2010). Automated detection of fish bones in Salmon fillets using X-ray testing. Proceedings - 4th Pacific-Rim Symposium on Image and Video Technology, PSIVT 2010, 46–51. https://doi.org/10.1109/PSIVT.2010.15

Summary of progress report for Case Study 11

Date of report:	**27.4.2020**
Case Study name:	**Marine Fish Farming**
of relevane for WPs	**1, 2, 7, 9**

Abstract/Summary

CS 11 activities were running according schedule until March 2020. Task 11.1 aimed to produce Brazilian flounder in RAS, this task was not supposed to begin before October 2020. However, as juvenile flounder production for Task 11.2 was carried out in RAS, this info will work as a preliminary study for Task 11.1. Task 11.2 had one experiment planned: determine the protein requirement of juvenile flounder. Task 11.3 aimed to evaluate the protein sparing effect of lipid on the protein requirement for juvenile flounder. During 2019 FURG, CCMAR, and CIIMAR worked together to establish the experimental design and to formulate the experimental diets. Task 11.2 (protein requirement) was carried out during the months of February and April 2020. At the same time, Covid 19 reached Brazil, and we were fortunate to be able to finish the experiment, collect and store samples for further analysis. We were not able to run the experiment planned for Task 11.3 due to the restrictions to operate the hatchery and analytical laboratories, but these experiments were not expected to be finished during the first 12-months.

CST 11.1 Production of Brazilian flounder in RAS

Task Leader Ricardo Vieira Rodrigues, FURG

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
11.1	Production of Brazilian flounder in RAS	T1.2, T1.3, T1.4, T2.2	FURG	10%	✓	M17	M7	M42	0	4	7

Introduction

Marine fish culture in Brazil is a very new activity. There are a few farms producing fish in near-shore cages, but the use of RAS is not a common practice among fish farmers. Therefore, we proposed to use the experimental hatchery at FURG for larviculture and growout of Brazilian flounder in existing RAS. The system for larviculture was already operational, but for the growout phase, some modifications were needed, as we wanted to produce flounder in a race-way. This Case Study Task did not have activities planned for the first 12 months of the project, but the team at FURG worked on the search for funds to make the race-way RAS operational. We also worked on a preliminary flounder larviculture in a RAS with the objectives:

- to optimize the protocol for larviculture of Brazilian flounder in RAS;
- to evaluate the performance of Brazilian flounder growth in RAS, fed on existing commercial diets in the Brazilian market.

Methods

Flounder broodstock were maintained in a RAS under controlled temperature and photoperiod environment (Figure 11.1.a), in order to achieve gamete maturation during the summer months. The system is composed of a circular tank (4 m diameter, 0.8m high) attached to a biological filter, UV sterilization, sand filter, skimmer, and sump. Fish were fed daily on fresh food (fish, crustacean and mollusc) along with dry diets. Natural spawning was not observed and as such females were induced

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to spawn using carp pituitary extract (5mg/kg), males did not receive hormones as running milt could be obtained after simple abdominal massage. Artificial fertilisation was performed and fertilised eggs were incubated. Newly hatched larvae were transferred to larviculture tanks. Larviculture was performed in 300L tanks attached to a RAS equipped with sump, sand filter, bag filter, UV steriliser, and skimmer (Figure 11.1.b). Larvae were fed live feed (microalgae, rotifers and Artemia) and weaned into dry diets.

Results

As this task did not have activities planned for this period, only preliminary data was obtained. Therefore, there is no experimental data to report or discuss. The focus was on learning how to produce Brazilian flounder juveniles in RAS (Figure 11.1.c). The output of this activity was the production of a few hundred juveniles for the experiments in Task 11.2.

Figure 11.1.a: General view of RAS systems at the Federal University of Rio Grande in Brazil.

Figure 11.1.b: General view of RAS systems at the Federal University of Rio Grande in Brazil.

Figure 11.1.c: Juvenile flounder produced in RAS at the Federal University of Rio Grande in Brazil.

Discussion

None (see results)

Progress, deviations, problems & next 12M

Progress: Despite no actual data was registered for this task, and it was not planned to begin before M17, we assumed a 10% completion of the task, because we learned how to operate the flounder larviculture in a RAS, and this had not been done before.

Deviations & Problems: as far as task 11.1 is concerned, there were no deviations and problems during the first 12-months. However, as the Covid-19 pandemics arrived in Brazil, it is uncertain how we will be able to operate from now on.

Outlook: We are assuming we will be able to begin activities in this task as planned in M17 (subject to changes due to the pandemic).

CST 11.2 Determine the protein requirement for juvenile Brazilian flounder

Task Leader Marcelo Borges Tesser – FURG

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
11.2	Determine the protein requirement for juvenile Brazilian flounder	T3.2	FURG, CCMAR, CIIMAR	20%	✓	M1	M1	M21	0	4	0

Introduction

The Brazilian flounder *Paralichthys orbignyanus* is a Pleuronectiformes. It is being considered for aquaculture, and shows important characteristics for intensive production, such as high tolerance for ammonia and nitrite toxicity (Bianchini et al., 1996), and acidification as well (Wasielesky et al., 1997). A protocol for reproduction and larviculture has been proposed by Sampaio et al. (2008). It has been determined this is an euryhaline species (Sampaio and Bianchini, 2002). Actually, larvae do not tolerate salinity below 10‰, but as soon as they metamorphose into juveniles, they will survive in freshwater (Sampaio et al., 2007). Protein is known as an important item in fish diets, it is important for growth (Lee et al. 2002; Kim et al. 2004; Martinez-Palacios et al., 2007), but due to its high cost, it also influences the economics of aquaculture (Lee et al, 2002). The protein requirement of other Pleuronectiformes species varies from 450 g Kg^{-1} CP for olive flounder (*Paralichthys olivaceus*) (Lee et al., 2002) to 515 g Kg^{-1} CP for Southern flounder (*Paralichthys lethostigma*) (Gao et al., 2005). However, nutritional requirements for juvenile Brazilian flounder are lacking in the literature.

Therefore, in this Case study Task, we aimed to determine the optimum dietary protein level for Brazilian flounder when fed isoenergetic diets and reared in a recirculating aquaculture system (RAS). We were able to conduct the protein requirement trial before the proposed timeframe. The second experiment in this task will be carried out as planned from M13 onwards, but this is subject to change, since we have to wait for clearance to work at the hatchery in the course of the pandemic.

Methods

Experimental procedures

Juvenile Brazilian flounder were produced at the Marine Fish Hatchery of the Federal University of Rio Grande (Southern Brazil), following the protocol described by Sampaio et al (2008). This study was approved by the Ethics Committee on Animal Experimentation #029/2020.

This trial was conducted in a RAS composed of 15 tanks (50L), biological filter, protein skimmer, sump, mechanical filter, and UV sterilisation. A group of 195 fish (6.47±0.97g) was collected from the

production tank and transferred to the experimental units (13 fish per tank). Photoperiod was set at 12h light per day, and water was continuously aerated in the sump and the tanks.

Water quality parameters were measured daily. Temperature and oxygen were measured with an oxymeter (YSI 50A, Yellow Springs, OH, USA), salinity using a handheld device (YSI 50A, Yellow Springs, OH, USA), and pH using a pHmeter (YSI® -pH100, Yellow Springs, OH, USA). Ammonia was measured following UNESCO (1983) and nitrite following Aminot e Chaussepied (1983). Nitrate and phosphate were measured weekly accordingly García-Robledo et al. (2014).

Temperature was set to 24°C (24.34 ± 0.06°C), and salinity at 34‰ (34.11 ± 0.01). Average dissolved oxygen was equal to 6,14 ± 0,14mg/L and pH 8.02 ± 0.03. Regarding nitrogenous compounds, total ammonia averaged 0.29 ± 0.09mg/L and nitrite 0.18 ± 0.00mg/L, while nitrate reached 19.76 ± 1.24mg/L. Phosphate at the end of the trial was equal to 2.16 ± 0.75mg/L.

Diet preparation and proximate composition analysis

Five protein levels were evaluated (420, 470, 520, 570, 620 g Kg^{-1}), all diets had the same lipid level (7%) and were isocaloric (488$kcalg^{-1}$). The composition of the experimental diets is shown in Table 11.2.a. Fish meal and fish oil were used as the main protein and lipid source respectively. All dry ingredients were mixed, homogenised, and subsequently oil was added. The mixture was humidified with distilled water at 50°C, it was forced through a meat grinder with 2mm-diameter holes. Extruded pellets were dried in an oven at 60°C for 24h and sieved through 2mm sieves. Diets were kept at -20°C until use.

Proximate composition of diets was determined as follows: moisture was measured after drying samples at 105°C up to constant weight. Ash content was determined after incineration at 550°C. Lipid content was determined by ether extraction with a Soxhlet extractor. Crude protein content was determined by the Kjeldahl method (N x 6.25). All analyses followed the AOAC (1995) standard procedures.

Fish samples were obtained at the beginning and the end of the trial. Fish were pooled, grinded, homogenised, and had their body composition analysed accordingly to the same procedure described for the diet analyses (AOAC 1995). Diets and whole-body fish composition were performed in triplicate.

Feeding trial

The experimental design was entirely randomised, with five treatments tested in triplicate. Fish were fed four times per day up to apparent satiety at (9:00, 11:30, 14:00, and 16:30 h). The amount of food consumed in each tank was registered daily.

All fish were measured at the beginning and at the end of the experiment. Fish were anesthetised with 50ppm benzocaine, and individually measured and weighed. Dead fish were removed and counted whenever found. At the end of the experiment, all surviving fish were counted to estimate survival.

Performance parameters

Effects of different dietary protein levels were evaluated as follows:

Survival (S) = [(initial number of fish – number of dead fish)/(initial number of fish) x 100],

Weight gain (WG) = (final weight – initial weight),

Specific growth rate (SGR) = [(ln final weight – ln initial weight)/(day) x 100],

Feed conversion (FC) = feed intake/ weight gain.

Statistical analysis and estimation of protein requirement

Before statistical procedures, data were tested for homogeneity of variance and normality using Levene and Kolmogorov-Smirnov tests respectively. Data were subjected to one-way analysis of variance (ANOVA) followed by the Tukey's HSD test when significant differences were detected. A significance level of 95% was adopted in all tests. The relationship between SGR and weight gain with dietary protein was analysed using the Broken Line Model in order to ascertain the protein requirement for juvenile Brazilian flounder.

Results

Growth of Brazilian flounder was significantly influenced by dietary protein, fish fed the diet with lowest protein content grew significantly less than those fed a diet containing 620 g Kg^{-1} CP ($p<0.05$). On the other side, survival was unaffected by the content of protein in the diet ($P>0.05$) (Table 11.2.b).

Feed conversion rate was improved as the protein content of the diets increased ($P<0.05$), but total feed consumption was not altered by the diet ($P>0.05$) (Table 11.2.b).

The protein requirement estimated by the Broken Line Model using the SGR (Figure 11.2.a) and WG (Figure 11.2.b) were equal to 500gKg^{-1} CP.

Table 11.2.a. Formulation and proximate composition of experimental diets.

Ingredient (g Kg^{-1} diet*)*	Dietary protein level (g Kg^{-1})				
	420	470	520	570	620
Fish meal	520	580	640	700	760
Dextrin	356	296	239	182	125
Cellulose	30	30	30	30	30
Fish oil	57	54	51	48	45
Min. and vit. premix	10	10	10	10	10
Carboxymethyl cellulose	30	30	30	30	30
Proximate composition					
Dry matter (DM, %)	91.11	90.28	89.88	90.78	91.30
Crude protein (% DM)	42.8	47.2	52.4	57.2	62.1
Crude fat (% DM)	6.9	6.8	6.9	6.9	6.8
Ash (% DM)	8.4	8.3	10.3	10.7	12.3
Energy (Kcal g^{-1})	480.86	484.62	488.38	492.14	495.91

Table 11.2.b. Growth performance, feed utilization, and survival of juvenile Brazilian flounder Paralichthys orbignyanus fed diets containing graded levels of protein for 60 days.*

Parameters	Dietary Protein (g Kg^{-1})				
	420	470	520	570	620
Initial weight (g)	6.45 ± 1.09	6.50 ± 0.85	6.47 ± 1.08	6.46 ± 0.97	6.48 ± 0.89
Final weight (g)	15.88 ± 0.28^{b}	19.51 ± 2.46^{ab}	20.75 ± 2.11^{ab}	20.37 ± 0.93^{ab}	22.77 ± 2.79^{a}
WG[1]	9.43	13.01	14.28	13.91	16.31
SGR[2]	1.58 ± 0.08^{b}	1.82 ± 0.21^{ab}	1.94 ± 0.18^{ab}	1.91 ± 0.07^{ab}	2.08 ± 0.21^{a}
FC[3]	1.19 ± 0.08^{c}	0.99 ± 0.04^{b}	0.86 ± 0.01^{ab}	0.85 ± 0.01^{a}	0.79 ± 0.05^{a}
TFI (g)[4]	145.87 ± 9.5	167.00 ± 29.8	160.81 ± 21.8	154.24 ± 12.6	167.95 ± 19.6
S (%)[5]	97.0 ± 4.4	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0

* Data are mean values (± standard error) of three replicates.

