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Microalgae cultivation as a food source in aquaculture applications

Diploma thesis

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Declaration

I declare that I have elaborated my diploma thesis "Microalgae cultivation as a food source in aquaculture applications" independely under the supervision of the thesis supervisor and consultant and using literature and other information sources cited in the thesis and listed in the bibliography at the end of this document. In addition, as the author of the thesis I declare that I have not infringed the copyrights of third parties.

In Prague, 23.07.2020 _____

Acknowledments

I would like to express my sincere gratitude to my advisors RNDr. Milan Skalický, Ph.D. and Ing. Karel Douda, Ph.D. for the continuous support and encouragement received through the collaborative work, I greatly appreciate the enriched discussions and lessons that guided me through this process.

Gracias a mi familia (Agatha, Lucas y Roque en el cielo incluidos) los extraño por montones, cada recuerdo con ustedes es perfecto e invaluable, vamos con toda los “tri” amigos.

Juan Felipe haces que todos mis días sean los más hermosos muero de ganas por seguir construyendo nuestra familia, te amo infinitamente.

Microalgae cultivation as a food source in aquaculture applications

Summary

Microalgae play a very important ecological role in both aquatic and terrestrial ecosystems and represent one of the most efficient converters of solar energy to biomass. Because of those characteristics, microalgae have been used in plenty of industrial and biotechnological processes and have attracted interest for their potential to generate valuable products in high volumes. Some of those applications include the use of microalgae cultured in intensive systems for aquaculture and animal nutrition. The majority of microalgae world requirements for aquaculture goes to mollusks, as economically and ecologically important filter feeders there has been an increased interest in research for algae production focused in this group, however much of the research is sparse and varied with no single general trend, moreover the majority of studies are focused on marine bivalves, with freshwater bivalves having much less attention. In this thesis we aim to review the current knowledge on the use of unicellular and simple multicellular microalgae in aquaculture especially for rearing bivalves, as well as finding general trends and patterns of research in the topic. Additionally, we developed a protocol for the establishment of a pilot culture of algae aimed to feed freshwater mussels and assessed the algal culture early growth using image analysis techniques under different growth conditions. The bibliometric analysis retrieved a total of 287 peer reviewed articles clustered in 6 groups the most important ones consisting of (*i*.) terms related to potential harmful consequences of the microalgae, (*ii*.) nutritional value of microalgae diet, (*iii*.) nutritional elements necessary to rear bivalves. A total of six bivalve species are the most co-occurent of the 287 peer-reviewed articles, all of them inhabit marine ecosystems and are reared because of their meat (except for *Pinctada margaritifera*, Margaritidae), regarding the algae species the search found a total of 13 most relevan species, all of them naturally distributed salty or brackish water.
Concerning the pilot culture, our protocol using *Ettlia oleobundans*, produced a viable mid-size culture of size 2.28 x10^7 cells/mL and a 90% of viability after two months of culturing. On the other hand, early growth assessed through image analysis showed that for the group of pictures taken after 20 days a stronger contrast was identifiable, the Anova found a significant effect of the media used during acclimatization (Pr(>F) <0.01), algae that were acclimatized in agar before the start of small-scale experiment were significantly greener, and hence, denser, than algae acclimatized in liquid media. The mean Hue value for algae acclimatized in agar was 68.49 ± 5.80, while the liquid acclimatized algae 62.39 ± 4.46. Additionally, the mean Hue value for the three different evaluated concentrations was as follows: A=65.99 ± 5.37, B=66.79 ± 8.23, and C=63.41 ± 3.21. The concentration of F/2 media showed no significant effect (Pr(>F) 0.22). The method seemed promising for evaluating growth in algae, especially in early stage although the presence of some outliers in the data produced by the limitations of the image taking and processing algorithm deserve further research.

Keywords: bibliometric networks, *Ettlia oleoabundans*, bivalve rearing, culture setup, Image analysis, Hue value.

Content

1 Introduction

The term algae is routinely used to indicate a phylogenetically diverse group of O_2-evolving, photosynthetic organisms (Barsanti & Gualtieri, 2006), they contribute to half of the globe's photosynthetic activity (Day et al., 1999; Field et al., 1998) and are the base of over 70% of the world's biomass food webs, including all marine, freshwater, and a small portion of terrestrial food webs (Raven & Giordano, 2014). Microalgae play a tremendous ecological role supporting the aquatic primary production (carbon dioxide assimilation and oxygen production), and currently are widely used on different human-productive direct and indirect applications, such as biofuels, bio-derived goods like coloring agents, anticancer drugs, antimicrobial drugs, poly-unsaturated fatty acid, or directly as human or animal food and food supplements (Khan et al., 2018).

The volume required to carry out all these productive activities is immense; it is estimated that around 20 tons of microalgae are produced annually (Tredici et al., 2016), today's market is dominated by 5 genus: *Arthrospira* with more than 12,000 tons per year, followed by *Chlorella* with about 5000 tons, *Dunaliella salina* (marketed for its β-carotene) with 3000 tons and finally *Aphanizomenon* with 1500 tons per year (Levasseur et al., 2020), the number is likely to increase as new biofuel production technologies increasingly require biomaterials to supply the demand for new markets. It is for this reason that technological advances seek to optimize the space and supplies used for cultivation.

These advances can also be applied to the cultivation of microalgae with the ultimate goal of optimally feeding aquatic animals in captivity, microalgae play a significant role in mariculture as the basic diet for many molluscs, crustaceans and some fish (Muller-Feuga, 2000). Photobioreactors (PBRs) are devices for the mass cultivation of microalgae, they have the advantage of maintaining a stable medium (temperature, pH, low O_2 concentration) and providing the necessary nutrients for growth, including light. There are two opposite approaches to the design of this type of device: (*i*.) Open reactors prioritize economy by accepting poor control of the environment, while (*ii*.) closed PBRs manage to closely control all the optimal conditions that allow microalgae to grow at an optimal speed in exchange for a higher cost (Posten, 2009). The use of these devices when cultivating different species of freshwater bivalves represents a great advantage, either for the industry or when the objective is the study of different experimental conditions including studies in toxicology, ecology, development, behavior and conservation of species of bivalves. Microalgae are cultivated as food for the different stages of rearing bivalves of some economic interest (Helm & Bourne, 2004). Until recently, live algae were the only source of food for bivalve larvae and juveniles in hatcheries, but this situation is now changing as recent research on the development of appropriate artificial and inert diets (Mazón-Suástegui et al., 2017; Nevejan et al., 2007). Although it represents between 20 and 50 percent of the total production and operating costs of the hatcheries, the production of live algae will continue to be a fundamental aspect in the success of hatchery management in the immediate future, even if it is only as a live food that complements the most novel foods (Michael M. Helm & Bourne, 2004),

2 Scientific hypothesis and aims of the thesis

1. The aim of the thesis is to review the current knowledge on the use of unicellular and simple multicellular microalgae in aquaculture specially for rearing bivalves.

    1.1.1. There has been an inceasing interest in the culture of microalgae in the past few years to investigate the applications for bivalve feeding and aquaculture research.
    1.1.2. The latest studies have shown that feed bivalves with fresh microalgae are a better source of nutrition over other methods.

2. Perform a pilot laboratory-scale experiment dealing with the selection of the model algae strain, and the setting of a small-scale culture for potential utilization in filter-feeding bivalves.

    2.1.1. *Ettilia oleabundas* can be cultured successfully in small and medium scale in the lab for long time bilvave feeding.
    2.1.2. The use of indirect methods such as image analysis to follow the development of the culture during the first phases to avoid the risk of infections is correct and reliable.

3 Methods

The methodology was divided into two phases according to the stated objectives. The first phase includes a bibliographic search focused on relevant information of the ecology, physiology and microalgae uses, focusing the bibliometric analysis on the cultivation of microalgae to use them in bivalve rearing and farming, including a potential use in conservation.

The second phase involves the setting of a small to medium scale microalgae culture for the malacology lab of the Faculty of Agrobiology, Food and Natural Resources of the Czech University of Life Science in Prague. Starting form the selection of the an algae species suitable for mussel feeding and its posterior cultivation, for this porpuse the selected algae strain was carefully scaled up from the inicialfrom a single tube purchased in the UTEX collection to 8 mL test tubes, 200 mL erlenmeyers up to a 10 L carboy; a volume that could either be used to increase the production, or as the source of fresh food for filter feeding bivalves in the malacology laboratory.

This phase also includes the materials selection and subsequent assembly of an appliance using an available incubator that could provide the optimum conditions to the chosen microalgae species in terms of temperature, proper light and mixing, and an automatic and fast technique of assessing the density of the culture based on image analysis when the culture is to week to do a proper cell counting.

3.1 Bibliographic review of algae culture and bibliometric analysis of its use in bivalves

3.1.1 Data sources and selection criteria

The bibliographic search focused on relevant information of the ecology, physiology and microalgae uses was complemented with a visual approach to analyze the bibliometric networks on the topic of cultivation of microalgae to use them in bivalve rearing and farming, including a potential use in conservationin.

The search was performed in the Web of Science database, the Science Citation Index-Expanded (SCIE) of Thomson Reuters' Web of Science provides comprehensive data of publications and is considered the ideal database for bibliometric analysis as the use of additional biomedical databases does not significantly increase the yield of relevant journals (Aggarwal et al., 2016; Gao et al., 2017; Garrido-Cardenas et al., 2018). The manuscript types were restricted to original articles. All searches were performed on a single day, May 20, 2020, to avoid changes in the number of publications and citations as much as possible. The retrieval strategy was:

TS= (bivalve* OR mussel* OR oyster* OR clam) AND TS= (Microalgae*) AND TS= (culture* OR aquaculture OR hatchery OR photobioreactor OR conservation) language English and Only peer-reviewed articles were included (Article).

3.1.2 Data collection

The data of all eligible publications related to the use of microalgae as food source in filter feeding bivalves including title, year of publication, authors' names, nationalities, affiliations, name of publishing journal, keywords, abstract, times of citation, country and H-index were downloaded. The data was imported into VOSviewer version 1.6.15 (van Eck & Waltman, 2010). VOSviewer provides distance-based visualizations of bibliometric networks.

3.2 Small scale experiment

In this section, the preparation and assembly process of the small and medium-scale culture will be described starting with the assembly of a small and precise appliance using an available incubator in which temperature, light, photoperiod, and aeration can be controlled allowing the cultivation of fresh algae directly in the malacology laboratory. And continuing with the selection of the appropriate algae to feed bivalves and the medium for its cultivation, The algae acclimatization process and subsequent inoculation and transfer from 8 mL test tubes to 200 mL Erlenmeyers and a finally a 10 L carboy.

3.2.1 Selection of light source

LED lights

Different types of light sources have been used in horticulture and indoor cultivation, see Figure 1. Light emitting diodes provide some advantages for indoor culture, their small size and low heat-loss rate permit optimal matching of the culture vessel and the LED mounting surface. LED lignts with cool spectrums happened to have a suficient amount of all required wavelengths to culture photosynthetic organisms such as plants or microalgae. When compared with different lamps according to their PAR efficiency (of 1.91 μmol-ph s− 1 W− 1 in commercially available LEDs) the results indicate that LEDs would be the most suitable lamps for microalgae cultivation (Blanken et al., 2013). An advantage of this type of light source is that they do not heat, which is perfect when the temperature control is an important factor, LEDs also work faultless at 25°C, (our setting was aiming to permanently maintain a temperature of 25°C, *Ettlia oleoabundans* perfect growth conditions).

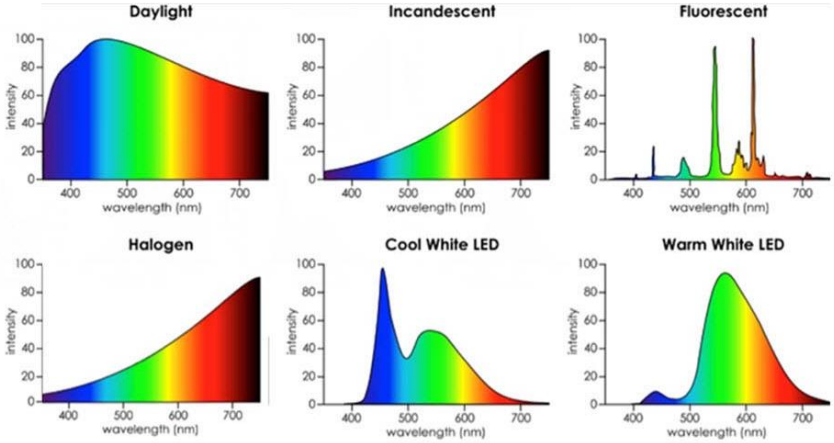

Figure 1 Emission spectra from daylight and typical incandescent, fluorescent, and LED lights. Image from: (Heiting, n.d.)

The LEDs selected for the setting had the following characteristics: 9,6W / m, 12V without protection IP20, day white color temperature -3100K Color temperatures between 3100K and 4500K are referred as “cool white”. In the Figure 2 the instructions for LED lights setting can be observed, the lights were cut and wired to fit the malagology lab incubator.