[1] WG (Weight gain) = Final weight – Initial weight

[2] SGR (Specific growth rate) = (ln final weight – ln initial weight)/days x 100;

[3] FC (Feed conversion) = Feed intake /weight gain;

[4] TFI (Total feed intake) = Sum of feed ingested during the trial;

[5] S (Survival) = (Final number of fish/ initial number of fish) x 100.

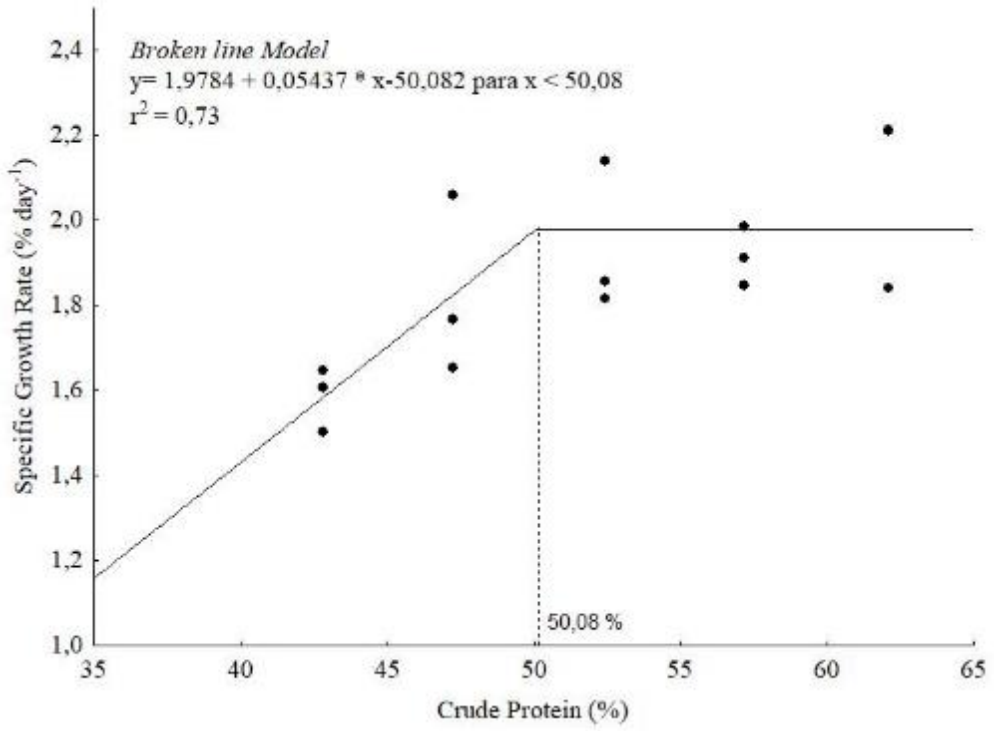

Figure 11.2.a. Protein requirement for juvenile Brazilian flounder (Paralichthys orbignyanus) estimated by the Broken-line Model, considering specific growth rate.

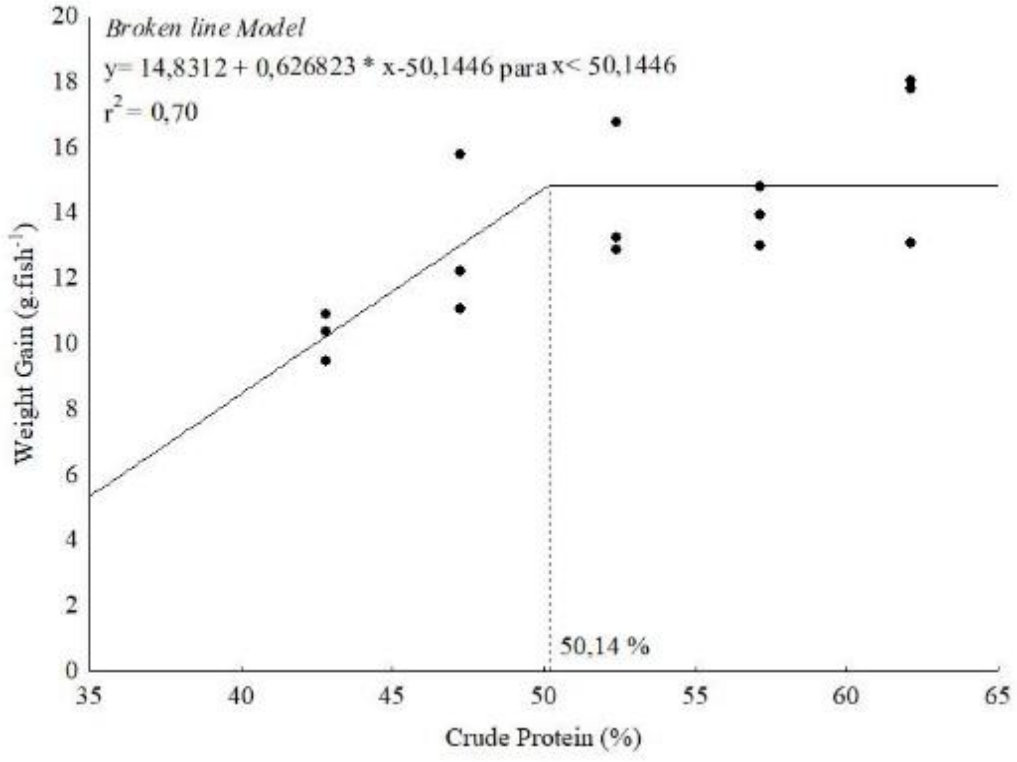

Figure 11.1.b. Protein requirement for juvenile Brazilian flounder (Paralichthys orbignyanus) estimated by the Broken-line Model, considering weight gain.

Discussion

Protein content of the diet clearly influences growth of juvenile Brazilian flounder. Growth reaches a plateau when they are fed a diet containing 470gKg^{-1} CP, but increasing the protein content of the diet up to 620gKg^{-1} CP does not reach a harmful level. Martínez-Palacios et al (2007) observed reduction of growth for *Menidia astor*. They hypothesize the energetic cost of protein deamination in a diet with protein level above the optimum, would result in less energy available for growth.

Energy content is considered one of the major criteria controlling feed intake in fish (Glencross 2006; Mohanta *et al*. 2008), but fish size and temperature also influence feed intake. However, when working with isoenergetic diets, feed intake is most likely controlled by dietary protein content, in an attempt to adjust feed intake to meet their protein requirement (Horn *et al*. 1995; Martínez-Palacios *et al*. 2007). We did not find evidence of alteration of feed intake for juvenile Brazilian flounder fed diets with different protein content.

Feed efficiency of juvenile flounder was improved when dietary protein level was increased in the present study, reaching its maximum as the protein content reached 570 and 620gKg^{-1} CP, lower dietary protein resulted in poorer feed efficiency. This is contrasting with the response of feed efficiency observed by Kim et al (2004) for olive flounder, they found an inverse relationship of feed efficiency with protein content of the diet, as dietary protein was increased above 50gKg^{-1} CP.

Dietary protein requirement varies between species, but comparison of protein requirement among fish species is complicated due to differences in fish size, diet formulation and culture conditions tested (Elangovan & Shim 1997). However, the result obtained in this experiment with Brazilian flounder is comparable to the protein requirements reported for juveniles of other *Pleuronectiformes*, as olive flounder (*Paralichthys olivaceus*) 450gKg^{-1} CP (Lee et al., 2002), turbot (*Scophthalmus maximus*) 494gKg^{-}1 CP (Lee et al., 2003), Southern flounder (*Paralichthys lethostigma*) 515gKg^{-1} CP (Gao et al., 2005), and starry flounder (*Platichthys stellatus*) 500gKg^{-1} CP (Lee et al., 2006). In general, protein requirement of flatfishes is higher than omnivorous species, such as ayu (*Plecoglossus altivelis*) 380gKg^{-1} CP (Lee *et al*. 2002), jundia (*Rhamdia quelen*) 373gKg^{-1} CP (Meyer & Fracalossi 2004), milkfish (*Chanos chanos*) 400gKg^{-1} CP (Jana *et al*. 2006), and mullet *Mugil platanus* 350gKg^{-1} CP (Carvalho et al. 2010).

Brazilian flounder has a high protein requirement (500gKg^{-1} CP), which is not different from other *Pleuronectiformes*. Therefore, in order to increase the likelihood of successful aquaculture for this species, it is important to develop studies towards the reduction of protein requirement, and look for alternative sources of protein, in order to spare fish meal and make it a more sustainable activity.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained so far and detailed above, we consider to have achieved around 20% completeness for this task.

Deviations & Problems:

This experiment was carried out before it was scheduled. The trial with fish ended well and carcass samples were collected and stored frozen. We did not have the opportunity to finish analysing these samples, but we are expecting clearance of the university in order to finish processing these samples. Once this is done, the task will be completed and the manuscript will be ready for publication.

Outlook: In the next 12-months we expect to finish running the biochemical analysis planned for task 11.2.

CST 11.3 Determine the protein sparing effect by lipid for juvenile Brazilian flounder

Task Leader Luís André Sampaio – FURG

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
11.3	Determine the protein sparing effect by lipid for juvenile Brazilian flounder	T3.2	FURG, CCMAR, CIIMAR	0%	✓	M3	M3	M21	0	4	7

Introduction

Protein is an important item in the diet of flounder. We have found the protein requirement for Brazilian flounder to be equal to 500gKg^{-1} CP when the lipid level is equal to 7%. In the planned trial we will increase the energy content of the diet by increasing lipid level up to 10% in an attempt to reduce the protein requirement, thus sparing protein.

Methods

We are planning a factorial design for this trial with three protein levels and two lipid levels. Lipid levels for *Pleuronectiformes* are low, Lee et al (2002) used diets with 7% lipid content for juvenile *P. olivaceus* and *P. flesus*. However, for *S. maximus*, the lipid content is close to 10% (Lee et al 2003). Diets will be formulated taking into consideration the protein requirement established in task 11.2 and these lipid levels used for other flatfish species.

Results

Nothing to report yet

Discussion

Nothing to report yet

Progress, deviations, problems & next 12M

Progress: This task has not been initiated, therefore it has 0 % completeness.

Deviations & Problems: Due to Covid-19 this task was put on hold and this experiment was not started. We produced the fingerlings needed for this experiment, but February 2020, Covid-19 made its way to Brazil and we had to stop running research activities. These fish had to be discarded following the recommendations of the Ethics Committee on Animal Experimentation. We will have to produce a new group of fingerlings in order to run this experiment. If we cannot run a larviculture by the end of this summer, we will have to postpone the experiment for M30-32, as the breeding season would be over after M23.

Outlook: We are waiting for clearance of the university to allow us to work with this experiment. As of today, we are working in the hatchery to keep the broodstock in good conditions, so that larviculture can start as soon as possible.

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Summary of progress report for Case Study 12

Date of report:	**30.03.2020**
Case Study name:	**Use of by-products**
of relevane for WPs	**WP2, WP3**

Abstract/Summary

The overall aim of CS12 is to reduce the wastes produced in shellfish aquaculture and marine fishery activities by valorising them following circular economy principles. CST12.1 deals with the inclusion of shellfish aquaculture in the carbon trading economy, which involves showing that shellfish $CaCO_3$ is a CO_2 sink and identifying uses that ensure a long-term preservation of this $CaCO_3$. During the first 12 months, we have elaborated a methodology to obtain accurate biological CO_2 budgets for shellfish aquaculture and applied them to the extensive Galician mussel aquaculture (NW Spain). A research paper by Álvarez-Salgado et al (2021). is in preparation. In addition, we reviewed the environmental, agricultural and industrial applications of shellfish $CaCO_3$ focusing on optimising the triad low carbon footprint – high added value – long inert time. A research paper by Alonso et al. has been submitted (accepted on August 2020). CST12.2, which is focused on an environmental (ocean alkalinisation) and an industrial (ecological paint) application of shellfish $CaCO_3$ have started on M12, in the middle of the COVID lockdown, and we have not conducted any activity on this task. CST12.3 aims at valorising side-streams of fisheries and shellfish aquaculture activities to produce feed ingredients for marine finfish aquaculture. During the first 12-months we produced hydrolysed proteins from fishery discards of Galician trawlers at the pilot plant scale and started to document, develop and test the methods to extract proteins and oil from fish frames and non-commercial mussels at the laboratory scale. Finally, in CST12.4, devoted to formulate and test diets for Senegalese sole juveniles with the ingredients obtained in CST12.3, starting on M9, we have arranged the roadmap for preparation and testing of the diets.

CST 12.1 Shellfish valorisation: assessing carbon sequestration and trading

X. Antón A. Salgado, CSIC

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
12.1	Shellfish valorisation: asssesing carbon sequestration and trading	T2.3	CSIC, StellU	50%	✓	M2	M2	M24	M24	2	6

Introduction

Calcium carbonate ($CaCO_3$) is the major by-product of marine bivalve aquaculture as shell represents from 60% to 95% of the total fresh weight of bivalves and from 90% to 99% of bivalve shell is $CaCO_3$ (Alonso et al., 2021) [40]. Currently, most of the bivalve shells end in urban incinerators, where the CO_2 fixed as $CaCO_3$ returns back to the atmosphere. Since 1kg of $CaCO_3$ sequesters 440g of CO_2, an effort have to be made to substitute non-renewable mineral $CaCO_3$ by sustainable bivalve shell $CaCO_3$ in applications that ensure a long-term preservation of this by-product. Apart from prioritizing applications that avoid the release of CO_2 to the atmosphere, the inclusion of bivalve aquaculture in the carbon trading system should be considered too. It implies demonstrating that the biological CO_2 budgets of these cultures are favourable to CO_2 sequestration. This is still a controversial issue because

[40] Alonso, A. A., Álvarez-Salgado, X. A., and Antelo, L. T. (2021). Assessing the impact of shellfish aquaculture on the carbon trading economy. Journal of Cleaner Production 279, 123873.

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement No 818173. This report reflects only the authors' view and that the Commission is not responsible for any use that may be made of the information it contains.

of the lack of consensus on the biological processes to include or how to include them in the CO_2 sequestration budget, on the variety of approaches to estimate those processes, and on the relevant scale to apply, from the individual to the ecosystem level[41,42,43]. In line with these two goals, long-term preservation of bivalve shell $CaCO_3$ and CO_2 sequestration of bivalve aquaculture, during the fist 12 months of AquaVitae we have i) reviewed the environmental, agricultural and industrial applications of bivalve shell $CaCO_3$ focusing on optimising the triad low carbon footprint – high added value – long inert time; and ii) elaborated a methodology to obtain accurate biological CO_2 budgets for shellfish aquaculture, applying it to the extensive Galician mussel aquaculture (NW Spain).

Methods

CST 12.1 is essentially based on a literature review of the environmental, agricultural and industrial applications of $CaCO_3$ and their associated carbon footprint, added value and long-term preservation. Global, continental and regional analyses of the $CaCO_3$ demand of each application compared with the bivalve production has also been reviewed. Furthermore, existing data on the biological processes involved in the CO_2 budget of Galician mussel aquaculture (NW Spain) have been collected to quantify the different carbon fluxes contributing to that budget. Finally, we are adapting the two-structures Dynamic Energy Budget (DEB) growth model by Fuentes-Santos et al. (2019)[44] to simulate mussel carbon fluxes and CO_2 budget.

Results

Starting on M2, the literature review of the environmental, agricultural and industrial applications of $CaCO_3$ has resulted in a research paper submitted to Journal of Clear Production in M12 (May 2020), which was accepted in August 2020 and published in January 2021 (Alonso et al., 2021)[1]. The overall idea is that shell $CaCO_3$ is a sustainable biomaterial that could partly replace the presently dominating non-renewable mineral sources in some applications. Although bivalve $CaCO_3$ contribution to CO_2 sequestration during shell formation is still controversial, the integration of bivalve aquaculture in the carbon trading market would demand that shell $CaCO_3$ is employed in activities that keeps the integrity of CaO_3 for prolonged time periods. We have shown that, ideally, these activities should combine i) providing the maximum added value to the biomaterial; ii) producing the lowest carbon footprint during their life cycle; and iii) preserving the $CaCO_3$ integrity as long as possible. Furthermore, we have matched global, continental and regional demands of $CaCO_3$ with their respective mussel aquaculture productions in an effort to scale up offer and demand. Table 12.1.a and Figure 12.1.a summarise the main uses of $CaCO_3$ together with the carbon footprint during their life cycle and the time during which $CaCO_3$ remains inert.

[41] Filgueira, R., Byron, C. J., Comeau, et al. (2015). An integrated ecosystem approach for assessing the potential role of cultivated bivalve shells as part of the carbon trading system. Mar. Ecol. Prog. Ser. 518, 281-287.

[42] Filgueira, R., Strohmeier, T., and Strand, Ø. (2019). "Regulating Services of Bivalve Molluscs in the Context of the Carbon Cycle and Implications for Ecosystem Valuation" in Goods and Services of Marine Bivalves, eds. A. Smaal, J. Ferreira, J. Grant, J. Petersen and Ø. Strand (Springer, Champ.), 231-251.

[43] Morris, J. P., and Humphreys, M. P. (2019). Modelling seawater carbonate chemistry in shellfish aquaculture regions: Insights into CO2 release associated with shell formation and growth. Aquaculture 501, 338-344.

[44] Fuentes-Santos, I., Labarta, U., and Álvarez-Salgado, X.A. (2019). Modelling mussel shell and flesh growth using a dynamic net production approach. Aquaculture 506, 84-93. doi: 10.1016/j.aquaculture.2019.03.030

*Table 12.1.a. Consumptions of $CaCO_3$ (per application sector and per year), associated carbon footprints and immobilisation times of bivalve $CaCO_3$ applications: *Immediate (transformed into CaO during manufacturing); Very short: 1-5 years; Short: 5-25 years; Medium: 25-75 years; Long: > 75 years. [a] value is computed as a fraction of Spain contribution to the EU-28 Construction GDP1 (6.55%). [b] value is computed from Table 1 in Quina et al. (2018) as the ratio Spain/EU production in 2013 (742.6 t/10,933.3 t). [c] value is computed as a fraction of Spain contribution to the EU-28 Agriculture GDP2 (7.6%). [d] value is computed as a fraction of Spain plastic production value (4% of the total national manufacturing GDP e 34,615 million €[3]) and the manufacturing GDP of the EU-28 (2,254,640 million €[4]). [e,f,g] values are computed based on the $CaCO_3$ content range in construction glasses (5%-12%) and in the global[e], European[f] and Spanish[g] glass production data obtained from literature. [h] value is computed based on the $CaCO_3$ production directed to paper industry in Europe (33%). [i] only 50% of the amended $CaCO_3$ is immobilised at the long term. [j] if $CaCO_3$ in amended eggshell is valorised, the immobilisation time can be extended accordingly. [k] except short-term applications, e.g. clothing. Taken from Alonso et al. (2021).*

	Consumptions of $CaCO_3$, per sector and per year				Cradle to gate carbon footprint and inertization times of bivalve $CaCO_3$ applications		
Sector of application	Worldwide (10^7 t)	Europe (10^6 t)	Spain (10^6 t)	References	Carbon footprint (kg CO_2 eq/t of product)	References	Inertization time*
Agriculture (Soil amendment)	23.0[1]	4.5[c]	0.4[c]	[a]Minerals Yearbook (2018); [b]IMA Europe (2018)	370–1360 (crop production)	Cheng et al. (2016), Jaswal et al. (2020)	LONG
Construction (aggregates)	17.0	3.5	0.4[a]	Adams (2005)	500 (cement), 930–1100 (concrete)	Andrew (2018), NRMCA (2012)	LONG
Paper industry	13.5[1]	2.6[?]	2.7[?]	[a] [illegible] (2001); [b]Minerals Yearbook (2018); [c] Adams (2005a); [d] Adams (2005b)	1200 (white paper of 80 g/m[2])	Das et al. (2007)	VERY SHORT
	11.0 – GCC	[illegible] – GCC	[illegible] – GCC		1200–1770	AFPA (2017)	MEDIUM
	3.5[?] – PCC	1.5[?] – PCC	0.7[?] – PCC				
Poultry (eggs production)	1.01	2.3	0.1[b]	Quina et al. (2018)	45–410 (CO_2 after [illegible])	[illegible] (2015), [illegible] et al. (2015)	VERY SHORT[j]
Plastics	11	2.3	0.26[d]	Adams (2005), Minerals Yearbook (2018)	4260	Zheng and Suh 2020	SHORT
	7.5 – GCC						
	3.5[1] – PCC						
PET	0.?	N/A	N/A	Adams (2005c)	2430	Zheng and Suh 2020	LONG
Glass production:					500–700	[illegible] (2008), Schmitz et al. (2011)	IMMEDIATE
Low $CaCO_3$ content	2.9[e]	1.6	0.2[g]				
High $CaCO_3$ content	7.1[e]	1.8[f]	0.5[g]				
Paints	5.0	1.8	0.03[h]	Adams (2005a)	1650–3700 (solvent based),	[illegible] (2004),	MEDIUM
		1.18 – GCC	0.024 – GCC		1700–2700	[illegible] (2012),	
		0.42 – PCC	0.006[?] – PCC		5200–6200 (solvent based)	[illegible] (2005)	
					1300–1700 (water based)		
Polyolefins and polyester	2.6	N/A	N/A	Adams (2005a)	7700–8600	Chen et al. (2006); [illegible] et al. (2015)	LONG[k]
Sealants and adhesives	N/A	1.9	0.03[h]	Adams (2005a)	1000–3000 (water based)	https://www.[illegible]	SHORT
					2700–9000 (hot melt)		
					2000–5000 (solvent based)		
					4700–10,000 (reactive)		

[1] https://www.statista.com/[illegible]
[2] https://ec.europa.eu/info/sites/info/files/food-farming-fisheries/farming/documents/agri-statistical-factsheet-eu_en.pdf.
[3] https://[illegible]/manufacturing-production
[4] https://[illegible] https://[illegible]
[5] https://www.statista.com/topics/[illegible]/glass.
[6] https://www.[illegible]/industries.
[7] http://www.[illegible]/wp-content/uploads/2015/04/DOCUMENTO-Economia-Circular-Vidrio-Espana-2017.pdf

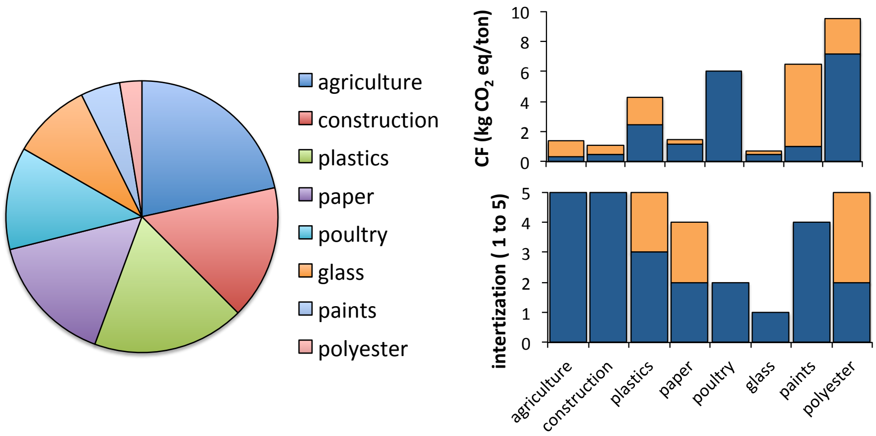

Figure 12.1.a: Main agricultural and industrial uses of $CaCO_3$ and the respective carbon footprint and $CaCO_3$ long-term preservation. Blue bars indicate minimum and beige bar maximum carbon footprint and log-term preservation.

The cradle-to-gate carbon footprint of bivalve shell $CaCO_3$ is in the range of the footprint produced by limestone processing, suggesting that the impact of manufacturing $CaCO_3$ from a geological or a biological resource is similar. However, from the viewpoint of the applications that mobilize immediately the CO_2 in $CaCO_3$ to the atmosphere (*e.g.* glass manufacturing, applications demanding CaO), while extraction of $CaCO_3$ from mines implies the eventual mobilisation of a CO_2 that has been formed and preserved millions of years ago (non-renewable source), the CO_2 trapped in bivalve shell $CaCO_3$ was synthesized in the last few months to decades (sustainable source). From the viewpoint of the applications that immobilises the CO_2 in $CaCO_3$ for longer periods (*e.g.* aggregates for construction,

paints, PVC), using bivalve shell $CaCO_3$ implies the removal of CO_2 from the current atmosphere, mitigating global warming and giving bivalve aquaculture a chance in the carbon trading market.