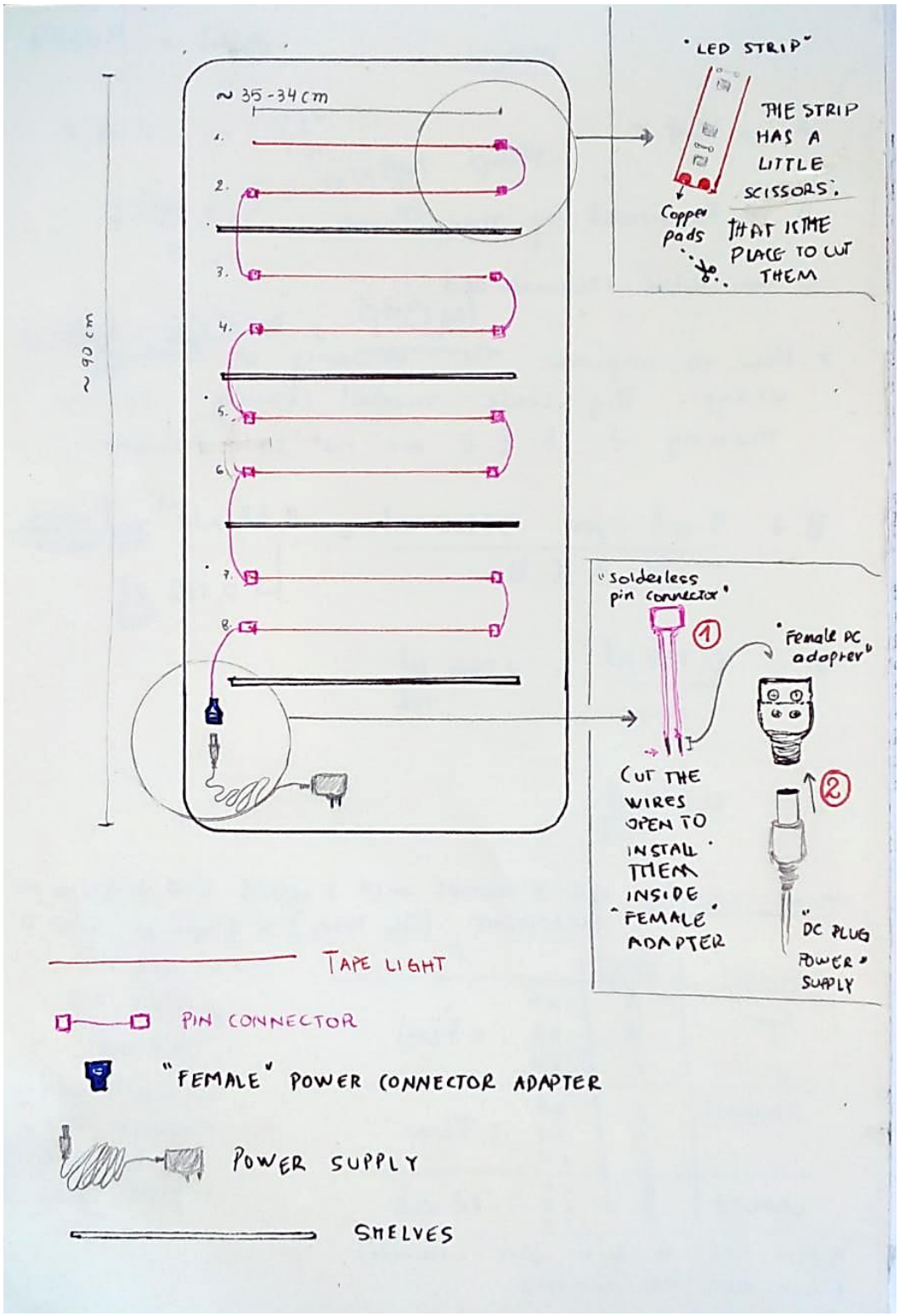

Figure 2 LED setting and materials used during the planning of the culturing appliance.

3.2.2 Algae species selection

For the selection of the appropriate microalgae species, a bibliographic search was carried out considering interesting characteristics (resistance to extreme conditions, ease of cultivation, high protein content, possible uses in trophic ecology studies, among others) the previous use in the culture and rearing of filtering molluscs was also taken into account. Three species were preselected and *Ettlia oleoabundans*, was chosen and ordered to the UTEX Culture Collection of Algae (UTEX Number 1185).

Ettlia oleoabundans (S. Chantanachat & Bold) J. Komárek 1989

Classification:
Empire Eukaryota; Kingdom Plantae; Subkingdom Viridiplantae; Infrakingdom Chlorophyta; Phylum Chlorophyta; Subphylum Chlorophytina; Class Chlorophyceae; Order Chlamydomonadales; Family Chlamydomonadales (incertae sedis); Genus *Ettlia*

Figure 3 Ettlia oleoanundans cells.

Image from: UTEX web site - 1185 Neochloris oleoabundans

3.2.3 Selection of media

The algae strain was transported in Agar Soil Extract media. Subsequently to its arrival the strain was subjected to an acclimatization process trying to emulate the conditions of the UTEX laboratory (20 °C and a photoperiod of 12 hours of light and 12 hours of darkness). After one week and a visual inspection, a transfer to a new media was performed under sterile conditions (all the necessary materials were autoclaved 45 min. at 120°C and 20 psi): the strain was inoculated into four 10 mL tubes of Liquid Soil Extract medium also bought from the

UTEX Culture Collection with the idea of transfer the algae into F/2 medium and making the transition less stressful for the algae from liquid to liquid.

The establishment of the sample in the new soil extract medium was allowed, resulting into two stock cultures, one growing in Agar and one growing in Liquid medium. From this point, the algae was inoculated from both sources into a commercial Guillard's 1975 F/2 formulation liquid medium (Proline F/2 Algae Food, Part A and Part B) as described in the numeral below: "*3.2.4 First inoculation for 8 mL tubes".*

3.2.4 First inoculation for 8 mL tubes

Three different concentrations of a commercial F/2 medium (Proline F/2 Algae Food, Part A and Part B) were used in combination with two algae sources (previously mentioned Agar origin and Liquid origin Soil Extract medium) to start the culture in the malagology lab. Three different concentrations were tested in order to optimize the amount of culture medium that would be used for future transfers into bigger vessels and in general for the cultivation of microalgae in the malacology laboratory of the faculty. Because the goal is to use the algea as a fresh food source it is required that the addition of it doesn't drastically change the chemichal composition of the water therefore the target was to reduce the amount of nutrients added to the bivalves' tank.

The experiment had a total of 6 treatments and six replicates per treatment (36 test tubes in total), and the concentrations tested were (*i*.) 75% of the recommended concentration, (*ii*.) the recommended concentration which is equal to 5 mL per 37800 mL of water, (*iii*.) and 150% of the recommended concentration. The complete design of the experiment is presented in the following Table 1:

Table 1 Complete experimental design for 2 factors with 3 levels and 6 replicates per treatment

Treatment	**Source**	**f/2 Concentration**	**Code**	**No. Replicates**
1	Agar	75%	Ag.A	X6
2	Agar	Recommended*	Ag.B	X6
3	Agar	150%	Ag.C	X6
4	Liquid	75%	L.A	X6
5	Liquid	Recommended*	L.B	X6
6	Liquid	150%	L.C	X6

All the materials were autoclaved for 45 min. at 120°C and 20 psi (except for the B-part which was filtered using 0.45 μm syringe filter), the procedure was done in sterile conditions: 500 mL of water plus 112.5 μl autoclaved A-part and 112.5 μl of filtered B-part were added into an Erlenmeyer, the solution was stirred to homogenize, Figure 4. Of this first solution 96 mL were used to fill the first set of tubes with 8 mL each: this first set of tubes has 150% the

recommended concentration. The twelve tubes were marked with a C letter and the source of inoculum (agar or liquid soil extract: Ag.C, Cl.C and Op.C).

In the same way the rest of the solutions with the desired concentrations were prepared, and twelve tubes per treatment were filled with 8 mL each to transfer the algae inoculum, those tubes were also marked as proposed in the Table 1 with the B letter (Ag.B, Cl.B and Op.B) and A letter (Ag.A, Cl.A and Op.A) to be subsequently inoculated, see Figure 4.

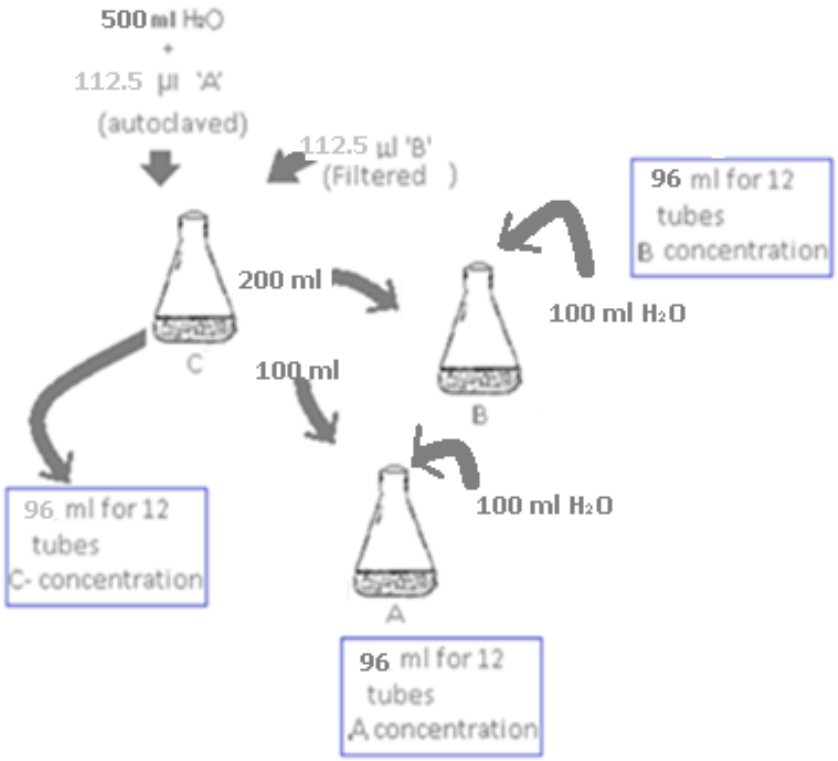

Figure 4 Summary of medium preparation 8 mL tubes

3.2.4.1 **Analysis of cell density using image analysis techniques**

Because of the reduced population size and cell density of the algae culture in the initial small-scale culture, no direct quantifications or samples were taken to avoid possible infections on the tubes. Instead an indirect and non-invasive method based on image analysis techniques was used for studying the effect of the media used during the acclimatization period and the different media concentration treatments. This analysis consisted in two steps, in the first step the photos from the tubes in the small-scale culture were taken on days 14 and 20 after inoculation. The second stage, here referred as the validation stage, was carried out after the small culture phase was finished and the algae were successfully transferred to bigger vessels. A subsample was then taken form an advanced stage of the culture several months later (22.67 mill. Cells/ml) and used to create a dilution (Table 2), each solution of the series was then photographed using the same protocol described below and the cell density was counted using a cell counting chamber BLAUBRAND Neubauer Counting Chamber (Sigma-Aldrich BR717805-1EA). The goal of this second phase was to validate that changes detectable trough image analysis are correspondent with variation in cell density.

Table 2. Percentage concentration and cell density of the algae solutions for the validation phase of the image analysis experiment.

Algae concentration of the solution (%)	Cell density (cells/ml)
100	$2.27x10^7$
87.5	$1.96 x10^7$
75	$1.90 x10^7$
62.5	$1.76x10^7$
50	$1.26 x10^7$
37.5	$1.08 x10^7$
25	$7.30 x10^6$
12.5	$4.10x10^6$
6.25	$2.60 x10^6$
0	0

Photo capture process

The photos were taken by placing the tubes into a specially designed box with standard conditions of light. The box was made from white polystyrene sheets glued together with dimensions 15 x 20 x 10 cm. Each box was equipped with a 3 cm LED white light bar to provide enough and standardized illumination inside the box. A custom-made wire holder was inserted inside the box, this holder had a stable base and a rubber coated ring to hold the tubes firmly during the image acquisition process. The images were taken using a digital camara and stored as .JPG files for analysis. Each tube was photographed from a standardized distance, three times from the side and three times from the bottom. However, because of the low contrast of algae with respect to the environment in the side picture, only the results for the bottom pictures were considered (Figure 5).

Figure 5. Left: polystyrene box for image acquisition without tube holder, right: Tube holder and sample tube loaded.

Image analysis protocol

Images were stored in JPG format and analysed using the Fiji distribution for the software ImageJ (Schindelin et al., 2012, Schneider 2009). Image J is a powerful suite for image analysis of biological data, that includes many plugins. Fiji in the other hand is a distribution of Image J that includes plugins for more complex image analysis methods like conversion of color spaces in images.

The analysis protocol had four steps, first each image was cropped and just the area of interest (bottom of the tube) was kept. In the second step, the images were transformed from the RGB color space to HSB. A color space is the way in which computers represent color, normally by a combination of three or more values (or channels) in each pixel. This step is important because, despite that standardized light conditions were used during the image acquisition phase, the default way of most cameras to display colors (the RGB color space) is very prompt to variation caused by changes in light conditions, in RGB images each color is represented by a combination of values for Red, Green and Blue, this means that the same color can have many different combinations of values depending on how dark or illuminated the picture is. The HSB color space also uses combination of three values to represent different colors, however only one of these channels, the Hue or dominant wavelength, is directly linked to the color, while the other two values control non color related aspects of the image like; saturation (S) and brightness (B), therefore, the HSB color space allows for color comparison between pictures that are dependent only in one value and are somehow more stable under variation of light conditions (Yang et al., 2015).

After the images were transformed to HSB the third step consisted in eliminating the saturation and brightness channels, to leave only the values for the Hue. Finally, in the fourth step the mean value of the pixels in the area of interest was obtained. Green color corresponds roughly to Hue values ranging from 60 to 140. The following figure represents the complete process.

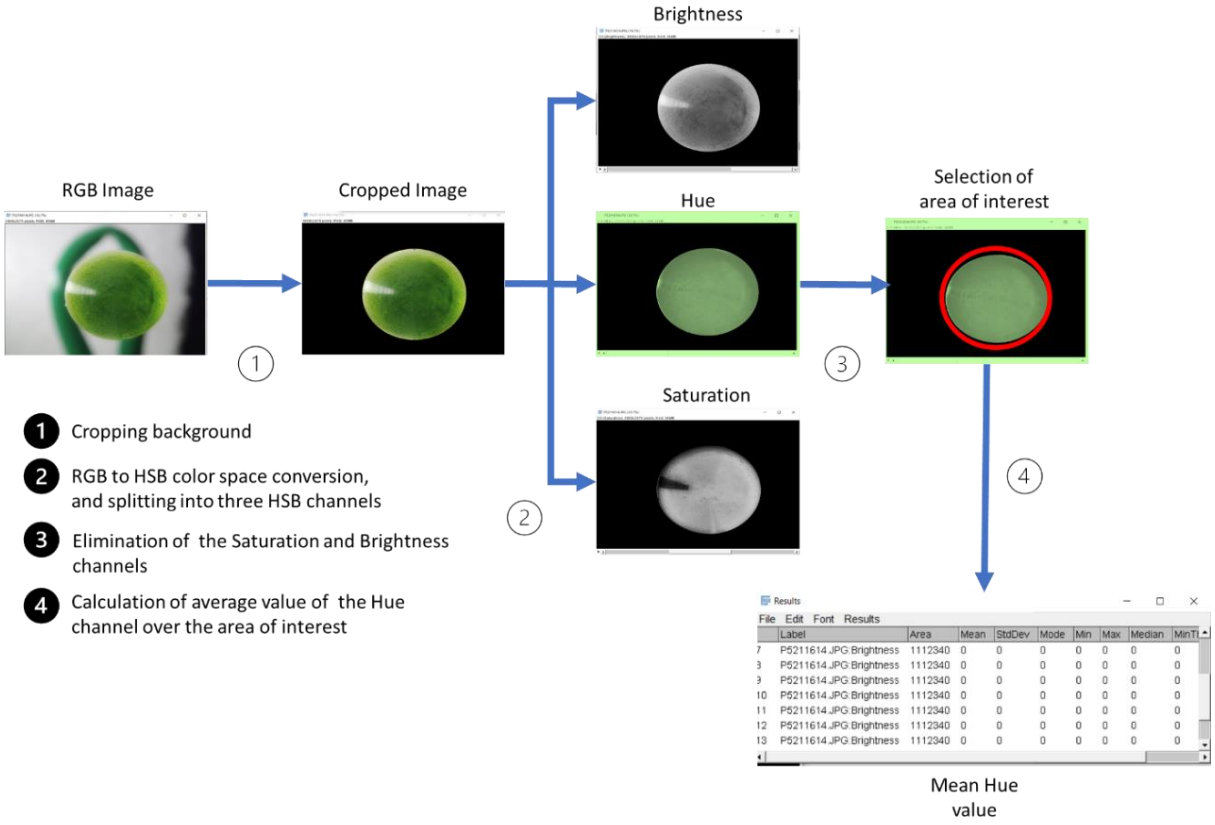

Figure 6. Description of the Image analysis protocol.