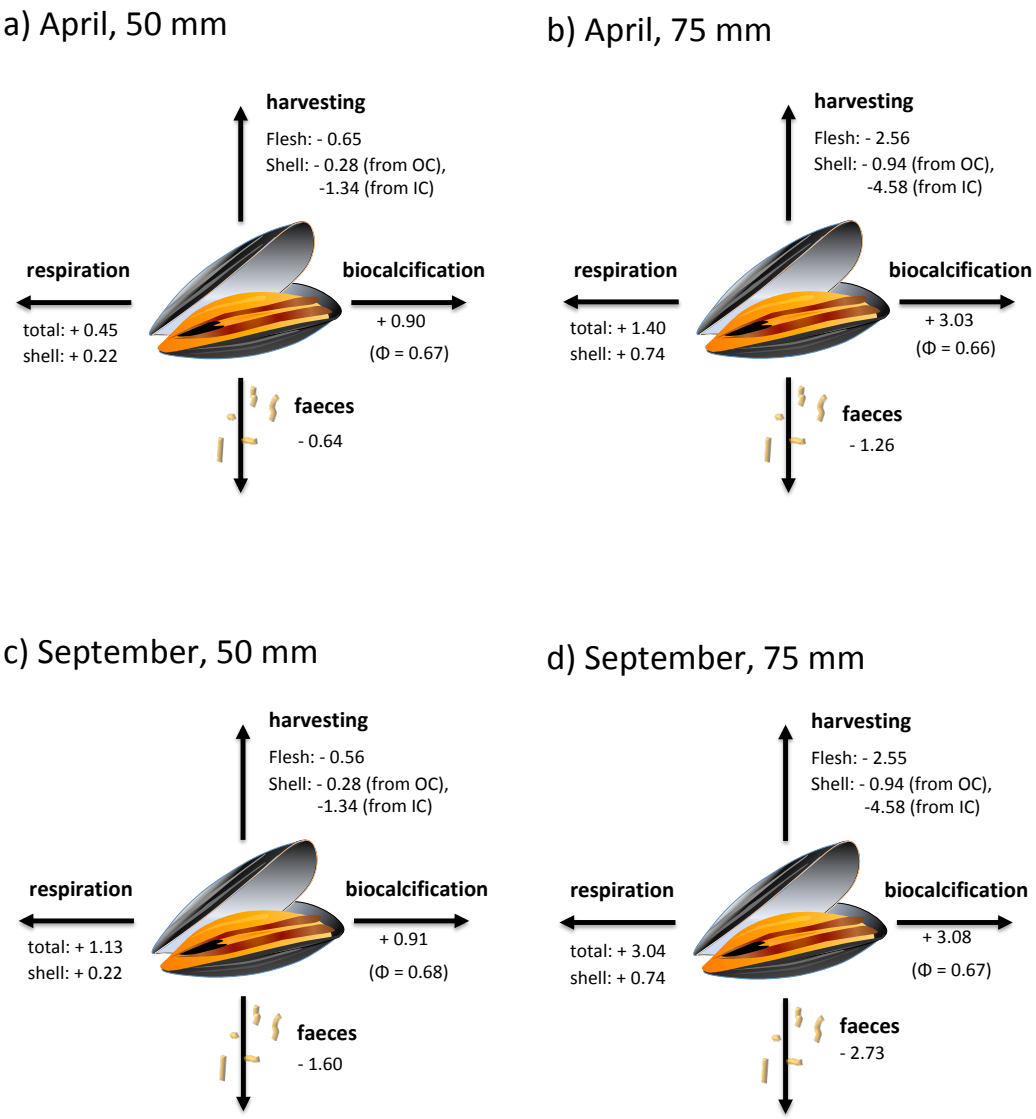

Figure 12.1.b: CO_2 fluxes (in g CO_2 $indv^{-1}$) associated to the biological processes involved in mussel growth for the four scenarios tested: a) 50mm mussel / seeding in April (120-days to harvesting); b) 50mm mussel / seeding time September (300-days to harvesting); c) 75mm mussel / seeding time April (180-days to harvesting); and d) 75mm mussel / seeding time in September (390-days to harvesting). OC: organic carbon; OI: inorganic carbon.

Recommending a methodology to obtain accurate biological CO_2 budgets for shellfish aquaculture activities requires to i) identify all relevant biological processes involved in the removal or production of CO_2 during bivalve growth; ii) quantify those processes; iii) obtain a CO_2 budget at the individual scale; and iv) upscale the individual budget to the ecosystem scale[2,3]. From M4 on we have identified the biological processes involved in the CO_2 budget at the individual scale and quantified those processes for the Galician mussel aquaculture (NW Spain). Figure 12.1.b summarises the CO_2 fluxes associated to these processes as a function of the seeding time and harvesting size.

Finally, from M8 on we have started the adaptation of the two-structures Dynamic Energy Budget (DEB) growth model by Fuentes-Santos et al. (2019)[5] to simulate mussel carbon fluxes and CO_2

budgets. It has implied to convert all the simulated biological processes (ingestion, egestion, excretion, respiration, bio-calcification, and shell and flesh growth) to g CO_2 and to model their impact on the CO_2 system variables (inorganic carbon, alkalinity, pH and pCO_2) of the water parcel where the mussels are cultured. Table 12.1.c summarises the conversion factors that we have compiled to do the conversion to g CO_2.

Table 12.1.c. Conversion factors implemented in the two-structures Dynamic Energy Budget (DEB) growth model by Fuentes-Santos et al. (2019)[5] to simulate CO_2 budgets. Taken from Pájaro et al. (in prep).

Variable	Description
Shell $CaCO_3$ content (g)	Calculated from the shell dry weight (model output) assuming that 95% of the shell is $CaCO_3$
Shell inorganic C content (g)	Calculated from the shell $CaCO_3$ given that C is 12% of $CaCO_3$ weight. Atomic weight (a.w.) C, 12 g mol^{-1}; molecular weight (m.w.) $CaCO_3$, 100 g mol^{-1}
CO_2 eq of shell $CaCO_3$ content (g)	Calculated from shell $CaCO_3$ given that CO_2 is 44% of $CaCO_3$ weight. m.w. CO_2, 44 g mol^{-1}; m.w. $CaCO_3$, 100 g mol^{-1}
Shell organic matter content (g)	Calculated from the shell dry weight (model output) assuming that 5% of the shell is organic matter
Shell organic C content (g)	Calculated from the shell organic matter content assuming that 52% of the shell organic matter is C
CO_2 eq of shell organic C content (g)	Calculated from shell organic C given that C is 27.3% of CO_2 weight. a.w., 12 g mol^{-1}; m.w. CO_2, 44 g mol^{-1}
Flesh organic C content (g)	Calculated from flesh organic matter content (model output) assuming that 9.6% of the wet mussel shell is C
CO_2 eq of flesh organic C content (g)	Calculated from flesh organic C given that C is 27.3% of CO_2 weight. a.w., 12 g mol^{-1}; m.w. CO_2, 44 g mol^{-1}
Faeces organic C content (g)	Calculated from faeces organic matter content (model output) assuming that 38% is C
CO_2 eq of faeces organic C content (g)	Calculated from faeces organic C given that C is 27.3% of CO_2 weight. a.w., 12 g mol^{-1}; m.w. CO_2, 44 g mol^{-1}
CO_2 eq released by bio-calcification (g)	Calculated from CO_2 eq of shell organic C content (g) multiplied by 0.69 (ratio of CO_2 release to $CaCO_3$ fixation during calcification)
CO_2 eq of mussel respiration (g)	Calculated from mussel respiration (in ml O_2) assuming a C:O_2 respiration quotient of 0.85 mol C mol O_2^{-1}, multiplying by the m.w. of CO_2 (44 g mol^{-1}) and dividing by 22.4 l/mol O_2
TA change due to mussel flesh production	Calculated from flesh organic C content divided by 12 g mol^{-1} and multiplied by 0.31 eq mol^{-1}
TA change due to shell $CaCO_3$ production	Calculated from shell inorganic C content divided by 12 g mol^{-1} and multiplied by -2 eq mol^{-1}
TA change due to shell organic C production	Calculated from shell organic C content divided by 12 g mol^{-1} and multiplied by 0.31 eq mol^{-1}
TA change due to NH_4^+ excretion	Calculated from ammonium excretion (model output) divided by 14 g mol^{-1}
TA change due to faeces mineralisation	Calculated from faeces organic C content divided by 12 g mol^{-1} and divided by 6.7 eq mol^{-1}

Discussion

The research paper by Alonso et al. (2021)[1] constitutes a fundamental step for the consecution of our overall key exploitable result of promoting the inclusion of the bivalve aquaculture in the carbon trading economy. In this regard, the paper has clarified that only the applications that imply a long-term immobilisation of the bivalve shell $CaCO_3$ are valid to be considered as a CO_2 sequestration mechanism. The paper also explains that the production of bivalve $CaCO_3$ is adjusted to the offer and demand of some applications, which is also crucial to have a change in the carbon trading economy. Another important issue that we also developed in this task is the identification and correct evaluation of the biological processes that should be accounted in the biological CO_2 budget of cultured bivalves. In this regard, we have done an unprecedented work identifying and quantifying all these processes.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained so far we have completed 50% of the activities programmed for CST12.1.

The review and analysis of the environmental, agricultural and industrial applications contributing to the long term immobilisation of bivalve shell $CaCO_3$ by Alonso et al. (2021)[1], the recommendation of a methodology to obtain accurate biological CO_2 budgets for shellfish aquaculture and its application to the extensive Galician mussel aquaculture (NW Spain) and the adaptation of the growth model of Fuentes-Santos et al. (2019)[5] to simulate biological CO_2 fluxes are in good progress to our key exploitable result of promoting the inclusion of shellfish aquaculture in the international carbon trading economy

Deviations & Problems: none

Outlook: We have to finalise the calculation of the biological CO_2 sequestration budget of Galician mussel aquaculture and publish the results in a peer-reviewed journal. We also have to finalise the implementation of the CO_2 module of the mussel growth model and publish the code and the interpretation of the results in a peer-reviewed journal. Finally, we have to extend our budget and model results to the shellfish aquaculture of Sweden (CS8) and Denmark (CS9).

CST 12.2 Shellfish valorisation: inclusion in the coating industry

X. Antón A. Salgado & Luis T. Antelo, CSIC

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
12.2	Shellfish valorisation:inclusion in the coating industry	T2.3, T3.2	CSIC, StellU	5%	✓	M20	M12	M48	M24	2	7

Introduction

Two applications that ensure a long-term immobilisation of the CO_2 trapped in bivalve shell $CaCO_3$ are ocean alkalinisation (environmental use) and paints (industrial use). For the case of ocean alkalinisation, depending on the carbon chemistry of the waters amended with bivalve shell $CaCO_3$, it can contribute to reduce the pCO_2 of the waters when they are carbonate ion unsaturated or remain as $CaCO_3$ for decades to centuries when they are carbonate ion oversaturated. In CST 12.2 we propose a protocol for the alkalinisation of the coastal waters of Galicia (NW Spain) that can be transferred to other areas. For the coating industry, we propose to include bivalve shell $CaCO_3$ as a component of ecological paints. Since the activities of CST12.2 have started on M12, in the middle of the COVID-19 lock down, we have not any significant progress to inform but just our first trials of organic matrix removal and grinding of mussel shells that we had initiated in advance by M10.

Methods

Thermal (calcination at 400ºC in an muffle for 12h) and chemical (immersion in diluted sodium hypochlorite 0.1% for 48h) procedures have been tested to remove the shell protein matrix as a first step to purify the $CaCO_3$ in mussel shells provided by PROINSA (IRG of CS12). Subsequently, shell $CaCO_3$ has been ground in a ball mill.

Results

Our very preliminary trials, at the beginning of M10, just a few days before the COVID-19 lock down in Spain, indicated that thermal decomposition of the protein matrix was more efficient than the chemical method. Furthermore, we were able to obtained variable $CaCO_3$ grain sizes depending on the grinding intensity and time programmed in the ball mill.

Discussion

Whereas the carbon footprint of thermal decomposition of the shell protein matrix is 60 kg CO_2 eq per ton of $CaCO_3$, for the case of the chemical method we estimated that it is < 20 kg CO_2 eq per ton of $CaCO_3$. Therefore, it has to be explored if $CaCO_3$ treated with the chemical method is adequate for some paint applications because it produces a lower carbon footprint. In the same way, carbon footprint associated to grinding varies from 10 to 210kg kg CO_2 eq per tonne of $CaCO_3$, increasing with deceasing grain size, revealing the importance of balancing grain size and carbon footprint.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained so far we have completed 5% of the activities programmed for CST12.3.

Deviations & Problems: none

Outlook: We have to produce a draft of the protocol for ocean alkalinisation of the Galician coast and document, develop and test the method to produce a bivalve shell $CaCO_3$ based paint at the laboratory scale.

CST 12.3 Fishery by-catch valorisation: ingredients

Luis T. Antelo, CSIC

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
12.3	Fisheries by-catch valorisation: ingredients	T3.2	CSIC, StellU	30%	✓	M2	M2	M18	M18	3	7

Introduction

Fisheries and shellfish aquaculture activities and their associated industrial processing generate substantial amounts of biomass not usable for human consumption. Among them are fishing discards, fish frames from the fishery transformation industry, and non-commercial mussels (undersized or broken) from the mussel transformation industry. In this CST 12.3, we propose the utilisation of these by-products as feed ingredients in marine aquaculture to replace the conventional ingredients used in this sector.

The Common Fisheries Policy (CFP) of the European Commission introduced in 2013[45] a discard mitigation strategy which states that all catches of species subjected to catch quotas and/or Minimum Conservation Reference Size (MCRS) will have to be landed and will be counted against quota. This so-called Landing Obligation (LO) has being gradually implemented, since 2015 to 2019 when all EU fisheries were required to land all catches except a set of *de minimis* percentage of catches that are yearly set based on the scientific data of catches acquired from on-board observers and landing notes together with survival studies for different species.

Important amounts of individuals below the minimum legal size of various species subject to Total Allowable Catch (TAC) are landed and cannot be destined for direct human consumption, so they must be properly managed following a different commercialisation and management route than usual. For this fraction we denoted by FNHC (*Fish for Non-Human Consumption*), together with those specimens above MCRS that lack of quality enough to be sold and those fish side-streams generated in land during

[45] European Commission, 2013. Regulation (EU) No 1380/2013 of the European Parliament and the Council of 11 December 2013 on the Common Fisheries Policy, Amending Council Regulations (EC) No 1954/2003 and (EC) No 1224/ 2009 and Repealing Council Regulations (EC) No 2371/2002 and (EC) No 639/ 2004 and Council Decision 2004/585/EC.

elaboration and transformation processes, a wide range of available technological alternatives exist[46, 47] but not all of them may be equally feasible.

Fish meal obtained after a thermal process of fish by-products, to coagulate the protein and separate the oil, is the most common and extended process but the biomass undergoes a low valorisation level (generally obtaining low quality products), the fish wastes must be transported from fishing ports to meal plants and the environmental impacts (air pollution, odours, high water etc.) of those plants is huge. Thus, alternatives for a best use of these new biomasses from LO maximising the obtaining of compounds of high commercial interest in diverse sectors of application must be studied, as it can be seen in Figure 12.3.a.

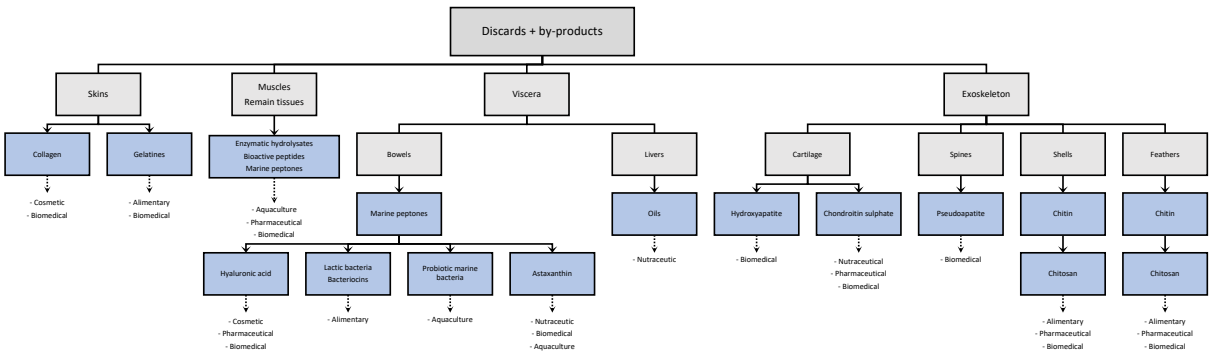

Figure 12.3.a. Simplified flow diagram of a marine biorefinery of previously discarded fish biomass and processing fish by-products.

Valorisation processes directed by enzymatic hydrolysis to produce fish protein hydrolysates (FPHs) including the recovery of essential nutrients[48] and bioactive compounds could be an excellent and viable practice to efficiently upgrade this new FHNC biomass. The preparation and characterization of FPHs covering different species[49], enzymes[50], or hydrolysis conditions[51] have been extensively studied. FPHs have demonstrated excellent functional properties as antioxidants against free radicals, antihypertensive pharmacological agents and antimicrobial properties. On the other hand, since FPHs are rich in soluble proteins and with high digestibility, they can be also employed as ingredient of aquaculture diets[52] with very promising results.

From M2, we have developed an effective valorisation strategy based on enzymatic hydrolysis (EH) for the fishery discards, particularly blue whiting from Galician trawlers, carrying out lab and pilot plant productions. Chemical and functional properties of the obtained FPHs have been also determined. Furthermore, on M8 we have initiated the study of the optimal conditions of enzymatic hydrolysis of fish frames (mainly from canned sardine industry) and non-commercial (undersized or broken) boiled

[46] Iñarra, B., Bald, C., Cebrian, M., Antelo, L.T., et al. (2019). What to do with unwanted catches: valorisation options and selection strategies. In: Uhlmann, S., Ulrich, C., Kennelly, S. (Eds.).

[47] Pérez-Martín, R.I., Antelo, L.T., Vázquez, J.A., Mirón, J. (2020). An on-land management and valorisation approach for biomass associated with landing obligation compliance, Marine Policy, 116, 103506

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[49] Chalamaiah, M., Dinesh Kumar, B., Hemalatha, R., Jyothirmayi, T. (2012). Fish protein hydrolysates: proximate composition, amino acid composition, antioxidant activities and applications: a review. Food Chem. 135, 3020e3038.

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mussels based on a literature review of existing methods and previous knowledge of the partners involved in the project. In this report, we will focus on the production and characterisation of FPHs for fishery discards. As a consequence of the COVID-19 lock down in South Africa and Spain, our progresses towards FPHs production from fish frames and boiled mussels have been very preliminary at M12, therefore we will describe them in the next report (M13 to M18).

Methods

1. Previously discarded biomass quantification and characterization

Regarding biomass from fishing activity, all fish species, classified as discards by Galician fishing fleets operating in ICES areas 6, 7, 8c and 9a, have been captured in the North Atlantic Ocean: Blue whiting (*Micromesistius poutassou*), Mackerel (*Scomber scombrus*), Red scorpionfish (*Scorpaena scrofa*), Pouting (*Trisoreptus luscus*), Gurnard (*Trigla* Spp.), Grenadier (*Macrourus* spp.), Megrim (*Lepidorhombus bosci*i), European hake (*Merluccius merluccius*), Boarfish (*Capros aper*) and Atlantic horse mackerel (*Trachurus trachurus*). Discard rates of Galician trawlers are highly variable, depending on the fishing ground, the time of the year and the target specie/s. In general, discards rates are low in some specific fisheries, but moderate to high in most trawling trips, ranging between 15% and 75% (kg discards/kg total capture).

2. *Optimisation of enzyme hydrolysis of blue whiting discards*

The combined effect of pH and temperature (T) on the digestion of these species individuals by Alcalase 2.4L (2,4 AnsonUnit/g, AU/g enzyme, Novozymes, Nordisk, Denmark) has been evaluated. For this purpose, rotatable second order designs of two variables have been carried out (with 5 replicas in the centre of the experimental domain). The rest of the experimental conditions remained constant: agitation, (S:L) ratio and enzyme concentration. These experiments have been carried out in a pH-Stat system equipped with a 100mL enzyme reactor with temperature and agitation control. Secondly, the individual effect of enzyme concentration has been studied using the same experimental equipment and maintaining constant (in the optimal values obtained in the previous factorial plans), the rest of experimental conditions. In the same way, the individual effect of (S:L) ratio on blue whiting hydrolysis has also been finally tested. In all optimisation experiments, after hydrolysis (4 h) the mini reactors have been centrifuged (15,000g/20 min) and the sediments (mainly bones) and supernatants quantified. Additionally, the degree of hydrolysis (H, as %) has been determined in all hydrolysis kinetics by the pH-Stat method employing mathematical models previously reported in the literature[12].

3. Production of fish protein hydrolysates (FPHs) at lab and pilot plant scale

Lab-scale hydrolysis have been carried out in a controlled pH-Stat system with a 5L glass-reactor (suspending 1kg of milled discards in 2L of distilled water, (S:L) ratio of 1:2 w/v) using 5M NaOH as alkaline reagent for pH-control. Optimal conditions obtained in the previous section for BW have been applied for all fish discards: 60°C, pH 8.65, agitation of 200rpm and 1% (v/w) of Alcalase 2.4L. At the end of the hydrolysis (4h), the content of the reactors has been filtered (100µm) to remove bones, the liquid hydrolysates have been centrifuged (15,000 g/20 min) to recover oils (adding a step of decantation for 5min) and final FPHs have been quickly heated (90°C/15 min) for enzyme deactivation. In Figugre 12.3.b, a schematic flowchart of FPHs processing from fish discards is shown. After the sterilisation (121°C/15 min) and centrifugation (15,000g/20 min) of FPHs, the recovered liquid phases were denominated as fish peptones.

Pilot plant trials at the iDVPs facilities in the port of Marín (described in the next section) have been performed in a stainless reactor of 500L equipped with control of temperature, agitation, reagent

addition and pH (pH-Stat system). Hydrolysis have been executed following the same experimental conditions as at lab scale but with initial loads of fish discards of 50-150kg.

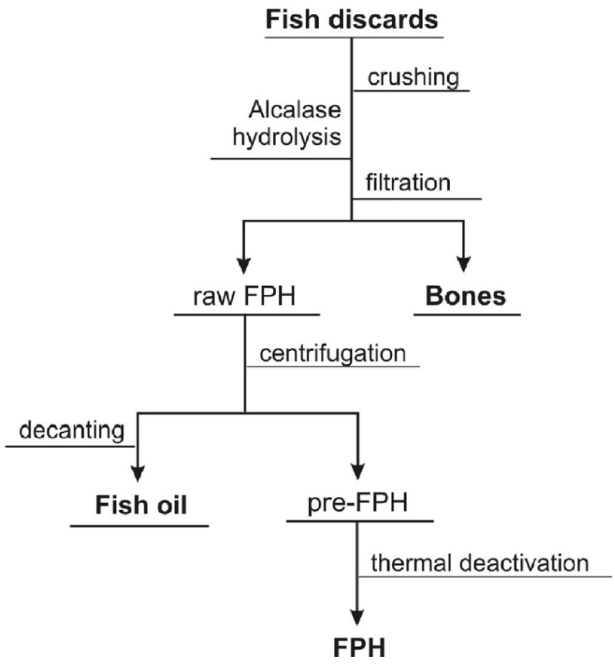


Figure 12.3.b. Schematic flowchart of previously discarded fish biomass processed by enzymatic hydrolysis

4. Chemical analyses

The profile of fatty acids from fish oil have been analysed by GC chromatography after chemical methylation. FPHs have been stored at -18°C until analysis. The basic analyses of FPH have been: 1) total soluble protein; 2) total sugars; 3) total protein as total nitrogen x 6.25; 4) amino acids content (quantified by ninhydrin reaction, using an amino acid analyser); 5) *in vitro* digestibility (pepsin method) and 6) molecular weights of FPH distribution determined by Gel Permeation Chromatography (GPC).

Results

1. Previously discarded biomass quantification and characterization

We have taken advantage of our access to the confidential fishing data obtained by human observers on-board commercial vessels of trawling fleets based in Vigo (NW Spain) and operating in the four ICES areas above mentioned (ICES areas 6, 7, 8c and 9a) during 62 on-board campaigns in the framework of LIFE iSEAS project from 2015 to 2018.

Regarding the catching profile of the 24 trawling vessels operating in the Sole bank (ICES area 6 and 7), it is quite different from the other two considered fleets. This is due to both the target species of this fleet as well as to the long distance from the base port (trips take 10-14 days). This implies that species with poor conservation capacities as the case of blue whiting (whose quality and freshness degrades very quickly) are all discarded. Regarding other species like horse mackerel or mackerel, they are fully discarded due to the low commercial value that will have at the end of the trip. Haddock and boarfish are fully discarded because Spain has no quota assigned to catch this species while discards of target species (megrim, hake, black bellied angler and skates) are mainly due to specimen under minimum conservation reference size (MCRS), i.e. small individuals. For the case of coastal trawlers operating near the base port, the catch profiles are quite different since now the fishing trips ranges from 1-2

days (62 vessels working on ICES area 8c) to 6-days (2 vessels operating in ICES area 9a). Both kind of trawlers target a set of different demersal species (horse mackerel, mackerel, blue whiting and hake) and the discarded fractions of these species are mainly due to three factors: i) specimen under minimum conservation reference size (MCRS); ii) in order to not down the price of fish at auction, they reduce the volume of catch retained to be landed to get a better price and; iii) no quota available.

2. Optimisation of enzyme hydrolysis of blue whiting discards

On basis of the previous analysis, blue whiting has been the chosen species to carry out the factorial experiments because it is the most discarded by the fishing fleets that work in the North Atlantic Ocean.