Analysis of data

For the validations stage, the mean Hue value was compared with the measured cell density using a scatter plot, a linear regression was fitted to the data after assessing the presence of outliers with a Cleveland plot (Cleveland, 1993), using the cell density as an dependent value and the mean Hue value as independent variable.

For the data of the small scale experiment the mean Hue value for the images was analysed using an ANOVA for each day of assessment (14 and 20 days after inoculation). For both sets of data the independent variables were the media type used during the acclimatization period (either, Agar or liquid media) and the concentration of F/2 media added to each tube during inoculation (Either A: 0.099 µl/ ml, B:0.132 µl/ ml, C: 0.198 µl/ ml). The analysis was carried out in R (R Core Team, 2019).

3.2.5 Transfer to 200 mL Erlenmeyers

After one month and aiming to increase the volume of the culture, the tubes that displayed a more intense and darker green after a qualitative inspection (at least two tubes per treatment) were selected to continue with the scale up. The quantities in the following paragraph were calculated to adjust 200 mL of medium per Erlenmeyer. In a big (1 L) sterilized Erlenmeyer with autoclaved water (1000 mL) 285 µl of A-part were added using a pipette. In the same flask, 285 µl of the previously filtered B-part were also added and stirred until homogenization. This

first flask has 150% the recommended concentration, four-small Erlenmeyers marked with the C letter (Ag.C and L.C) were filled with 200 mL of this medium. In a second big (1 L) sterilized Erlenmeyer with autoclaved water (1000 mL) 150 µl of the B-part with 150 µl of A-part were added. The second mixture "B-solution" has the manufacturer recommended concentration, 200 mL of this solution were poured to the 4 clean Erlenmeyers marked with the B letter (Ag.B, L.B).

Finally, in a sterilized Erlenmeyer with autoclaved water (1000 mL) 122.5 µl and 122.5 µl of the B-part previously were also added to reach a concentration of 75% the recommended concentration, for the remaining erlenmeyers marked with the A letter (Ag.A, L.A), see Figure 7.

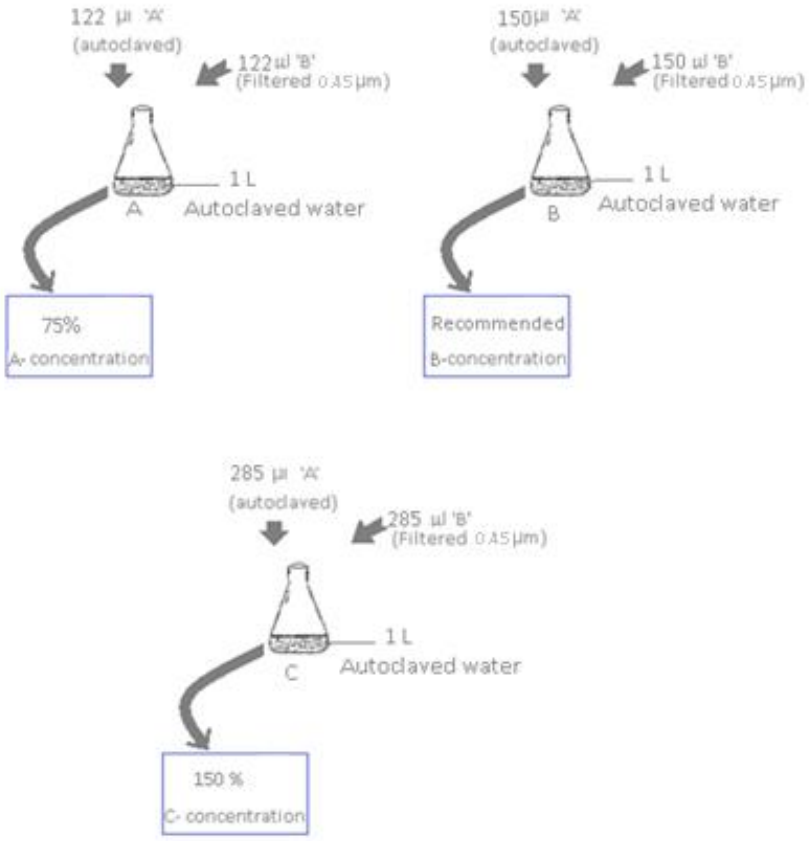

Figure 7 Summary of medium preparation 200 mL Erlenmeyer

3.3 Medium-scale culture setting

The final step was to stablish a 10 L culture that could be constantly harvested to feed the animals in the lab, the setting for this culture (see Figure 8) included two ¼" autoclavable silicone tubes plus one 3/8" vinyl hose. The vessel used was a 10 L polypropylene autoclavable carboy (C4442 Sigma Nalgene®), an analog time switch to control the photoperiod of the LEDs (12 hours day – 12 hours of night) was also used, one air pump for mixing that was connected to a 0.2 µm filter to avoid any external contamination, and one vinyl 3/8" hose closed with with a 3/8" Female autoclavable Quick Disconnect fitting for inoculation. This was placed inside an incubator at 25°C with LED lighting arranged like in the Figure 2.

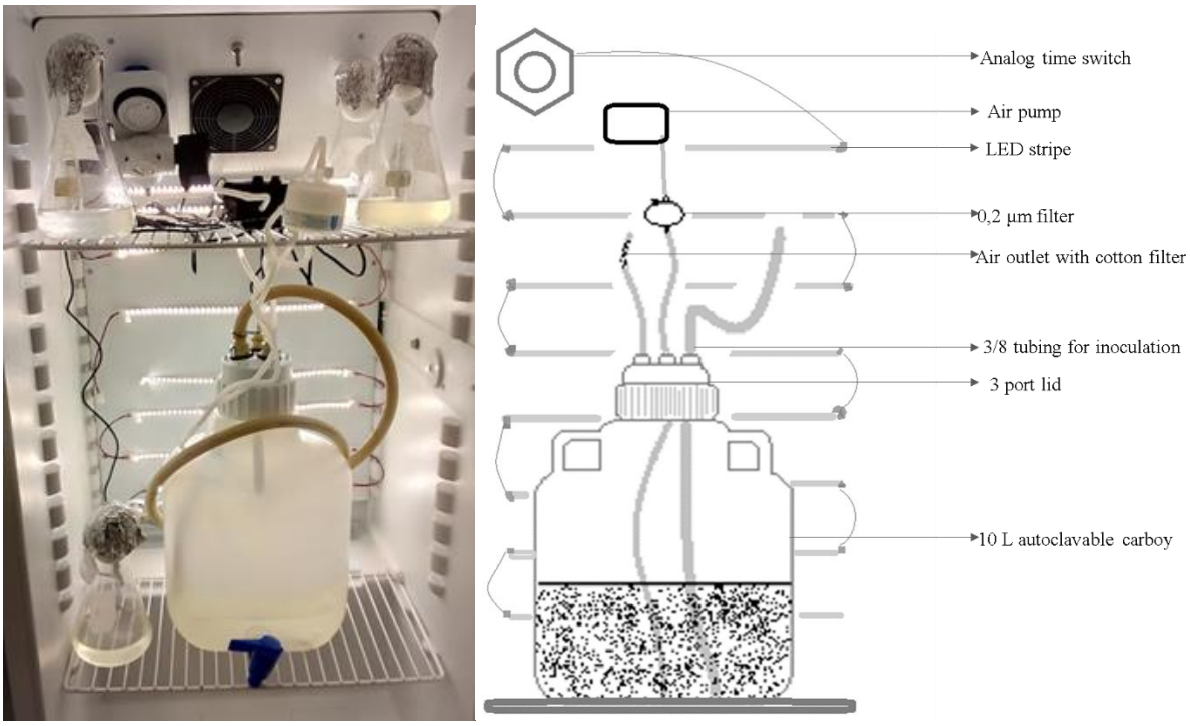

Figure 8 Final design for a medium setting for potential utilization in filter-feeding bovalves

3.3.1 Transfer to 10 L Carboy

Again, after one month on the 200 mL Erlenmeyers the culture was ready to be reinoculated with fresh nutriens into a bigger volume vessel. The final step was also carried out in sterile conditions, all the materials were again autoclaved as mentioned before. Assuming the medium was completely depleted, the manufacturer's recommended concentration (B concentration) was prepared for a starter volume of 5 L (660 µl of A-part were added using a pipette plus 660 µl of the previously filtered B-part were also added and stirred until homogenization). A qualitative inspection was made and the two greenest Erlenmeyers were selected to scale up the culture, see Figure 9.

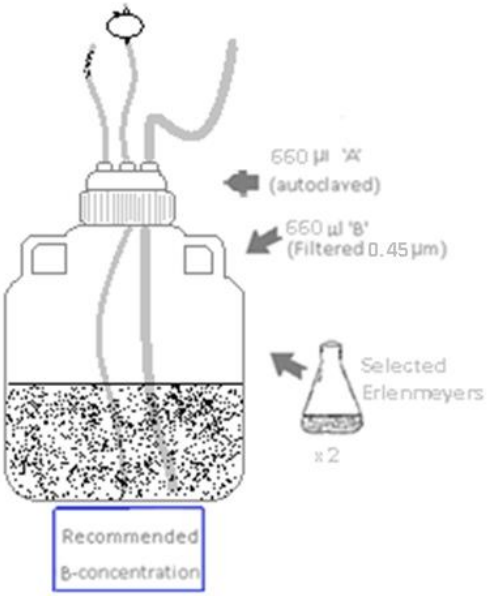

Figure 9 Summary of medium preparation fpr 5 L carboy

4 Results

4.1 Bibliographic review and bibliometric visual analysis

4.1.1 Introduction to microalgae

Microalgae are a polyphyletic group of oxygenic photosynthetic microorganisms. "*This definition includes cyanobacteria and eukaryotes in a number of clades with the eukaryotic algae originating from a symbiosis between a non-photosynthetic eukaryotic cell and a photosynthetic cyanobacterium*" (Raven & Giordano, 2014). Even though from a systematics point of view, there is a division amongst prokaryotic cyanobacteria and eukaryotic green algae, the term comprehends any organism with chlorophyll a and a thallus not differentiated into roots, stem, and leaves (Lee, 2008). Thus, including cyanobacteria and eukaryotes. of enourmous ecological importance (Mata et al., 2010) (Richmond, 2004), that vary greatly in their physiology, morphology, and environmental range. The Eukaryotic autotrophic microalgae can be divided (for practical approaches) according to their light-harvesting photosynthetic pigments: Rhodophyta (red algae), Chrysophyceae (golden algae), Phaeophyceae (brown algae) and Chlorophyta (green algae) (Masojídek et al., 2013). Other systems of classification are based for example in the origins of simple and complex plastids via primary and secondary endosymbioses (Barsanti & Gualtieri, 2006; Sitte, 1993), and modern molecular phylogenetic studies that form the framework for understanding the group's evolutionary history (Bhattacharya & Medlin, 1998). Additionally, the remaining Cyanobacteria or blue-green algae form a natural group because of being the only prokaryotic microalgae, generally unicellular (some can arrange in aggregates or filaments), their cyanobacterial cell has 70S ribosomes, a nucleoplasm rich in DNA fibrils not enclosed inside a distinguishable membrane and a chromoplast that contains sheets of photosynthetic membranes, typically arranged in parallel in the most outer portion of the cell near the plasma membrane. Chlorophyll a is their main photosynthetic pigment (Lee, 2008; Masojídek et al., 2013).

Microalgae play a tremendous ecological role supporting the aquatic primary production (carbon dioxide assimilation and oxygen production) and contributing to half of the globe's photosynthetic activity (Day et al., 1999; Field et al., 1998; Raven & Giordano, 2014) these organisms are the foundation of all marine food webs, some freshwater food webs and a small portion of terrestrial food webs (Raven & Giordano, 2014), forming the source of food for over 70% of the world's biomass (Wiessner et al., 1995) (Andersen, in Hunter-Cevera & Belt, 1996).