Figure 12.3.c shows the graphical results of the different studies of optimisation. The optimal values that maximise the process of hydrolysis has been calculated by numerical derivation: T_{opt} *= 59.5 °C and* pH_{opt} *= 8.61* to obtain the maximum degree of hydrolysis (H_m) and T_{opt} *= 60.5 °C and* p_{Hopt} *= 8.69* to reach the maximum ratio of digestion/liquefaction of raw material to liquid phase (V_{dig}).

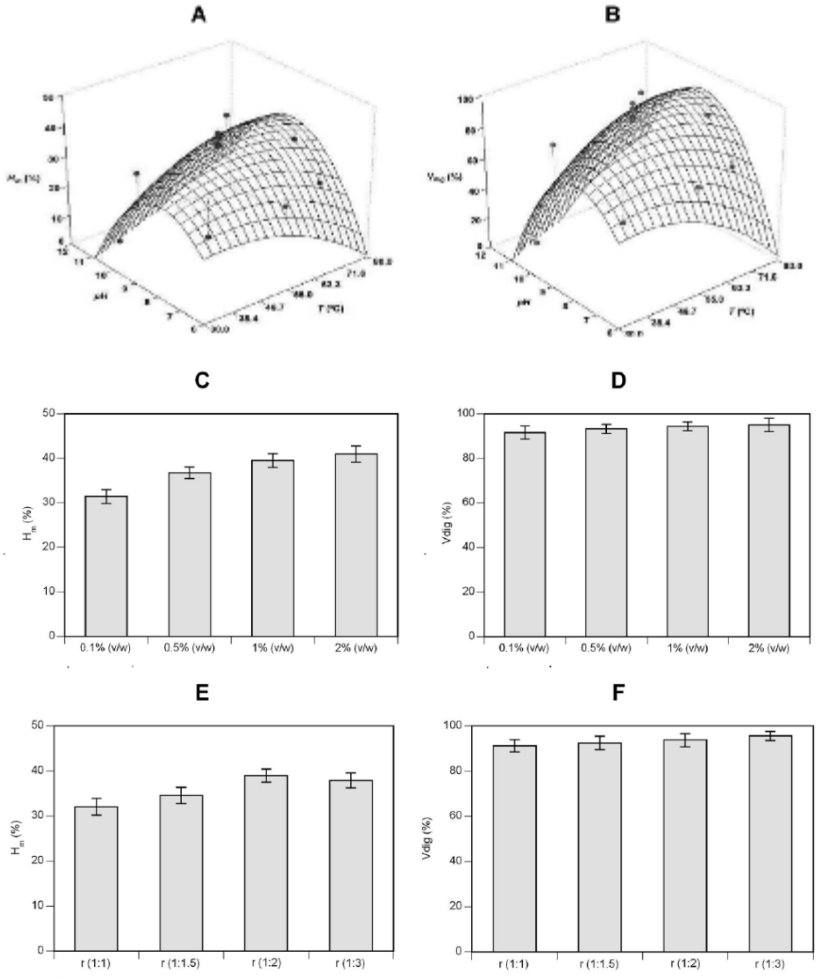


Figure 12.3c: Optimisation studies of Alcalase hydrolysis of blue whiting discards. A: Experimental and predicted response surfaces describing the simultaneous effect of pH and T on H_m response. B: Experimental and predicted response surfaces describing the simultaneous effect of pH and T on V_{dig} response. C: Individual effect of Alcalase concentration over H_m. D: Individual effect of Alcalase concentration over $V_{dig.}$ E: Individual effect of S:L ratio over H_m. F: Individual effect of S:L ratio over V_{dig}. Error bars are the confidence intervals for n = 2 and α = 0.05.

Then, and using the average values (60°C, pH 8.65), the individual effects of Alcalase concentration and S:L ratio on the hydrolysis process have been evaluated (Figure 12.3.c). The difference between

the concentrations of Alcalase 1% and 2% (39.5 ± 1.6% and 40.9 ± 1.8% for H_m and 94.3 ± 2.0% and 95.0 ± 3.0% for V_{dig}, respectively) were not statistically significant ($p > 0.05$) but they were higher for H_m response and equal for V_{dig} response than employing 0.1% and 0.5% of Alcalase. Taking into account V_{dig} as dependent variable, the effect of increasing (S:L) ratios was not significant ($p > 0.05$). For H_m, 1:2 and 1:3 ratios led to higher degrees of hydrolysis than 1:1 and 1:1.5 ratios.

3. Production of fish protein hydrolysates (FPHs) at lab and pilot plant scale

The discarded biomass of blue whiting has been hydrolysed at the pilot plant scale based on the conditions defined in the previous section. To carry out the pilot scale productions of FPHs, we take advantage of the availability of two pilot plants (iDVP1 and iDVP3) that were designed and built in the framework of the LIFE iSEAS project, coordinated by the leaders of CST 12.3. In these facilities, located in a very important fishing port of Galicia (Port of Marín, NW Spain), processes have been performed by applying the bio-refinery concept to manage and upgrade fishery discard biomass, as depicted in Figure 12.3.d.

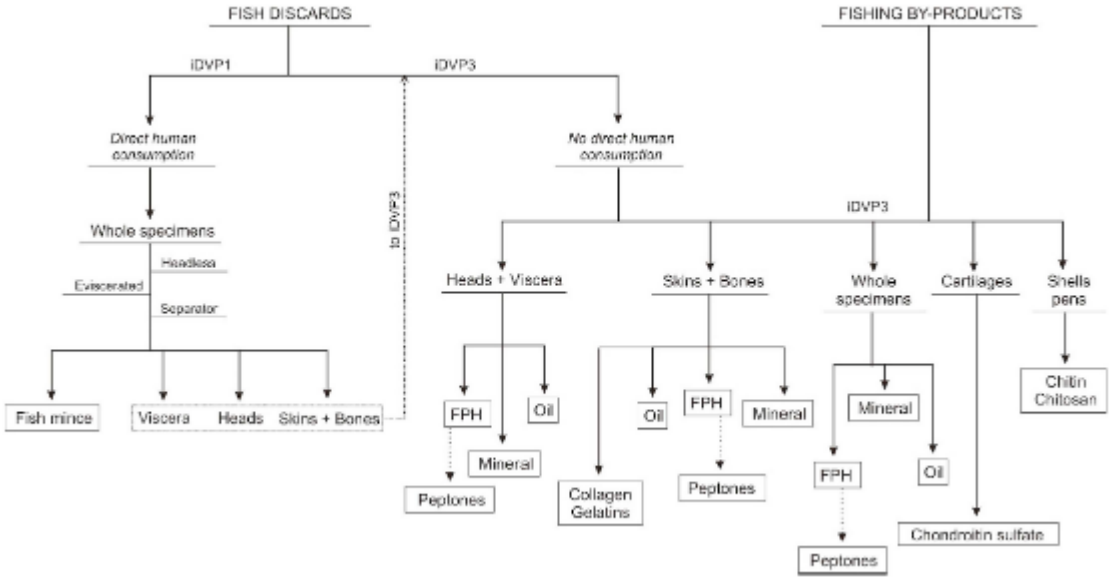


Figure 12.3.d: Valorising processes integrated in iDVP1 and iDVP3 (raw materials and final products obtained).

The pilot plant is divided into three different rooms: a) a chilled room for storage; b) a food processing area (iDVP1, Figure 12.3.e) and; c) a non-food product processing room (iDVP3, Figure 12.3.e). FHC is used to name the fraction of the landed catch composed of legal sized individuals, regardless whether they are subject to the TAC regulation, that can be used for direct human consumption but, for commercial reasons, do not generate interest for direct sale at the fish market. This fraction is treated at iDVP1 for the production of minced fish blocks. These blocks can be used later as a raw material for the production of food (restructured products) for human consumption or for pet food. Similarly, FNHC is used to name the fraction of individuals below the MCRS of any species subject to the TAC. This fraction is managed differently from the rest of the catch and processed at the iDVP3 plant together with by-products generated at iDVP1 (head, viscera, skin, and bone) and industrial fish by-products, minimizing the impacts of a possible lack of supply of undersized specimens as raw material. iDVP3 includes production lines to obtain valuable biocompounds, such FPHs, mineral supplements, collagen/gelatine, fish oil, marine peptones, chondroitin sulphate or chitin/chitosan, among others. Our blue whiting FPHs have been processed at the iDVP3 plant.

Figure 12..e: iDVP1 (left) and iDVP 3 (right) installed in the Port of Marín (Galicia, NW Spain).

4. Chemical analyses

During the production of FPHs from blue whiting discards, the obtained FPHs (like the ones depicted in Figure 12.3.f) showed levels of soluble protein higher than 33 g/L with 97% of in *vitro* digestibilities.

Figure 12.3.f: Steps of enzymatic hydrolysis of fish discards to produce FPH and oil in iDVP3.

The maximum degree of hydrolysis of FPHs has been greater than 30% and the liquefactions of the solid blue whiting substrates to the liquid FPHs (digestion of organic material) have been higher than 90%. Further, other added-value compounds of interest such as a fish oil fraction (rich in omega-3 fatty acids) together with solid bones, that can be used as mineral supplement (rich in calcium and phosphorus) for food/feed applications, are obtained.

The most abundant amino acids are glutamic and aspartic acids followed by leucine and lysine. Several studies have reported the same predominance of glutamic and aspartic acid in fish hydrolysates of several fish species. Essential amino acids (Ile, Leu, Val, Lys, Met, Phe, Thr, His and Arg) are also significantly present.

Discussion

In CST 12.3, an enzymatic process has been optimised for the hydrolysis of previously discarded (for commercialization and human consumption) fish species caught by North Atlantic fishing fleets, particularly for blue whiting. As a previous step, a complete characterization of these fish biomass side-streams has been carried out in order to clearly state the availability of raw material towards an industrial implementation at landing-ports of this value chain based on enzymatic hydrolysis to obtain valuable hydrolysed proteins and oil from them, that are the first Key Exploitable Result of CST 12.3. It has been stated that FPHs obtained by the proposed methodology (both at lab as well as at pilot scale in the iDVP) exhibited a remarkable concentration of proteins and the profile of amino acids (including

essentials) is very interesting to be used as ingredients of fish feed diets to be developed and tested in CS12.4.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained so far we have completed 30% of the activities programmed for CST12.3.

We are very close to completing our first key exploitable result (SCTP12.3.1 production of ingredients from fishery discards) and a bit delayed with the tests for the production of ingredients from fish frames (SCTP12.3.2) and non-commercial mussels (SCTP12.3.3) because of the lockdown due to COVID19 in South Africa and Spain on March 2020. We will describe our progress towards SCPT12.3.2 and SCTP12.3.3 in the next report (M13 to M18).

Deviations & Problems: The test of methods to obtain hydrolysed proteins and oil from fish frames and non-commercial mussels at the laboratory scale have been stopped in March 2020 because of the COVID-19 lockdown. At the moment, the delay is minimal but, if the current situation persists, this would imply changes in the associated tasks.

Outlook: We have to make the scaling of the methods to obtain hydrolysed proteins and oil from fish frames and non-commercial mussels towards their implementation at a real pilot plant like the iDVP. We aim to produce these ingredients from both mentioned side-streams in the amount required to formulate and test the diets of CST12.4.

CST 12.4 Fishery by-catch valorisation: aquafeeds diets and tests

Cláudia Aragão, CCMAR

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
12.4	Fisheries by-catch valorisation: aquafeeds diets and tests	T3.2	CCMAR	5%	✓	M12	M9	M48	M48	4	7

Introduction

The suitability of the ingredients produced in CST 12.3 for finfish aquaculture diets is tested in CST 12.4. The use of these alternative diets will decrease the carbon footprint of feed for finfish aquaculture and the nitrogen and phosphorous emissions to the environment. In CST 12.4 Senegalense sole juveniles will be feed with ingredients derived from fishery discarded biomass and fish frames from the fishery transformation industry. CST 12.4 started at M9 with the elaboration of a route map for preparation and testing of diets from the fishery ingredients with an estimation of the amounts needed of each ingredient to formulate and test the diets.

Methods

CST 12.4 is essentially based on a fish growth trial to test diets incorporating high-quality ingredients from side-stream fisheries activities. The growth trial is expected to start at M18. In this experiment we will test experimental diets, including fish protein hydrolysates obtained from Galician fisheries by-catch, produced by CST 12.3. To calculate these amounts, some tentative information on the growth trial is needed. The proposed experimental design is reported in Figure 12.4.a and this will be the basis for the calculations presented in the next section.

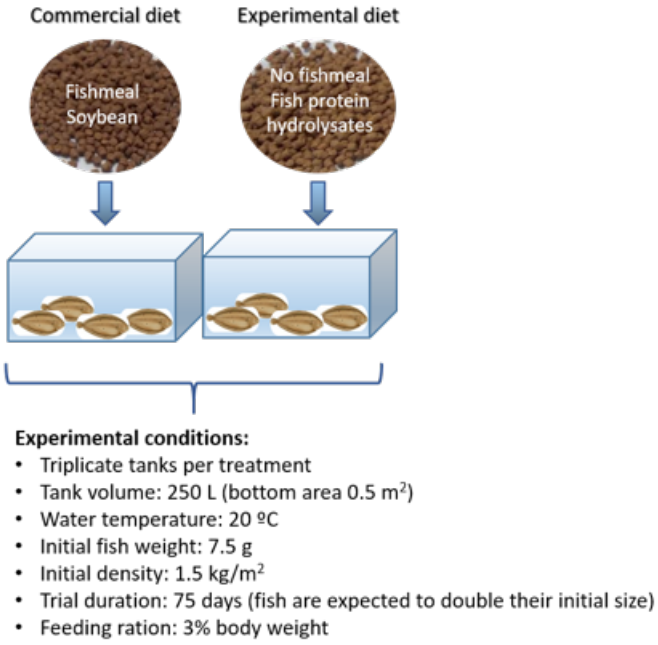


Figure 12.4.a: Experimental design for the growth trial to be performed in CST 12.4.

Results

A route map has been elaborated that will allow the involved partners to be fully aware of the Task developments and to better plan the interactions with the CST 12.3. This is reported in Figure 12.4.b.

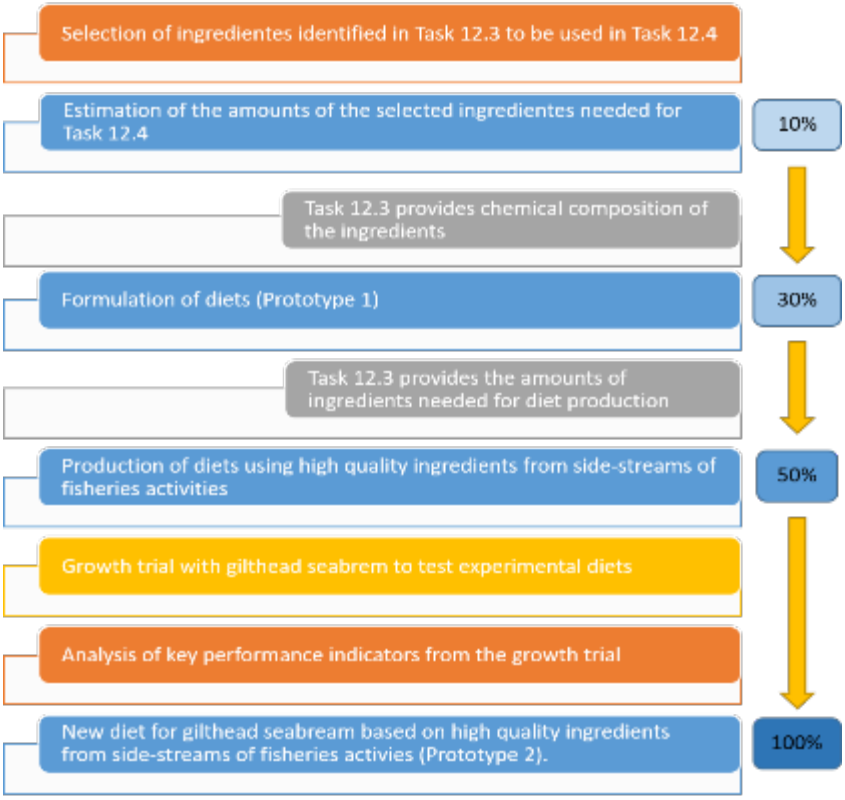


Figure 12.4.b: Route Map for CST12.4 with an estimation of the Task completeness at key points.

Based on the information provided at this time by CST 12.3, the experimental diet to be tested with Senegalese sole will include protein hydrolysates obtained from fisheries by-catch of Galician trawlers. If available on time, an additional experimental diet including protein hydrolysates obtained from fish frames from fishery transformation industry will be tested.

Table 12.4a shows the estimation of the amount of feed necessary for the growth trial, taking into consideration the conditions provided in Figure 12.4.a.

Table 12.4.a: Calculation of the amount of feed necessary to perform the CST12.4 growth trial.

Growth trial conditions	
Tank volume	250 L
Initial density	1.5 kg/m^2
Number of fish per tank	100
Initial fish weight	7.5 g
Initial tank biomass	0.75 kg
Expected final fish weight	15 g
Estimated final biomass	1.5 kg
Feed ration (%/day)	3.0
Trial duration (days)	75
Amount of feed (kg per tank)*	3.0 kg
Number of replicates per treatment	3
Total feed per treatment (kg)	9.0 kg

*amount of feed was calculated as: (initial biomass + final biomass)/2 x feed ration x number of trial days x 20% safety margin.

To estimate the amount of protein hydrolysates from fishery discarded biomass, it is assumed that diet formulation will include a maximum of 10% of this ingredient, to act as a feed attractant in a fish diet completely devoid of fishmeal. Therefore, since almost 10kg of each experimental feed are necessary to the growth trial, CST 12.3 will need to provide CST 12.4 at least 1kg of protein hydrolysates in order to proceed with the production of diets.

Discussion

N/A (results from growth trial will only be available after M18).

Progress, deviations, problems & next 12M

Progress: Based on the results obtained so far, we have completed 5% of the activities programmed for CST12.4 (Figure 12.4.a).

The task started on M9 to prepare the Route Map and to inform CST 12.3 about the amounts needed of each ingredient to formulate and produce the diets. After that, CST 12.4 have been inactive until CST 12.3 supply the ingredients by M18 the latest.

Deviations & Problems: none

Outlook: over the next 12M we have to receive the ingredients extracted from fishery discarded biomass and fish frames from fishery transformation industry produced by CST 12.3, formulate the diets, and start the nutritional experiments to test them in Senegalese sole juveniles.

CS13

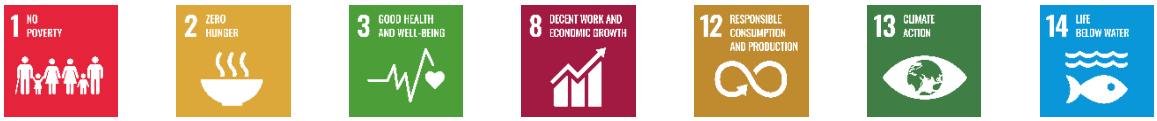
Summary of progress report for Case Study	**13**
Date of report:	**30.3.2020**
Case Study name:	**Low trophic feeds**
of relevane for WPs	**2, 3**

Abstract/Summary

There is a growing concern for the ability to produce enough food to feed the global human population in the future. If the global population reaches 9.6 billion by 2050, the equivalent of almost three planets will be required to sustain current lifestyles. To maintain the actual per capita average seafood consumption without further improvements expected from fisheries, aquaculture production will have to increase by 70% and this will depend on its capacity to expand while reducing environmental impact. New biomasses able to accommodate aquaculture expansion need to be explored, so tackling this food demand in a transdisciplinary approach is vital for a better protection of the environment for future generations.

The present work aims to increase system biological efficiency by including low trophic levels ingredients in diets to feed abalone, shrimp, freshwater, and marine fish. The novel concepts of AquaVitae such as harvesting low trophic levels species, that additionally can be produced as side species associated to aquaculture production; in IMTA systems; and included in animal diets to tackle this food demand, will be supported by the most advanced research and technology through the Atlantic Ocean. This challenge is being addressed in Brazil (EmBraPa, FURG, UFSC), Faroe Islands (ORF), Ireland (GMIT), South Africa (RhU, Marifeed), Spain (CSIC, ULPGC) and Portugal (CCMAR).

All the research will contribute to the national and international sustainability agendas, mainly by promoting the achievement of the UN SDGs 2 (Zero hunger), 13 (response to climate change) and 14 (Life below water), although the results will also benefit progress in SDGs 1 (No poverty), 3 (good health and well-being), 8 (Decent work and economic growth) and 12 (Responsible consumption and production).



The proposed aims present a high potential to implement the findings into innovative products and services for the aquaculture industry and to increase the competitiveness of worldwide aquaculture, promoting the concept of modern aquaculture as an environmentally and economically sustainable practice to society.

This project has received funding from the European Union's Horizon 2020 Research and Innovation Programme under Grant Agreement No 818173. This report reflects only the authors' view and that the Commission is not responsible for any use that may be made of the information it contains.

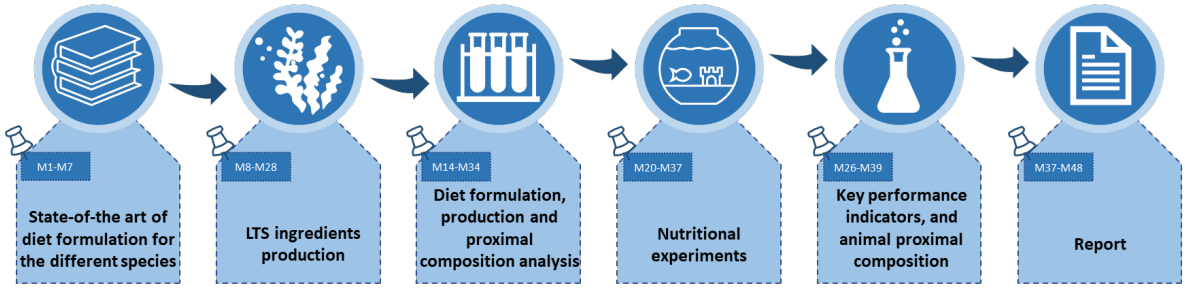


Figure 13: Illustration of the scheduled activities within CS13 for CST 13.1 and 13.2.

CST 13.1 Using macroalgae to improve feeding strategies for other low trophic species

Sofia Engrola, CCMAR

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
13.1	Using macroalgae to improve feeding strategies for other low trophic species	T2.4	CCMAR, ULPGC, ORF, GMIT, RhU, Mfeed	21%	✓	M1	M1	M48	M48	4	6

Introduction

Case Study Task 13.1 investigates the effect of introducing LTS-based ingredients coming from the CS2, CS3 and CS4 in new and existing feed formulas for two species of abalone, European abalone (*Haliotis tuberculata coccinea*) and South African (*Haliotis midae*). This research is being addressed in South Africa (RhU), Spain (ULPGC), Faroe Islands (ORF), and Ireland (GMIT).

Objectives: (1) to develop a diet for European abalone macroalgae-based and that supports an affordable final product; (2) to develop feed for South African abalone that include pathogen-free algae that enhances abalone growth and health; (3) to optimise the inclusion of algae, from various sources, in the diets of South African abalone to enhance growth and health.

The aim of this work will be to present the formulations for the production of abalone feeds that includes ocean harvested kelp or IMTA produced macroalgae as a dietary ingredient, to scale-up and manufacture these diets under commercial-feed production conditions, and to ultimately implement processes that makes them biosecure when the feed is cycled back into abalone production. Abalone diets will be formulated and manufactured and presented as a product as part of CC CS 13 (Figure 13.1.a).

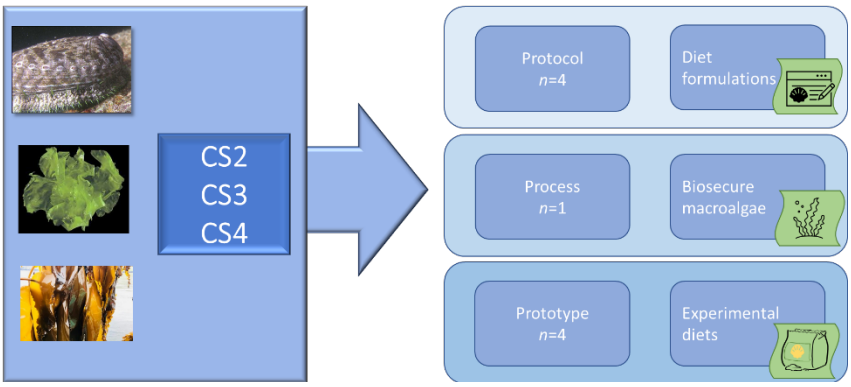


Figure 13.1.a: Diagram of the workflow and outputs of CST 13.1.

Two South African abalone feeds will be produced and will report as four products in cross cutting CS13; i.e. two feed formulations (CSTP13.1.3 and CSTP13.1.4). It should be noted that these

formulations will exclude proprietary information that was developed by partner Marifeed Pty Ltd ahead of this project. The other two products will be the two feeds that result from these formulations, i.e. CSTP13.1.8 and CSTP13.1.9 (Table 13.1.a), respectively, and which will be manufactured by Marifeed Pty Ltd. The fifth abalone feed product that will report as CSTP13.1.5 will draw on the first four products, only the IMTA produced algae in these diets will be subject to a newly developed biosecurity process, which will be developed and tested in CS4.