Microalgae represent one of the most efficient converters of solar energy to biomass (Masojídek & Torzillo, 2008) and are extensively distributed in terrestrial and in almost all aquatic (both marine and freshwater) ecosystems. Microalgae can be found in varied ranges of salinity, temperature, nutrients, light and pH (Hu et al., 2008), being successfully adapted to multiple ecological habitats and niches including extremes ones such as polar regions or deserts (Lyon & Mock, 2014; Schipper et al., 2019). Even though they are more commonly free-living, some microalgae live in symbiotic associations with a variety of other organisms, such as fungi

(lichens or not) (Gimmler, 2001), have noteworthy symbiotic relations with invertebrates including for example hermatypic corals where the algae provides glucose, glycerol, and amino acids, and the coral utilizes these products (resultant from photosynthesis) to build proteins, fats, carbohydrates, and produce calcium carbonate (Barnes & Hughes, 1999; Muller-Parker et al., 2015), other interesting symbiotic associations with invertebrate animals include species of large marine bivalves from the genus *Tridacna* that obtain their nutrition mainly from trans-located photosynthates from symbiotic zooxanthellae in their exposed mantle tissues (Holt et al., 2014; Ishikura et al., 1999; Klumpp & Lucas, 1994), or the sea slug *Elysia chloroticais* especialy notable because it can subsist for months depending exclusively on the energy produced by ingested plastids of the stramenopile alga *Vaucheria litorea* (Chan et al., 2018). Even more appealing symbiotic relations exist with vertebrates like the salamanders *Ambystoma maculatum* and *Ambystoma gracile* where some of cells of the algae *Oophila amblystomatis* live inside the egg capsules of these amphibians entering the embryonic tissues as well as individual salamander cells (Kerney et al., 2019), in this symbiotic association the salamander embryos benefit from increased oxygen concentrations provided by the algae and in return the algae benefits from the ammonia excreted by the embryos (Kerney, 2011). Just to name a few examples.

Because of their characteristics microalgae have been used in plenty of industrial and biotechnological processes and have attracted interest for their potential to generate valuable products in high volumes (Masojídek & Torzillo, 2008). Some of those aplications include: cultured in intensive systems for aquaculture and animal nutrition (Barnhart, 2006; Catarina & Xavier, 2012; Hemaiswarya et al., 2011; Sanjayasari & Jeffs, 2019), as eco-friendly organic fertilizer and soil stabilizer replacing chemicals such as nitrogen and phosphorus; prompting an increase of crop yields and plant growth (Guo et al., 2020; Osman et al., 2010; Renuka et al., 2018; Tiwari et al., 2017) (e.g. some studies carried out by the Food and Agriculture Organization of the United Nations (1981), suggested that in areas where chemic nitrogen fertilizers are not used for various reasons, including monetary ones, the application of blue-green algae in rice field soils can give to the farmers the benefit of low cost nitrogen input. Microalgae can also be used as bioremediatiors and possible global carbon emission reducers (Ho et al., 2011), or in water treatment due to their ability to use inorganic nitrogen and phosphorus to grow, but also for their faculty to remove risky elements as well as some toxic organic compounds (Abdel-Raouf et al., 2012). One of the aplications that has developed a lot of interest in the past few decades is microalgae as renewable stock for biofuels (Adeniyi et al., 2018; Mata et al., 2010) those findings also stimulated the research on algal oils with nutrional value for human consumption (Becker, 2013), some other application include the use of microalgae as a source of protein (which is the case of *Arthrospira platensis* known as "spirulina" and various commercial species of the unicellular green alga *Chlorella* that contain up to 70 % dry weight protein), vitamins, antioxidants, and other supplements for human diet (Jin et al., 2020; Wells et al., 2017), daily use products including cosmetics (Wang et al., 2015), natural dyes for food (Arad & Yaron, 1992), among other uses.

The Figure 10 depicts a summary of the main current uses of microalal biomass on the industry, that can be devided on (*i*.) direct uses (food for animals and humans), (*ii*.) biofuels,

and (*iii*.) bioproducts (to produce different kinds of medicines, vitamin supplements, inks, and others).

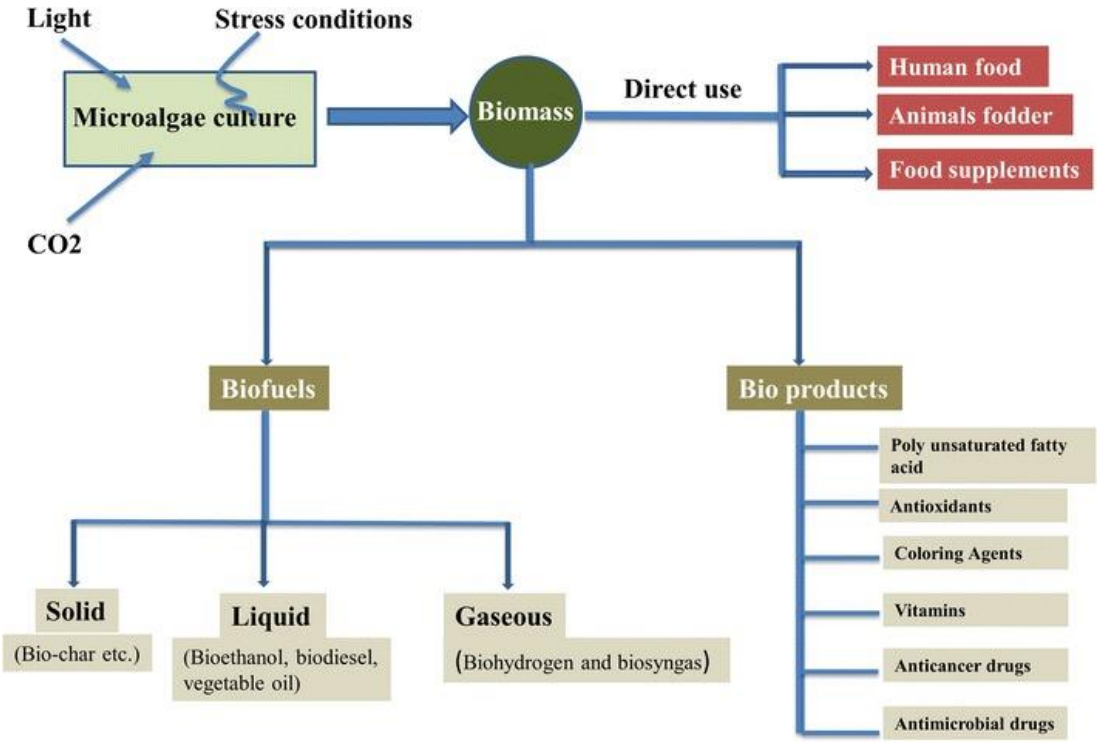

Figure 10 Summary of microalgae direct and indirect uses.

Image from (Khan et al., 2018)

Amongst the direct uses the most relevant one for the aim of the Thesis is the use of microalgae as animal food resource, the nutritional quality of microalgae mainly depends on many biochemical components such as polyunsaturated fatty acids, vitamins, sterols and carbohydrates (Dunstan et al., 1993). The second main criteria to select a microalgae species, is based directly on the specific requirements of the cultured animal; for bivalves the necessities change through the different stages of the life cycle (larval, post-larval, juveniles and adults), and are related for example with the size of the cell or cell wall thickness besides the nutritional requirements for the different species of bivalves (Sarkis, 2007). In the Table 3 the cell volumes, organic weight and gross lipid content of some of the most relevant algal species used as foods for bivalve larvae are depicted.

Table 3 The cell volume, organic weight and gross lipid content of some algal species used as foods for bivalve larvae.

Taken from: (Michael M. Helm & Bourne, 2004)

Species:	**Median cell volume (μm^3)**	**Organic Wt. ($\mu g\ 10^{-6}$ cells)**	**Lipid %**
Flagellates:			
Tetraselmis suecica	300	200	6
*Dunaliella tertiolecta**	170	85	21
Isochrysis galbana	40-50	19-24	20-24

Species:	Median cell volume (μm^3)	Organic Wt. ($\mu g\ 10^{-6}$ cells)	Lipid %
Isochrysis (T-ISO)			
Pavlova lutherii			
Diatoms:			
Chaetoceros calcitrans	35	7	17
Chaetoceros gracilis	80	30	19
Thalassiosira pseudonana	45	22	24
Skeletonema costatum	85	29	13
*Phaeodactylum tricornutum**	40	23	12

The species selected to start the culture in the Malacology lab has several interesting characteris that makes it suitable to use it as a reliable food source for bivalves, *Ettlia* is a green algal genus comprised of several economically important species that represent a taxonomic challenge. Currently there are 10 species registered in the AlgaeBase database, 7 of which have been accepted taxonomically (Guiry & Guiry, 2020). As type species of the genus *Ettlia* the Czech scientist Jiří Komárek designated *E. carotinosa*, the genus was described based on the Prague subculture (Praha-Ac. 93). *Ettlia* is characterized by "*cells spherical or subspherical, uninucleate, chloroplast parietal with pyrenoids. Zoospores with thin wall. Basal bodies of flagellar apparatus having clockwise orientation*" (Komárek, 1989).

Neochloris oleoabundans currently regarded as a synonym of *Ettlia oleoabundans* (S. Chantanachat & Bold) J. Komárek 1989; corresponds to a freshwater species sampled for the first time on top of a sand dune in Saudi Arabia (Lat. 21° 15'N, Long. 55° 15'E.). The species was fist described as a coccoid with a vegetative cell size of 6 - 25μm, without a gelatinous matrix and with a single spherical nucleus as the dominant cell type. The cell membrane always thin, the sexual reproduction of the species hasn't been observed, *E. oleoabundans* reproduces asexually by releasing oval zoospores from enlarged mother cells (diameter> 10 μm) to complete its cell cycle, see Figure 11, the zoospores have a diameter of 3.6 μm long and 2.7 μm wide. In young cells 1 pyrenoid can be detected, while 2, and rarely 3 excentric pyrenoids are observed in mature cells (Chantanachat & Bold, 1962), see Figure 3.

The desertic conditions from which this microalga was isolated forced it to be a highly flexible species, capable to deal with the daily salt, drought conditions and extreme temperature variations through the hot days and cold nights. The species also has a high growth rate (μ 2.2 D-1) and is resistant to highly alkaline conditions (up to pH 10), which reduces the risk of culture contamination (de Jaeger et al., 2018).

Amongs the interesting characteristics of the species, *E. oleoabundans* has been identified as a suitable food source for juvenile unionids (Gatenby et al., 2003; O'beirn et al., 1998), and was used by Barnhart (2006) to prove a compact system for rearing juvenile freshwater mussels. Jones (2016) compared algae diets of juvenile Unios and found that both *N. oculata* (marine microalgae from the genus *Nannochloropsis*) and *E. oleoabundans* promoted excellent growth

and survival of juvenile oyster mussels and rainbow mussels. *E. oleoabundans* can be easily ingested by newly metamorphosed juveniles due to its size see Figure 11, Beck & Neves, (2003) recorded evidence of selective feeding in captive juveniles of *Villosa iris* (Unionidae) as a function of particle size; the smaller cells of *N. oculata* (2.8 - 8.1 µm) were selected over larger colonies when used to feed captive mussels, the average size of *E. oleoabundans* range between 3 – 10 µm (de Jaeger et al., 2018). The study suggests that particle size of available food resources may limit filter-feeding bivalves. These results have an impact not only on aquacultural applications but also in the propagation and culture of endangered mussel species that could help to create effecient plans to enlarge the existing mussel populations and to reintroduce them to places that used to be part of their natural distribution ranges.

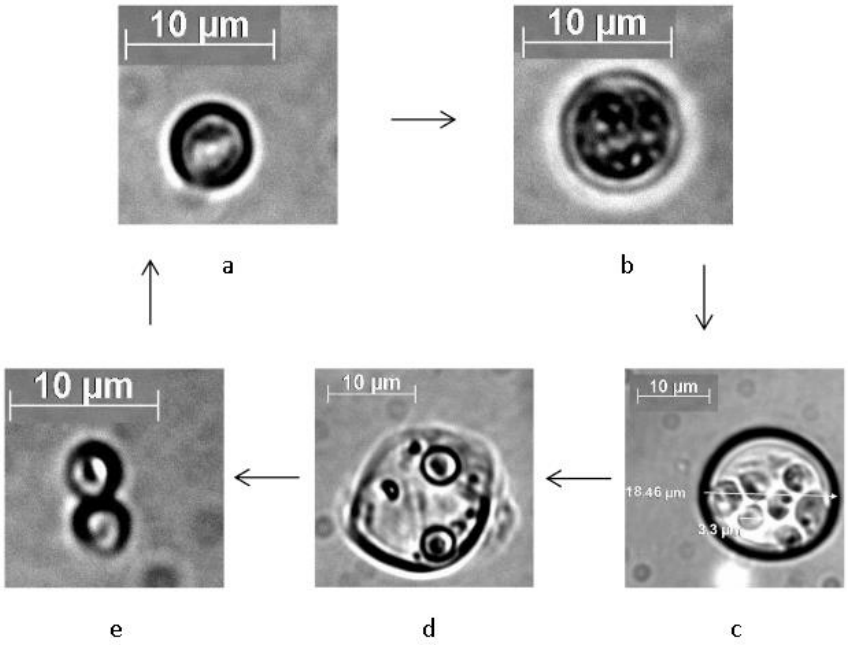

Figure 11 Life cycle of Ettlia oleoabundans: a. Vegetative single coccoid cell, b. Formation of zoospores in mother cell, c. Enlarged zoospore-enclosed mother cell. d. Release of zoospores from mother cell. e. Newly released zoospores. Different cell sizes can be appreciated depending on the stage.

Image from: (Y. Yang, 2013)

As mentioned above, given the diversity of ecosystems where microalgae can thrive one could not pinpoint a single set of ideal conditions for these organisms to grow, the optimal parameters as well as the tolerated ranges are species specific, but certainly the factors controlling more significantly the algal growth are light, nutrients, pH, aeration, temperature and salinity. (Food and Agriculture Organization of the United Nation, 1996) These optimal conditions must be taken into account when culturing microalgae: A culture has three distinct components, (*i*.) culture medium contained in an appropriate vessel; (*ii*.) the algal cells; (*iii*.) air, to allow exchange of CO_2 between medium and atmosphere (Barsanti & Gualtieri, 2006).

4.1.2 Optimal conditions for culturing microalgae

Light

Algae contain photosynthetic pigments as a fundamental part of the chloroplast lamellae (in the case of eukaryotic cells), or homogeneously distributed throughout chromatoplasm as in blue- green algae (Golterman, 1975). Photosynthesis occurs when the potential energy present in sunlight is utilized as energy source for carbon dioxide reduction. The process supports the growth of phototrophic bacteria, algae, and plants. Microalgae use the same part of the light spectrum as visible light called photosynthetically active radiation (PAR). PAR can be broken down into its colored components, from the highly energetic blue/violet (400nm) to the lower energetic red (700nm) (Yarish et al., 2012), see Figure 12. In photosynthetic cells the most commonly occurring pigment is chlorophyll a: present in brown (multicellular algae), green and blue-green algae and in higher plants, some other pigments can be found on the different algae groups, the composition and proportions of the different pigments are unique taxonomic features of the various classes of algae (Stoń-Egiert et al., 2019) and could even help to identify the species, see Table 4.

Table 4 Pigment composition of some algal groups modified after Dring, (1991)

Division	Common Name	Major Accessory Pigment
	All photosyntetic algae	chlorophyll a
Chlorophyta	Green algae	chlorophyll b
Charophyta	Charophytes	chlorophyll b
Euglenophyta	Euglenoids	chlorophyll b
Phaeophyta	Brown algae	chlorophyll c1 + c2, fucoxanthin
Chrysophyta	Yellow-brown or golden-brown algae	chlorophyll c1 + c2, fucoxanthin
Pyrrhophyta	Dinoflagellates	chlorophyll c2, peridinin
Cryptophyta	Cryptomonads	chlorophyll c2, phycobilins
Rhodophyta	Red algae	phycoerythrin, phycocyanin
Cyanophyta	Blue-green algae	phycocyanin, phycoerythrin

The underwater light environment changes with the distance from the surface, as light passes through water it is absorbed and scattered by water and other molecules, the intensity and spectral composition of this radiation varies depending on the depth: red wavelengths (which are longer and have lower energy) are usually absorbed near the surface, while blue wavelengths (shorter and with higher energy) can surpass longer distances in the water column (Yarish et al., 2012). Due to the intensity caused by high irradiances in the short-wave portion of the spectrum that can lead to the photodestruction of the photosynthetic centre, the algal cells produce photoprotecting pigments (carotenoids) that can process more energetic wavelenghts. This chromatic adaptation also affects the vertical relative presence of pigments in the water

column: being higher the occurrence of photoprotective pigments at the surface and decreasing with depth (compared to the relative concentrations of photosynthetic pigments (e.g. chlorophyll a) (Stoń-Egiert et al., 2019). Both light intensity and spectral quality affect the growth and metabolism of photosynthetic microalgae. Light intensity has also been suggested to influence algae capacity for lipids accumulation, which would be enhanced under strong illumination (Damiani et al., 2010; Zhukova & Titlyanov, 2006). This is because in the case of microalgae, light exposure activates a set of physiological processes in the cells, on both transcriptional (changes in the genes encoding light harvesting machinery) and metabolic levels (Lehmuskero et al., 2018). This is very important since algal growth and metabolism are essentially reliant on the conversion efficiency of light to chemical energy and depend on the consumption of chemical energy (ATP) and reducing power (NADPH) produced during the photosynthesis (Y. Yang, 2013).

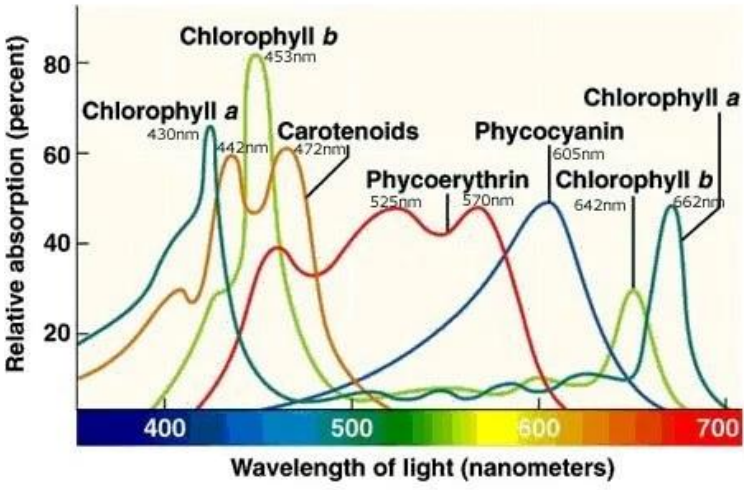

Figure 12 Relative absortion of select pigments.

Image from: Yarish et al. (2012)

Given the massive economic interest as promising feedstocks and the potential for the production of large volumes of bio-based materials, many studies have been done concerning the photosynthetic ability, light intensity, wavelength and photoperiod to culture microalgae, which are crucial for algal growth and metabolite synthesis (Benedetti et al., 2018; Patelou et al., 2020). Some of those studies have shown for example, that the natural day-night cycle and adjustment in light intensity corresponds with cyclic alterations in the pigmentation and cell diameter of diatoms (Stramski & Reynolds, 1993), the circadian clock provides organisms with an appropriate time frame to carry UV sensitive processes, such as DNA replication therefore cell division during the night, allowing cells to grow in size through the day, when light is available (De Winter et al., 2017).

Like in other autotrophic organisms light is one of the most importan aspects to grow, this has an impact on the outdoor farming or culture, mostly on the temperate or tepid climates, that generally have wider temperature ranges throughout the year and more distinct seasonal changes compared to tropical climates, where such variations are often small. As discussed

earlier, microalgae grow best when certain optimal conditions are reached and in colder climates the conditions for growth occur only through a part of the year. However, unlike most plants, microalgae can grow in a small space and can be cultured whitout soil. As a result, microalgae can be grown in specially equipped vessels or photobioreactors (PBRs), that allows not only the production of biomass for year-round, but also for the tuning of conditions such as light wavelength or intensity (Brzychczyk et al., 2020). In an optimal system with no other factors restricting the yield, the light availability limits the photosynthesis rate and therefore the productivity (Molina Grima et al., 1999). In the other hand, excessive light can produce a photoinhibitory response. These types of specially designed reactors will be discussed in greater depth in the following paragraphs.

Nutrients and CO_2

An autotrophic alga only needs light, CO_2, water, trace elements and nutrients to grow. By performing photosynthesis, the alga will be able to synthesize all the biochemical compounds necessary for growth. Only a few species of algae are completely autotrophic but the vast majority cannot synthesize certain biochemical compounds such as vitamins, that will have to be supplemented in the culture medium (Barsanti & Gualtieri, 2006).

Microalgae culture pass through different growth phases (e.g., lag, exponential, stationary, death). Different species may vary in their need for growth media, concentrations of cells in the cultures are usually higher than those found in nature, thus they need to be enriched with nutrients to make up for the nutrient insufficiencies in the water (Food and Agriculture Organization of the United Nation, 1996). However, the most important requirements are the same for almost all species and include essential nutrients, an organic or inorganic carbon source (HCO_3 or CO_2, nitrogen (especially nitrate, ammonia or urea), phosphorus as major nutrients (which account for 10–20% of algae biomass (Benemann & Oswald, 1996)). Other requirements for growth are the macronutrients like Na, Mg, Ca, and K; and micronutrients, such as Mo, Mn, B, Co, Fe, and Zn; and some additional trace elements (Grobbelaar, 2004).

Regarding to the principal source of carbon, microalgae can be either autotrophic or heterotrophic, see Table 5. If they are autotrophic, they use inorganic compounds as a source of carbon. Autotrophs can use light as a source of energy (photoautotrophic) or oxidizing inorganic compounds for energy (chemoautotrophic). In the other hand, if they use organic compounds to grow are heterotrophic. Heterotrophs are also divided in two categories, one including the microalgae that use light as a source of energy (photoheterotrophs, and one that contains the organisms that oxidize organic compounds as energy source (chemoheterotrophs) (Lee, 2008).

Table 5 Types of nutrition found in microalgae according to Lee (2008)

Type of nutrition		**Principal source of energy**	**Principal source of carbon**
Autotrophic	Photoautotrophic	Light	CO_2
	Chemoautotrophic	Oxidation of organic compounds	CO_2
Heterotrophic	Photoheterotrophic	Light	Organic compounds

Type of nutrition		Principal source of energy	Principal source of carbon
	Chemoheterototrophic	Oxidation of organic compounds	Organic compounds

Supplementing the microalgae culture with CO_2 could also represent an advantage not only as a carbon source but is preferred for pH control (Acién Fernández et al., 2013). In the case of high-density algal culture, the addition of CO_2 allows to lower the pH, when CO_2 is supplied, pH is modified as the microalgae grow, carbon dioxide is consumed through photosynthesis and the microalgae secrete OH^- ions as CO_2 and carbonate ions accumulate in the culture liquid (Kim & Kwak, 2020).

pH

The pH range for most cultured algal species is between 7 and 9, with the optimum range being 8.2 - 8.7 (Food and Agriculture Organization of the United Nation, 1996). Some of the consequences of maintaining a culture under pH stress conditions can be the disruption of many cellular processes and have direct physiological effects that can result in a complete culture collapse. Variation in pH can affect algal growth in several ways: changing the distribution of carbon dioxide species and carbon availability, alter the availability of trace metals and essential nutrients (Chen & Durbin, 1994).

Aeration and mixing

The main role of a gentle mixing in culture conditions is to prevent sedimentation and to guarantee that all cells of the population are uniformly receiving light and nutrients, the mixing helps to improve gas exchange between the culture medium and the air which is very important as the air contains the carbon source for photosynthesis in the form of carbon dioxide, the mixig also helps to avoid thermal stratification and the stacking of cells and to increase gas diffusion (Barsanti & Gualtieri, 2006).

Salinity

Most species grow best at a salinity that is slightly lower than that of their native habitat, but most marine microalgae are very tolerant to changes in salt concentration (Barsanti & Gualtieri, 2006). Prokaryotic microalgae are known to accumulate sucrose or α-glocosyl-glycerol as a response to stress conditions while eukaryotic microalgae approach this challenge with several different strategies such as are glycerol production, sucrose production, or amino acid accumulation (de Jaeger et al., 2018).

Temperature

Microalgae have no internal temperature control, the temperature and light of the environment influence the biomass content, metabolism, and metabolic rate of microalgae (Kim & Kwak, 2020). That is why the importance of temperature control cannot be understimated since most enzymatic reactions and physiological activities are highly dependent on

temperature. Most commonly cultured species tolerate temperatures between 16 and 27°C. Temperatures lower than 16°C will slow down growth, while those above 35°C are lethal for a number of species (Food and Agriculture Organization of the United Nation, 1996), and the response to the temperature is regularly species specific in terms of biomass accumulation and lipid production. In the case of *Ettlia oleoabundance* for example, the species can survive at 5 °C by entering dormancy (Chantanachat & Bold, 1962; Y. Yang, 2013). This could be explained because at low temperatures the efficiency of transport proteins embedded in the membrane happens with less fluidity, resulting in limited algal (Nedwell, 1999).

4.1.3 Photobioreactors (PBRs)

Photobioreactors (PBR) are devices *"...in which phototrophs (microbial, algal or plant cells) are grown or used to carry out a photobiological reaction"* (Tredici, 2004). No matter the final application of microalgae its production is based on the same optimal conditions of light quality, temperature, pH, inorganic mineral nutrients, carbon source, and other culture parameters. Photobioreactors are closed systems that can accurately control the most important culture factors and prevent the influence of one of the most limiting factos: optimum light all year long. Photobioreactor also have the advantage of reducing water losses, allowing to mantain a high cell concentration. This has a positive impact on the preparation of medium and therefore the costs, decreasing the amount of medium that needs to be prepared. One of the most advantageous aspects of the implementation of photobioreactors on the productive chain is that they can diminish the risks of contamination, contaminated cultures limit the value of the biomass, especially for the cosmetic and food markets (Tredici et al., 2016), the close environment provided by the PBR protects the culture from debris and infection invasion by unwanted species ensuring the cultivation of only desired strains.

Although microalgae have been studied over many centuries, the first investigations on microalgae production undercontrolled conditions started in the 1950s (Garrido-Cardenas et al., 2018). Over the following years, diverse types of photobioreactors were proposed, and many economic analyses on microalgal biomass production have been published. Most of them for open systems, or hybrid facilities where the photobioreactors are used in the first phases of production, while the majority of the process is carried out in open ponds (Tredici et al., 2016).

Open culture

In open photobioreactors the culture is in contact with the atmosphere. Being open they are susceptible to invasion by other organisms including other microalgae, these facilities try to compensate with low cost productivity due to little or no control of conditions such as pH or temperature making them especially suitable for robust and fast-growing species. Despite these drawbacks, most of the microalgae produced in the world come from this type of system. Its great advantage is that it is easy and economical to build them in large volumes, even hundreds of cubic meters.

There are two basic types of open photobioreactors: (*i*.) open ponds, which are simple receptacles of the appropriate size and shape, and (*ii*.) raceways ponds which, in addition, are capable of supplying agitation and mixing, facilitating gas exchange and even controlling pH to some extent. Large open ponds can be built on diverse shapes and sizes of different resistent materials such as plastic or concrete, and even more simplistic designs of compacted earth (Acién Fernández et al., 2013). These type of systems represent rather low construction and operating costs and the large ones can be constructed on degraded and nonagricultural lands that avoid use of high-value lands and crop producing areas (Tredici, 2004). However, open ponds have some intrinsic disadvantages closely related to the way they are constructed: (*i*.) poor light diffusion inside the pond, therefore, shallow ponds are more suitable for the culture as they have a low volume to area ratio; (*ii*.) mono-cultivation of one strain is difficult unless the desired microalgae is an extremophile species, one example of it is *Dunaliella salina* halophilic microalgae that grows in saline concentrations up to 100 g / L, which prevents the proliferation of other species, this fact has other implications, such as making the harvest unsuitable for use in food or pharmaceuticals; (*iii*.) growth conditions rely primarily on local weather conditions (light and temperature, ensuring culture stability and preventing celldeath caused by overheating or freezing), this represents a challenge in temperate zones and could induce a seasonal-dependent yield; (*iv*.) clean water is needed continuously; (*v*.) in addition tophotobioreactor design, the harvesting strategy is also a major factor determining the suitability of large-scale microalgae production; thisstep accounts for up to 30% of the overall production cost (Acién Fernández et al., 2013; Benemann & Oswald, 1996; Garrido-Cardenas et al., 2018; Lee, 2008; J. Masojídek & Torzillo, 2008; Tredici, 2004).