The algal ingredients that will be included in all of the above feeds will be produced and developed in other AquaVitae case studies (i.e. CS2, CS3 and CS4) and the performance of these diets in IMTA systems will be reported in the relevant IMTA case studies. More specifically, the kelp in CS13.1.8 will originate from kelp produced in the Faroe Islands in CS2. The macroalgae that will be included in CSTP13.1.6, CSTP13.1.7 and CSTP13.1.9 will be produced in IMTA systems (i.e. at ULPGC in CS3 and at Blue Ocean Mussel Pty Ltd as part of CS4). The abalone nutritional experiment will be done in CS3 (at Wildcoast Abalone Pty Ltd and at the ULPGC in Spain), as part of the IMTA production systems.

The first months (M1-M7) were dedicated to collect data regarding the state-of-the-art for feed formulation for both species, to design production systems, and to calculate the amount of low trophic species (LTS) ingredients needed to incorporate in the experimental diets for the in vivo studies (Table 13.1.a).

Table 13.1.a – Amount of diet needed for the in vivo studies in CST13.1.

Identifier	Application	Amount (Kg)
CSTP13.1.6	Diet for European abalone macroalgae-based	2
CSTP13.1.7	Diet for European abalone macroalgae- and vegetable-based	2
CSTP13.1.8	Diet for African abalone harvested kelp-based	2
CSTP13.1.9	Diet for African abalone IMTA macroalgae-based	2

The production of the macroalgae needed for the nutritional experiments in CS 13.1 will only be started in M13-M18 in CS2, 3 and 4. Also in CS4, the development of process to produce biosecure macroalgae for African abalone (CSTP13.1.5) was initiated by Partner RhU. Partner RhU also started some preliminary test of diet leaching with commercial feed to gather knowledge for the experimental diets formulations.

Methods

All isonitrogenous and isoenergetic diets will be formulated to include the LTS species. Diets will be manufactured by extrusion and supplemented with selected indispensable amino acids (IAA) and mono-calcium phosphate, whenever necessary, to fulfil the known nutritional requirements of abalone. Animals will be fed to apparent satiety. Parameters to be analysed include growth, feed conversion ratio, survival, animal, and diet proximal composition (crude protein, total lipids, ash, phosphorous, moisture and gross energy), amino acids and fatty acids profiles and gut bacterial community profile (African and European abalone). All chemical analysis will follow standard procedures of the Association of Official Analytical Chemists. Data will be presented as means ± standard deviation. All data will be checked for normal distribution and homogeneity of variances. Differences among dietary treatments will be identified by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test at $P < 0.05$ level of significance.

Preliminary abalone feed water stability and nutrient leaching testing using control diet:

Three standard abalone raceways (4.86m x 2.17m x 1.05m, l x b x h; 12,000L) containing 48 empty abalone baskets were prepared and aerated at the same rate used when stocked with abalone. Thirty grams of standard Abfeed S34® pellet (Marifeed Pty Ltd, South Africa) was added to each basket. At predetermined times of 0, 15, 30, 1, 4, 8, 12, 24, 36 and 48 h, three (3) randomly selected baskets with immersed pellets were removed from experimental tanks (one basket per tank) and allowed to drain for 10-15min. After draining, soaked pellets in each basket were rinsed with distilled water to wash away any dissolved salt and later transferred into pre-weighed petri-dishes for further processing. They were oven-dried at 65°C for 36-48h until constant weight was achieved. The calculated difference between the initial weight of pellet (before immersion) and final weight (weight after drying) was expressed as the percentage dry weight loss which represents a measure of water stability of the test pellets for the respective time interval. The percentage of dry matter leaching (DMi) at its respective immersion time was calculated by the equation of Carvalho and Nunes (2006): DMi = [1 – (Wdi /Wf)] x 100. Where: DMi = percentage of dry matter leaching at time i (%); Wf = dry feed weight before immersion in seawater (g), and Wdi = dry feed weight after immersion in seawater at time i (g), dry feed weight refers to feed weight after drying at 65°C for 48-hours. Triplicate samples of the dried pellets were analysed for nitrogen, phosphorus, and carbon composition.

Results

Currently the LTS production is ongoing (Figure 13).

Leaching trial with preliminary abalone feed: The control diet was water stable after 36 h. Highest stability (96.17 ± 2.71%, mean + SD) was recorded after 15min of immersion which was closely followed by 94.79 ± 0.31% at 30min. After 36h, pellet stability had reduced to 80.01 ± 0.15%. Moreover, nutrients in the pellets were also stable; after 36h of immersion in seawater, the nitrogen, phosphorus, and carbon composition of 4.93 ± 0.25, 1.00 ± 0.02 and 38.87 ± 0.46 % were similar to with those of the control (i.e. un-leached pellets): 5.31 ± 0.10, 1.15 ± 0.03 and 40.95 ± 0.15 %.

Discussion

Not applicable.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained so far, the CST13.1 is at 21% of completeness. CST 13.1 plays a crucial role in fulfilling the Specific Objectives SO1, SO3 and SO4. It will help developing new aquaculture species and the value chains (SO1), as new products for the aquaculture species (SO3) and all contribute to processes where nutrients are captured and recycled, using a circular economy approach (SO4).

Deviations & Problems: The status of work within CST 13.1 is considered green. However, due to the COVID-19 situation most of the countries involved in the task are in lockdown, if the current situation persists, this may imply some changes in the task. There were delays in receiving raw ingredients from the CSs in which they were to be producing material; and without this material we could not carry out proximate analysis on the raw ingredients and therefore could not produce diet formulations. Researchers working in CS13 have collaborated and coordinated with those in CS2, CS3 and CS4 to ensure that lost time is caught up as much as reasonably possible.

Outlook: For the next period M13-M24, the partner RhU will have some of the macroalgae needed to formulate and manufacture the diets for the nutritional experiments in abalone; the partner ULPGC will continue with the LTS production trials.

CST 13.2 Using LTS to improve the nutritional quality of new and existing feeds for high trophic species

CST #	Name	Reports to task(s)	Partners involved	completed by	Status	start month		end month		TRL	
						planned	actual	planned	actual	Current	by M48
13.2	Using LTS to improve the nutritional quality of new and existing feeds for high trophic species	T3.3	CCMAR, EmBraPa, UFSC, FURG, FCPCT, CSIC-IIM	21%	✓	M1	M1	M48	M48	4	6

Introduction

CST 13.2 investigates the effect of introducing LTS-based ingredients (microalgae, macroalgae, and mussels) in marine and freshwater fish, as well in marine shrimp (Figure 13.2.a). Macroalgae will be produced in CS1, while the mussel meal in cross cutting CS12.

Intensive research made possible to increase aquaculture production using low environmental footprint diets that rely partially on land produced ingredients. In addition, currently farming carnivores' species with formulated diets is more acceptable since this strategy ensure a high conversion efficiency of fish-in fish-out ratio. The replacement of fishmeal in fish and shellfish diets by low trophic levels ingredients is a possible approach to create an aquaculture industry independent of fishmeal and agriculture ingredients and simultaneously animal production may be brought down several trophic levels. This will increase not only the environmental sustainability of the sector, but also its economic and social sustainability by adding value to new biomasses, decreasing the imports of fishmeal, and fomenting the use of good practices in the sector.

Objectives (1) to develop a new diet that incorporates low trophic species to replace fishmeal and –oil in current diet formulation for marine and freshwater fish, and marine whiteleg shrimp; (2) to develop a diet for pirarucu broodstock that enhances reproductive performance.

The low trophic species will be included as a supplement or an ingredient in diets for marine (Senegalese sole, *Solea senegalensis*, Brazilian flounder, *Paralichthys orbignyanus*) and freshwater (tambaqui, *Colossoma macropomum*) fish juveniles, freshwater fish broodstock (pirarucu, *Arapaima gigas*), and marine whiteleg shrimp (*Litopenaeus vannamei*). This research is being addressed in Brazil (EmBraPa, FURG, UFSC), Spain (IIM-CSIC) and, Portugal (CCMAR).

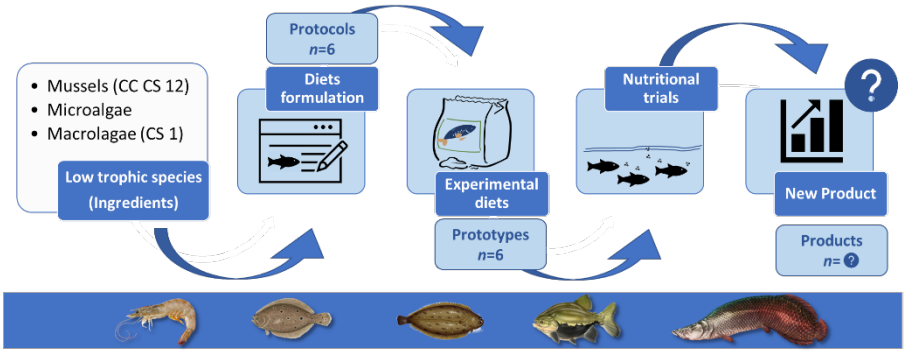


Figure 13.2.a: Diagram of the workflow and outputs of CST 13.2.

The first months (M1-M7) were dedicated to collect data regarding the state-of-the-art for feed formulation for all species, to design production systems, and to calculate the amount of low trophic

species (LTS) ingredients needed to incorporate in the experimental diets for the in vivo studies (Table 13.2.a). Several meetings were held between the task partners and other project partners to ensure the production of enough material such as macroalgae to include in the diets. Particular attention was giving to the pirarucu broodstock diet, due to the high amounts of macroalgae needed. For this reason, this in vivo trial will be the last to start.

Table 13.2.a: Amount of diet needed for the in vivo studies in CST13.2.

Identifier	Application	Amount (Kg)
CSTP13.2.7	Diet for Senegalese sole with inclusion of mussel meal	3
CSTP13.2.8	Diet for Senegalese sole with inclusion of mussel hydrolysates	3
CSTP13.2.9	Diet for Brazilian flounder with inclusion of algae	3
CSTP13.2.10	Diet for whiteleg shrimp with inclusion of algae	3
CSTP13.2.11	Diet for pirarucu with inclusion of algae	30
CSTP13.2.12	Diet for tambaqui with inclusion of algae	15

Methods

All diets will be formulated and manufactured and presented as a product as part of cross cutting CS13. Each diets will be isonitrogenous and isoenergetic and fed to apparent satiety. All fish experiments will be carried out in compliance with the Guidelines of the European Union Council (Directive 2010/63/EU) and national legislations for the use of laboratory animals. Parameters to be analysed include growth (weight, length, condition factor, daily gain, thermal growth coefficient), feed conversion ratio, survival, animal, and diet proximal composition (crude protein, total lipids, ash, phosphorous, moisture and gross energy), amino acids and fatty acids profile. All chemical analysis will follow standard procedures of the Association of Official Analytical Chemists. Data will be presented as means ± standard deviation. All data will be checked for normal distribution and homogeneity of variances. Differences among dietary treatments will be identified by one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test at $P<0.05$ level of significance.

Results

Currently the LTS production is ongoing (Figure 13) for Senegalese sole, Brazilian flounder, tambaqui, and pirarucu. One diet was already formulated and manufactured for whiteleg shrimp (*Litopenaeus vannamei*) at partner UFSC (Brazil). The diet has the inclusion of microalgae meal from *Nannochloropsis* spp. (Necton – PhytoBloom, Portugal). The *in vivo* trial will start in the following months.

Discussion

Not applicable.

Progress, deviations, problems & next 12M

Progress: Based on the results obtained so far, the CST13.2 is at 21 %of completeness. Currently from the 6 CSTPs related to diet formulation for the different species, the CTPS13.2.4 for whiteleg shrimp is done. The remaining 5 CSTPs related to diet formulation are waiting for the LTS ingredients production. Therefore, from the 6 CSTPs related to diet performance in animal experiments, the CSTP13.2.10 related to data collected from shrimp will initiate in the coming months.

CST13.2, plays a crucial role in fulfilling the Specific Objectives SO1, SO3 and SO4. It will help developing new aquaculture species and the value chains (SO1), as new products for the aquaculture species (SO3) and all contribute to processes where nutrients are captured and recycled, using a circular economy approach (SO4).

Deviations & Problems: The status of work within CST 13.2 is considered green. However, due to the COVID-19 situation most of the countries involved in the task are in lockdown, if the current situation persists, this may imply some changes in the task.

Outlook: For the next period M13-M24, the partner USFC will have the results for the *in vivo* trial in whiteleg shrimp; the partners CCMAR, FURG and EmBraPa (tambaqui) will have the LTS to include in the diets for the nutritional trials. The partner EmBraPa (pirarucu) will start the nutritional trail after this period due to the high amount of LTS needed to incorporate in the diets.