Closed photobioreactors

As formerly discussed, the main concern in microalgae culture is optimizing the performance to provide optimal conditions at minimal cost. Those optimal conditions are usually dependant on the medium, the temperature and pH, with a special emphasis on light availability to the cells, furthermore, providing optimal conditions at a big scale can represent a challenge (Acién Fernández et al., 2013). Smaller closed photobioreactors keep the harvest completely isolated from the outside environment. Typically, they are equipped with agitation, aeration, pH control, heat exchange, media and CO_2 addition systems. Closed photobioreactors are highly specialized devices, often specifically designed for a specific species. Different kinds of these type of reactors are widely used and are constantly modified to obtain the best results, some of them are (*i*.) columnar photobioreactors that consist of a bubbling column of transparent material, their cylindrical shape helps distribute light and well withstands pressure at the base. Its main problem is scaling, it is difficult to build them to support a large volume since increasing the diameter increases the proportion of dark volume very quickly, affecting the productivity. It is also not possible to make them very high since the pressure at the base makes bubbling difficult and causes hydrodynamic stress. (*ii*.) another type of closed photobioreactor are the flat plate reactors, which are similar to columns in their philosophy, they combine agitation and exchange of matter in the same space where light is captured, but they try to solve some of the problems of the columns: the optical path can be made as thin as necessarya and they can be built very simply and cheaply: a frame and a plastic covering are

sufficient. However, it is not easy to build flat PBRs that are too long due to the difficulty that this geometry has in supporting hydrostatic pressure. Furthermore, although they can be tilted, they remain vertical devices (they depend on bubbling) and are therefore poor light collectors at many times of the day if the light is not artificially provided. (*iii*.) Finally, tubular PBRs are the most sophisticated and the most specialized, but they are also the most expensive to build. The design distinguishes two parts: loop (part in which the capture of solar energy is carried out) and degasser (where the exchange of matter is carried out). The designs seek simplicity and economy and provides a compact shape while trying to make optimum use of the soil in order to be economical and in most cases the tubes are horizontally arranged.

4.1.4 Bibliometric visual analysis: Microalgae production to feed mussels

In total 287 original articles were found in the research engine Web of Science using the retrieving strategy mentioned in the methodology. Those articles are included mainly on the fields of Marine and Freshwater biology, Fisheries, Ecology, and interestingly also on the fields of Toxicology and Pharmacology.

Regarding the distance-based approach to visualize bibliometric networks, a total of 8495 terms were extracted from the title and abstract fields. The minimum number of co-ocurrent terms used was 5 and the final seleccion of the most relevant ones resulted in a total of 121 terms organized in 6 clusters, the network visual output using the VOSviewer software of the terms extracted from the titles and abstracts of publications is presented in the Figure 13. The tool allows to exclude some very general noun phrases from the co-occurrence network. VOSviewer therefore calculates for each noun phrase a relevance score, noun phrases have a low relevance score if their co-occurrences with other noun phrases follow a random pattern, and those noun phrases with a low relevance score also tend to be quite general. In the other hand noun phrases with a high relevance typically have a more specific meaning, and they co-occur mainly with a limited set of other noun phrases. VOSviewer allows to leave out noun phrases with a low relevance score and focus the analysis on the important co-ocurrent terms.

In the visualization presented in the Figure 13, each circle represents a term. The size of each circle indicates the number of publications that have the corresponding term in their title or abstract. Terms that co-occur more frequently tend to be located closer to each other in the visualization. The software has grouped the terms into six clusters, two of them are of significant size. The red cluster, located in the right-hand area in the visualization, consists of terms related to potential harmful consequences of the microalgae such as “bloom”, “toxin”, “sensitivity”, and “Okadaic acid” (which is a potent neurotoxin and phosphatase inhibitor produced by several species of dinoflagellates, and is known to accumulate in both marine sponges and commercially interesting shellfish (therefore in bivlaves), it is associated with seafood poisonings (Pyle & Reuhl, 2010).

The green cluster, located in the left area, covers terms related to nutritional value of microalgae diet, terms like “best growth”, “nutritional value”, “biochemical composition”, are part of this second most importan cluster, in this group can also be observed terms related to investigations on types of diets (“monospecific diet”, “mixed diet”, “experimental diet”, “concentrate”- referring to algal concentrates or pastes, “artificial diet”, and very interestingly the word “cornstarch” which can be used as a dietary supplement in conditioning broodstock and spat at a nurseries of the Pacific calico scallop, *Argopecten ventricosus* (Pectinidae) (Mazón-Suástegui et al., 2017) for example.

The purple cluster (closely related to the green-diets group) consist of terms associated to valuable nutritional elements necessary to rear bivalves and that can be obtained from the diet, such as carbohydrates, Docosahexaenoic acid (DHA), Polyunsaturated fatty acids (PUFAs) Eicosapentaenoic Acid (EPA) and Arachidonic acid (AA), that play an important role as biochemical constituents, taking part in several processes and acting like an energetic source under critical nutritional circumstances.

Closely linked with these green and purple groups (low, left-hand side) a blue cluster can be observed, this contains terms related with all development stages of the diverse most studied bivalve species, such as “gametogenesis”, “D larvae”, “metamorphosis”, “spawning”, and “pediveliger” which is a stage in the life cycle of a veliger when it is able to crawl using its foot.

A total of six bivalve species are considered to be the most co-occurent of the 287 peer-reviewed articles downloaded from the Web of Science research engine, all of them inhabit marine ecosystems and are reared because of their meat (except for *Pinctada margaritifera*, Margaritidae). In terms of the most relevant species of microalgae, the search found a total of 13 most relevan species, all of them naturally distributed salty or brackish water. Remarkably, *Skeletonema costatum* is part of the the most relevant species due to its connection with Harmful algal blooms, and feasibly because it is traditionally used to increase the mass of the bivalve tissues, see Table 6.

Table 6 Most relevant species found using the bibliometric networks analysis.

Species	**Family**	**General environment**	**Notes**
Algae			
Chaetoceros calcitran	Chaetocerotaceae	marine	
Chaetoceros muelleri	Chaetocerotaceae	marine/freshwater	
Tisochrysis lutea (T-iso)	Isochrysidaceae	marine	
Dunaliella tertiolecta	Dunaliellaceae	marine	Characteristic for its ability to outcompete other organisms and thrive in hypersaline environments
Isochrysis galbana	Isochrysidaceae	marine	
Haslea ostrearia	Naviculaceae	marine	
Nannochloropsis oculata	Monodopsidaceae	brackish	
Pavlova lutheri	Pavlovaceae	marine	
Pavlova salina	Pavlovaceae	marine	

Species	Family	General environment	Notes
Phaeodactylum tricornutum	Phaeodactylaceae	marine	
Skeletonema costatum	Skeletonemataceae	marine	Harmful algal bloom and traditionally used to increase the mass of the bivalve tissue
Tetraselmis suecica	Chlorodendraceae	marine	
Thalassiosira pseudonana	Thalassiosiraceae	marine	It was chosen as the first eukaryotic marine phytoplankton for whole genome sequencing
Bivalves			
Mytilus edulis	Mytilidae	marine	
Crassostrea gigas	Ostreidae	marine	
Crassostrea virginica	Ostreidae	marine	
Mercenaria mercenaria	Veneridae	marine	
Mytilus galloprovincialis	Mytilidae	marine	
Pinctada margaritifera	Margaritidae	marine	Farmed and harvested for pearls, the highest quality out of all the pearl oysters

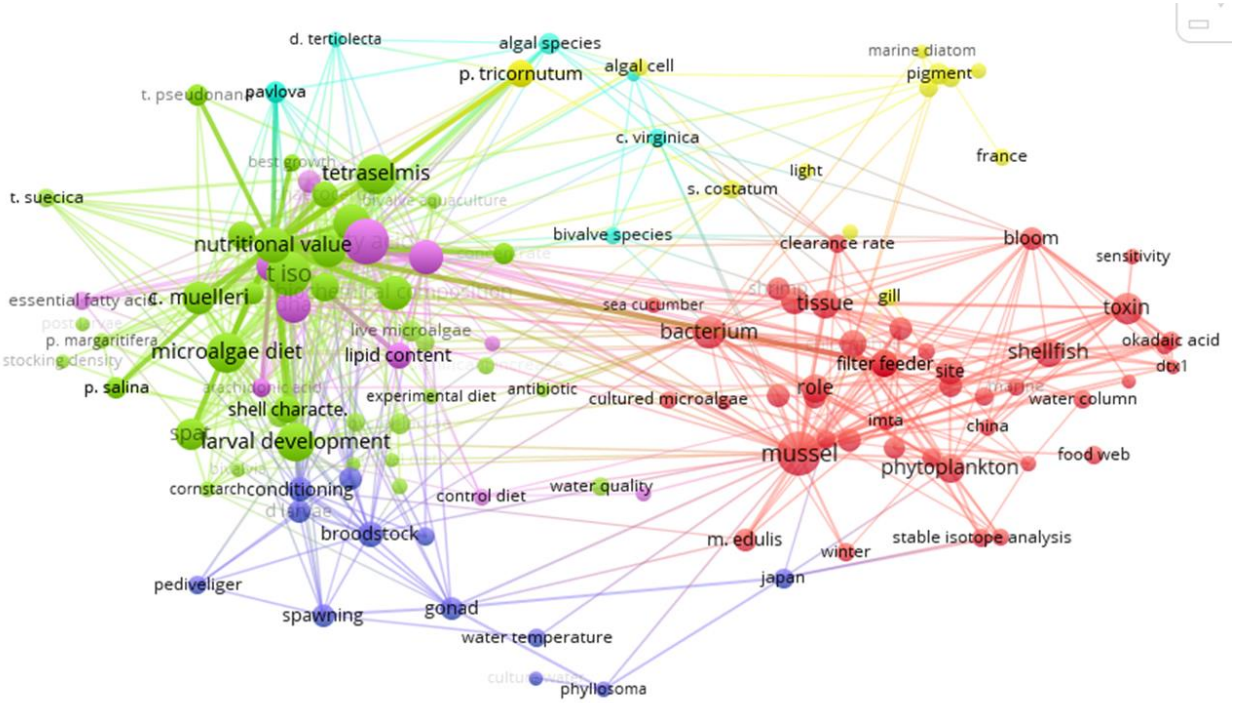

Figure 13 VOSviewer visualization of a term co-occurrence network where the six clusters can be observed; some of the most important species (both microalgae and bivalves) are presented, as well as the most occurrent terms found on the author's keywords and abstracts.

The second aproach to analyse the database allows to identify the current investigation trends, in the Figure 14, can be appreciated a shift on the investigation from nutritional and diet research to toxicity of the already mentioned Okadaic acid, but also investigations on dinophysistoxin-1 (DTX-1), diarrhetic shellfish toxin, which is a potent tumor promoter on mouse skin and is produced by *Dinophysis fortii*, an armoured bloom-forming, marine, planktonic dinoflagellate species (Fujiki et al., 1988). 2016 was the year with the majority of

the publications that met the retrieving strategy on Web of Science with 30 publications followed by 2019 with 22 on the fiel, and 2017 with 21 Figure 15. France, the United States of America and People's Republic of China leading the number of publications with 17.42%, 12.20% and 11.85% of the total 287 original research articles found on Web of science.

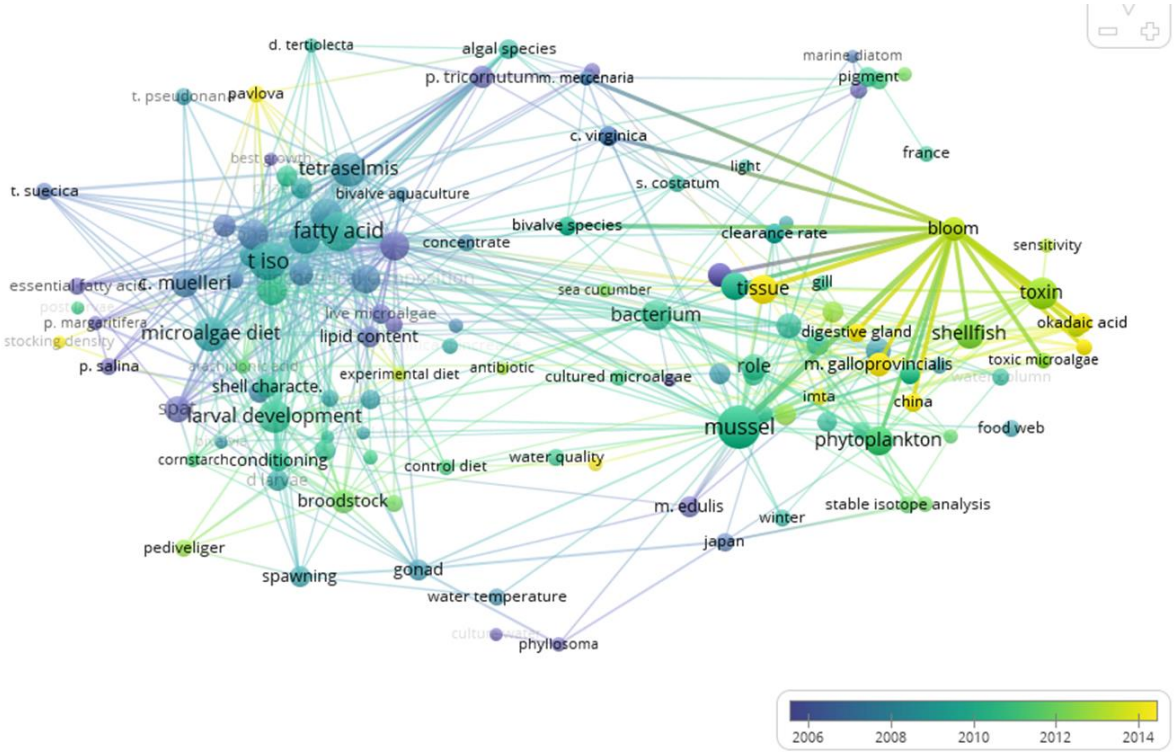

Figure 14 VOSviewer visualization of a term overlay visualization identifying the current investigation trends on the field.

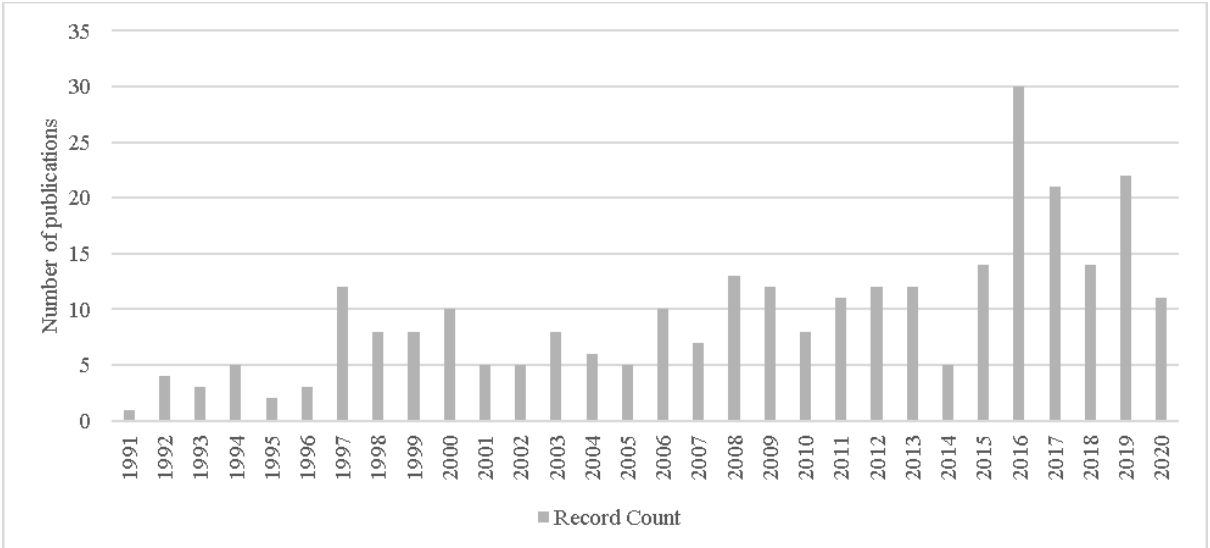

Figure 15 Growing research interest on the field of microalgae-bivalves relationship, the research articles include predominantly publications on microalgae as suitable food source and plausible detrimental effects of microalgae on bivalve rearing and posterior human health.

4.2 Small and Medium scale experiment

4.2.1 Preparation of culture settings

4.2.1.1 Image analysis to follow the development

Validation experiment

The validation experiment revealed a relationship between increase of the hue value and the cell density. The mean Hue value varied from 69.724 to 56.332 (range 13.392). In average each 5.67 units of increase of the Hue value corresponded to an increase of 1 Mill. Cells / ml in cell concentration.

Table 7. Results from the validation stage of the image analysis, actual concentrations of algae cells in the different solutions and the observed value of the integrated intensity.

Concentration of the dilution (mill. Cells/ml)	**Mean Hue value**
22.67	69.724
19.55	66.982
19	65.034
17.6	61.772
12.6	60.528
10.8	59.608
7.3	56.332
4.1	57.098
2.6	64.238

The Cleveland dotplot (Cleveland 1993) is a very useful graphical tool to visualize outliers (Zuur et al., 2010), in this case it depicts the position of each observation in the vertical axis and the value of Hue in the horizontal axis, this representation can be used to visualize data points that do not follow the general pattern of the dataset, points that stick out to the side are observed values that are considerable larger or smaller than most of the observations, and require further examination (Zuur et al., 2010). Plotting the row number (order of appearance) of an observation and not the interest factor (cell density for example) vs. the observation value (Hue value), has the advantage of allowing to easily differentiate which test tube resulted in an outlayer and requires particular attention. In the Figure 16, the first observation has a value outside the behaviour of the ones obtained from the other tubes in the data set, so it was eliminated in further analyses.

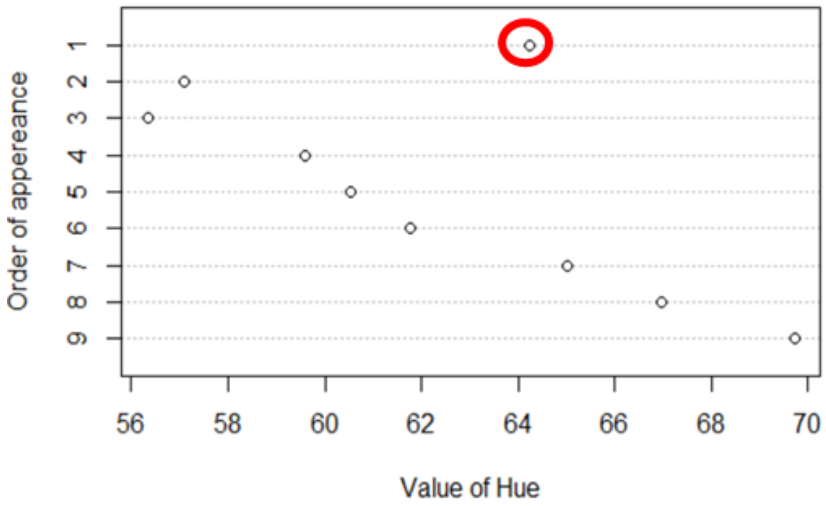

Figure 16. Cleveland dot chart of the observations for the mean value of Hue in the area of interest for nine test tubes of the dilution series.

The regression analysis revealed a strong positive relationship between the Mean Hue value and cell density (Figure 17). As can be seen in the summary table for the model (Table 8). The estimate coefficient for the Mean hue is 1.3, this value is significantly different from zero, as can be observed by the small p-value and the fact that the confidence interval for the estimate does not contain zero. The R2 and adjusted R2 are both above 0.85 suggesting a close correlation between the Mean Hue value and cell concentration in the tubes.

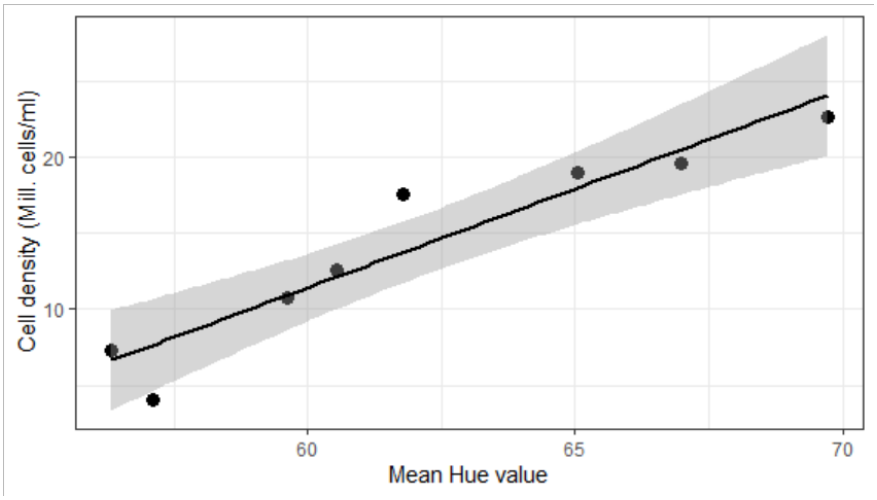

Figure 17. Comparison between Mean hue values and the cell density

Table 8. Coefficient value, confidence interval of the coefficient value and p value for the linear regression between Cell density and mean hue value.

	Cell density		
Predictors	*Estimates*	*CI*	*p*
Mean	1.3	0.85 – 1.75	**<0.001**
Observations	8		
R^2 / R^2 adjusted	0.892 / 0.874		

Small scale experiment

The analysis of the two sets of images for the small-scale experiment showed different trends in the behaviour of the factors studied. In day 14 the data were much more variable and no clear pattern was observed, Equally, no observable difference was observer between the tubes with algae that were acclimatized in agar or liquids media, the average value of Hue for the agar tubes was (mean ± SD) 68.65 ± 6.80, while for the liquid medium it was 70.34 ± 5.35. On the other hand, the average Hue value for the different F/2 media concentrations was 68.16 ± 3.90, 71.04 ± 4.92, 70.07 ± 80.20 for the concentrations A (0.099 µl/ml), B (0.132 µl/ml), C (0.198 µl/ml) respectively.

For the second group of pictures a stronger contrast was found. The mean Hue value for algae acclimatized in agar was 68.49 ± 5.80, while the liquid acclimatized algae had 62.39 ± 4.46. Additionally, the mean Hue value for the different concentrations was as follows: A=65.99 ± 5.37, B=66.79 ± 8.23, and C=63.41 ± 3.21.

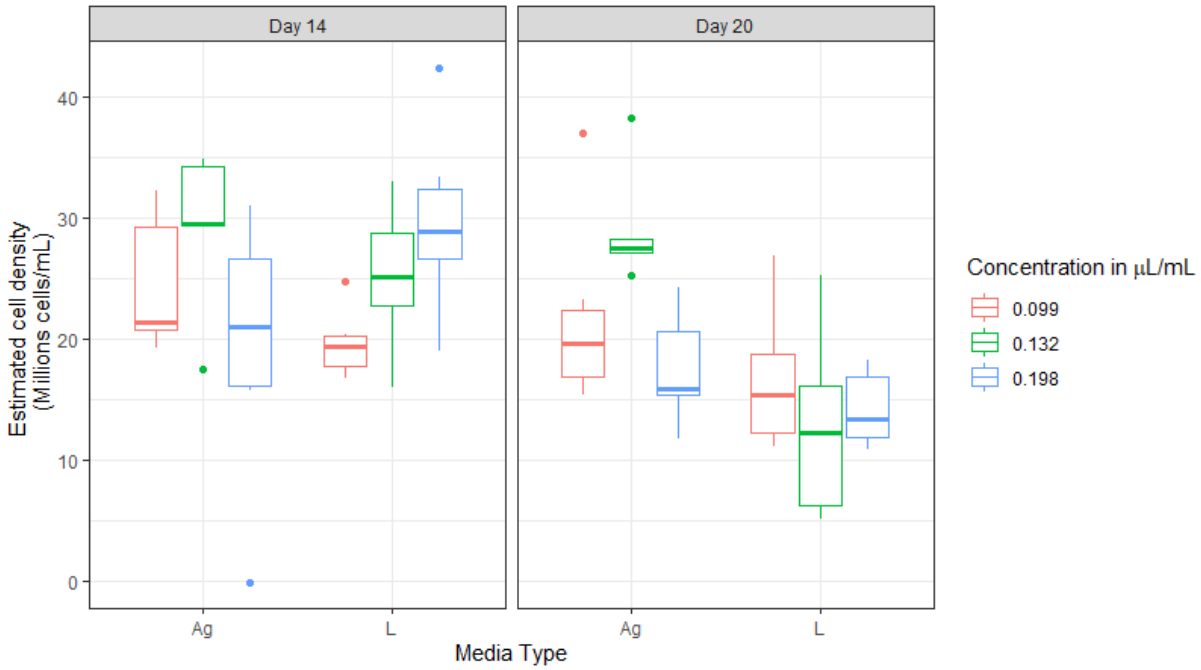

Figure 18. *Boxplots of the difference in mean Hue value for the tubes with different media used during acclimatization and in different concentrations of F/2 media.*

The Anova confirmed the previous observations, for the pictures of day 14 no significant effect was found for neither of the two factors evaluated. On the contrary for the second set of pictures of day 20, the Anova found a significant effect of the media used during acclimatization, specifically this means that algae that were acclimatized in agar before the start of small-scale experiment were significantly greener, and hence, denser, than algae acclimatized in liquid media. The concentration of F/2 media in the test tubes of the small-scale experiment, showed no significant effect of any of the concentrations tested on the mean Hue value.

Table 9. *Anova table for the two assessment days on day 14 and 20 after inoculation.*

Anova table Day 14					
Variable	*Df*	*Sum Sq*	*Mean Sq*	*F value*	*Pr(>F)*
Media accl.	1	4.19	4.19	0.11	0.74
Concentration	2	80.81	40.41	1.09	0.35
Residuals	31	1147.39	37.01		
Anova table Day 20					
Variable	*Df*	*Sum Sq*	*Mean Sq*	*F value*	*Pr(>F)*
Media accl.	1	325.65	325.65	12.71	**<0.01**
Concentration	2	82.31	41.16	1.61	0.22
Residuals	31	794.36	25.62		

Medium scale setting

After two months from the transfer of the selected erlenmeyers to the 10 L carboy a cell count was performed, the concentration of cells in the carboy was stimated in 2.28 $x10^7$ cells/mL. Using a colorimetric assay for determining the cell viability in the culture with Methylene blue, showed that 90% of the cells present in the sample at the moment of the count were viable. Therefore, the transfer of the culture from the 8 mL tubes, passing through the erlemeyers uo to the 10 L carboy was considered successful. This volume can be constantly harvested and scaled up or used as the basis for the initiation of a larger scale culture.

5 Discussion

Both trends on the research shown in the bibliometric visual analysis: (*i*.) algal toxicity and (*ii*.) algae to feed bivalves, have an industrial and economic impact:

Human interaction with microalgae is not always positive, the artificial addition of phosphorous and nitrogen to the water generated by human activities can result in the abnormal acceleration in the growth of photoautotrophic organisms (Bláha et al., 2009; Raven & Giordano, 2014). Algal blooms (HABs) represent a risk and their manifestations are correlated to a significant impact not only socioeconomic (millions of dollars are lost annualy due to freshwater and marine HABs on commercial systems, but the implications of this phenomena also extent to in human health (impact of eating contaminated animals) or algal blooms (especially in the cyanobacteria *Microcystis aeruginosa*) in drinking and recreational water (Sanseverino et al., 2016).

Blooms of cyanobacteria and other microalgae in fresh and marine water systems represent a major ecological problem: changing the diversity, disturbing the relationships between organisms, altering the oxygen concentration or compromising the light conditions of both marine and freshwater ecosystems (Bláha et al., 2009). The water contamination by toxins from algal blooms has also serious consequences for domestic animals and human health (Bláhová et al., 2007). In humans for example, the illness known as paralytic shellfish poisoning (PSP) is linked with the bioaccumulation of saxitoxins on marine and freshwater bivalves that accumulate the toxins when filtering in their tissues, saxitoxins are potent neurotoxins produced by marine dinoflagellates or by freshwater species of cyanobacteria (Wiese et al., 2010). The accumulation occurs primarily in the bivalve's viscera and can be fairly rapid, reaching concentrations dangerous to humans within 1 hr (Bricelj et al., 1990). This sort of consequences of algal blooms also embodies economical losses due to the closure of shellfish harvesting grounds worldwide.

Concerning the second most important aspect of the current investigation on microalgae and bivalves, it is important to indicate that the search for reliable nutritional sources for the farming of aquatic organisms has sparked interest in plants and particularly in microalgae, about 40 species of microalgae are currently used as a feeding source to fishes, molluscs, echinoderms, crustaceans and zooplankton (that is subsequently used to feed fish larvae) (Muller-Feuga, 2000; Raja et al., 2008), the most recurrent genus are *Chlorella*, *Tetraselmis*, *Isochrysis*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Nannochloropsis*, *Skeletonema* and *Thalassiosira* (Spolaore et al., 2006). By the bigining of this century the production of microalgae for aquaculture reached 1,000 tonnes, from this total yield approximately 62% is used to feed molluscs, 21% for shrimps and 16% for fish (Spolaore et al., 2006), the main application is associated with nutrition (as a fresh source or supplement), but also microalgae can be used for coloring the flesh of salmonids (with *Haematococcus,*or *Arthrospira)* or some other traditional coloring techniques like the oysters greening, that involves the use of the diatom *Haslea ostrearia* to produce a blue-green color on the gills and labial palps increasing the value by 40% (Muller-Feuga, 2000) (the importance of microalgae-origin pigments for the

aquaculture industry can be observed also in the Figure 13- the right upper-hand in yellow- since it is one of the co-ocurrent terms that meet the threshold).The microalgae are also used to cause the iniciation of other physiological and biological processes in the animals fed with them, (e.g) the use of the diatom *Skeletonema costatum* produced in high volumes in subterranean salt water to promote the increase of the flesh content of the oysters influencing the final value (Muller-Feuga, 2000).

The visual bibliometric analysis also revealed that as expected, most of the publications are related to mollusk species with some commercial value, for consumption as food source: like the the Pacific oyster, *Crassostrea gigas* (Ostreidae), which thanks to its characteritics (it is very easy to grow, very tolerant to environmental changes, and easily spread from one area to another) is currently the most widely farmed and commercially important oyster in the world (M.M. Helm, 2005). Another reccurent species shown by the bibliometric visual analysis is the blue oyster: *Mytilus edulis* (Mytilidae), also subject to commercial use and intensive aquaculture and the closely related *Mytilus galloprovincialis* (Mytilidae), also known as blue oyster and part of a three species complex of blue oysters (including the two formerly discussed and *Mytilus trossulus*), that can often hybridize with its sister taxa when they are found in the same locality (Rawson et al., 1996).

Mercenaria mercenaria (Veneridae) also appears as one of the relevant terms for the network analysis, this is also an edible species, native to the the Gulf of St. Lawrence in Canada through the northern Gulf of Mexico to Texas that differenciates itself from most economically interesting bivalves because it relies entirely on hatcheries for seed due to the lack any seed distributed naturally (Kraeuter, 2009). Lastly, the peral oyster *Pinctada margaritifera* (Margaritidae) farmed and harvested for pearls, considered to be the highest quality producer out of all the pearl oysters. It is unmistakable that even though inventigations on conservation of threathen species of bivalves are more and more frequent, the reseach that results in an economic advantage is the most common.

As mentioned earlier, the majority of microalgae world requirements for aquaculture goes to molluscs (Becker, 2013), for which no other dietary replacement is yet possible and involves several species. Some of them (*Dunaliella tertiolecta* and *Phaeodactylum tricornutum*) are of relatively poor nutritional value (Michael M. Helm & Bourne, 2004), see Table 3. The production cost of using only microalgae grown on site can represent up to 50% of the harcheries' costs, this has sparked a growing industry for nutritionally suitable alternatives that include dried diets and microalgal commercially available concentrates that can be refrigerated and are produced by centrifugation, this concentrates can also be prepared directly in the hatchery (Sarkis, 2007), it is important to mention that not all algae are suitable for concentrating, especially flagellates species are very susceptible to the process and can be easily damaged.

From a nutritional point of view, live microalgae have higher nutritive values and are more digestible if compared with substitutes (Hemaiswarya et al., 2011). The nutritional quality of food sources mainly depends on many biochemical components such as polyunsaturated

fatty acids, vitamins, sterols and carbohydrates (Dunstan et al., 1993). The second main criteria to select a microalgae species, is based directly on the specific requirements of the cultured bivalve, the necessities change though the different stages of the life cycle (larval, post-larval, juveniles and adults), and are related for example with the size of the cell or cell wall thickness besides the nutritional requirements among different species of bivalves (Sarkis, 2007).

Moreover, for a microalgae species to be suitable to be used as food source for commercial or conservation purposes it must meet several criteria. Undoubtedly it needs to be nontoxic, it must be easy cultured, needs to have the correct size and shape, and a digestible cellwall to make nutrients available to be ingested (Spolaore et al., 2006), but also the selected algae strain needs to have certain nutritional values and qualities directly related to the results obtained on the network visual analysis, those are (*i*.) protein content, (*ii*.) highly unsaturated fatty acids like Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA) (both considered to be essential for bivalves (Hendriks et al., 2003)) and Arachidonic acid (AA) content are of major importance for the growth and metamorphosis of the larvae of many marine animals (Becker, 2013; Prato et al., 2010; Spolaore et al., 2006).

Many marine organisms lack the ability to produce PUFAs and therefore rely on a dietary supply to obtain them (Nichols, 2003), they are also considered essential in bivalves because most of them are are unable to produce them from shorter chain precursors (Hendriks et al., 2003). More than half of the energy requirements for embryogenesis is met by lipids (PUFAs) and the remainig part is mostly met by proteins (Whyte et al., 1992). In general, PUFAs composition of the eggs is related to the fatty acids in broodstock diets. It is also known that seasonal changes in lipid contents (PUFAs) in adult bivalves are tightly related to the reproductive cycle and can be negatively influenced by the availability and composition of the diet (Prato et al., 2010).

Some other terms found in the nutrition cluster are for example Arachidonic acid (AA) which is a precursor for the synthesis of physiologically active hormone-like molecules, known to increase bivalves' resistance to stress, including various seawater salinities (Fokina et al., 2017). Eicosapentaenoic Acid (EPA) which regulates the membrane fluidity in response to cold conditions in *Placopecten magellanicus* (Pectinidae) (Hall et al., 2002), but is also used as a source of energy preferentially during embryonic development, and docosahexaenoic acid (DHA) is used as a source for structural compounds (M. M. Helm et al., 1991; Marty et al., 1992), both of them polyunsaturated fatty acids (PUFAs) of more than 18 carbons considered essencial for bivalves development. Currently, DHA is the only algal PUFA available commercially, and some of the species that have demonstrated industrial production potential of EPA are *Porphyridium purpureum, Phaeodactylum tricornutum* (one of the terms considered as relevant by the co-occurrence bibliometric analysis), *Isochrysis galbana* (also considered as for the visual output), *Nannochloropsis* sp. and *Nitzschia laevis* (Spolaore et al., 2006).

One additional cluster presented also on the visual analysis and closely related with (*i*.) the cluster of nutritional values of microalga, and (*ii*.) the one encompassing the valuable nutritional elements, was the cluster of (*iii*.) terms correlating the early development of the

animals, it was expectable that the three of them would be arranged very tightly given that the bivalve's proper development is highly influenced by the nutrition. Besides the instances discussed before (PUFAs composition of the eggs and EPA as energy source during embryonic development) another example of the diet importance on early stages of bivalve's development is the proportion of larvae reaching the D-stage, at which the first larval shell is developed, and larvae start feeding, is positively correlated with the initial lipid content of the eggs (Helm et al., 1973). After a decrease during the embryogenesis, lipid reserves of the larvae accumulate again throughout the following larval stage. In this stage the diet is vital to build up energy reserves for metamorphosis because during metamorphosis the larva is unable to feed (Whyte et al., 1990, 1992)

Nevertheless, it should be mentioned that the proportions of DHA, EPA and AA may be more important than their absolute quantities (Apt & Behrens, 1999). And some other important nutritional factors should be taken into account, this includes microalgal vitamin content also as it may be equally important.

Some of the reasons why *Ettlia oleoabundas* was selected to start the culture was the green algae's high percentages of C-18 polyunsaturated fatty acids (PUFAs) (Gatenby et al., 1996). Because this species contains high concentrations of protein, and lipids, feeding mussels with *E. oleoabundans* should provide a more complete diet. *E. oleoabundans* cell walls are composed of about 31.5% proteins, 24.3% carbohydrates, 22.2% lipids and 7.8% inorganic material (Rashidi & Trindade, 2018). About 45% of the dry wight of this species corresponds to protein, 20% to carbohydrates, 30% lipids and 10% of fatty acids (Gatenby et al., 2003).

Microalgae are an available resource with more than 25,000 species from which 15 are intensively used (Raja et al., 2008), as previously discussed the trending research topics are oriented to microalgal culture technology focused on the industrial and biotechnological processes for their potential to generate valuable products in high volumes.

For the majority of these applications, the marketplace is still on its early stages, and it is probable that the biotech use of microalgae will be extended to new areas. This comes conjoint with the development of a sophisticated culture and detection techniques so the microalgae biotechnology can keep up with the diverse demands of the industries (Benedetti et al., 2018; Hemaiswarya et al., 2011; Muller-Feuga, 2000; Raja et al., 2008). Which allows me to talk about the use of image analysis to follow the early development of the culture in the Malacology laboratory.

The use of image analysis to detect physiological and morphological changes in photosynthetic organisms has been more studied in plants of agricultural interest than in microalgae, some of the develpments on the field include processing of images to differenciate economically interesting crops from particular weeds using cameras and color photographs to first idendify the plant against a brown-black soil background, and then to development of crop-specific techniques to find segments on the images consisting of crop or weed plants (Andreasen et al., 1997). A number of more complex methods can even rectify issues such as pictures of

crops under different illuminations or changes in soil humidity (Burgos-Artizzu et al., 2011). Others methods worth mentioning are able to use color images to identify plants when they have been contaminated with materials (herbicides), which is useful when monitoring treatments; expressed by the loss of the greenness displayed during the pre-treatment stage (Gebhardt & Kühbauch, 2007), the damage in the crop can also be analyzed based on the same principle of loss of greenness. Some approaches involve the analysis of shape instead of color (Woebbecke et al., 1995), but in the case of the Malacology laboratory a procedure for greenness identification is more suitable, focused mainly on finding an indirect method that could help to assess the health and growth of the microalgae culture lowering the risk of loosing the first and only inoculum available at the time, the main concern was to decrease the possibility of infection but also to develop an easy and time-wise strategy to predict and compare the volume of cells in a sample for future projects.

Analogously to the methodology used to identify the different shades of green in the microalgae samples, the research work in real-time image processing for agriculture also recognizes some methods to solely distinguish the desired type-tonality of green and therefore type of crop (Guijarro et al., 2011). Almost every current detection method process the image in two steps: (*i*.) segmentation of vegetation in contrast to the background and (*ii*.) recognition of the vegetation pixels that represent other non-economically interesting plants on the field (Burgos-Artizzu et al., 2011), a similar step had to be taken to succesfully isolate the background form the sample.

Regarding the use of HSV color space it is important to mention that the HSB or HSV color space is an alternative representation of the RGB color model (which is defined by the three chromaticities of the red, green, and blue) designed in the 1970s by computer graphics researchers based on three attributes (i.) Hue "*attribute of a visual sensation according to which an area appears to be similar to one of the perceived colors: red, yellow, green, and blue, or to a combination of two of them*", (*ii*.) Brightness "*attribute of a visual sensation according to which an area appears to emit more or less light*" and (*iii*.) Saturation "*colorfulness of a stimulus relative to its own brightness*" (Fairchild, 2013). The HSB color space has been used in previous works for image analysis and computer vision methods. For example, Yang et al (2015), used HSB converted images to create a decision algorithm that could correctly separate corn seedlings from the very variable soil background. They found that using only the Hue channel for the images provided good results in many settings, however the best results were obtained when analyzing the three channels simoultaneosly. A similar approach could be applied to algae to be able to select correctly the area of interest without human intervention. On the same line, Bejo and Kamaruddin (2014) also used the HSB conversion of images to detect the change in sweetness of chokanan mango, their results found that the sweetness level of the fruit was closely related with the changes in the mean pixel Hue. In their study they found that the HSB-based analysis algorithm was able to correctly indetify the sweetness level of the mangos 86% of the times with lower sensitivity being related to the highest sweetness levels, were overall Hue change was smaller.

The benefits of using the HSB color space were also supported by further analysis (Jurio et al., 2010) that found that together with the CMY color space, the HSB showed the best results overall for image segementatiosn using different algorithms.

As previously mentioned, the different shades of green are characterized by specific Hue values. When the picture of the outlayer obtained in the results was reviewed, it was easily visible in the photo that in this tube the cell density was much lower compared to the pictures of other tubes, additionally these few microalgae cells were unevenly distributed at the bottom, which allowed the polystyrene cover prepared to standardize all the photos to be visible through the glass resulting in a greater number of gray and white color pixels, in the HSB color space white and grey colors are generated with variations of brightness at low saturations. Furthermore, because of the low saturation, Hue values contribute little to the formation of this colors, resulting in highly variable Hue values in these pixels of low saturation, this altered the calculation of the mean Hue value for that specific test tube.

One solution to overcome this issue could be to use a different backgrownd color to standardize all the pictures, preferably one that sits on the opposite polar coordinate of the green Hue values (purple), this could help to create a better segmentation process, but further investigations and new experiments need to be conducted. A similar issue was encountered by Ruiz-Ruiz et al. (2009) testing different color spaces based on hue for the environmentally adaptive segmentation algorithm (EASA) to analize the development of sunflower plants, in that case the color contrast (light green from small plants and light brown from the ground) was not enough to achieve satisfactory segmentation results. They recommend a series of steps including a vantage point perpendicular to the soil, large plant size, ground without pebbles and diffuse illumination conditions (since the incidence angle of natural light can create bright and dark areas with high contrast, making segmentation difficult) to obtain better results form the images. Most of those recommendations were adapted and applied for the Malacology lab conditions but for further experiments this series of steps can be improved the get better resuls.

A second option that does not requires changing the backgrouwnd color to take the pictures could be to use the mode or median of the Hue values instead of the mean, for symmetric distributions the mean is a good estimator of central tendency. However, for asymmetric distributions the mode and median could be a more robust estimate of central tendency when analyzing tubes that have the microalgae cells unevenly distributed in the bottom, thus highly impacting the mean Hue by the presence of extreme values.

In this study the use of the HSB color space and the comparison of the mean Hue, allowed a non-invasive and rapid assessment of algae growth under different conditions and this technique has great potential, however further analysis and experiments should be performed, specifically the use of the additional channels of the color space (Saturation and Birghtness) that could help to get better segmentation and analysis. Additionally, the use of a more developed standardization protocol, using highly hue-contrasting backgrounds (like purple in the case of green) will allow for comparison between two sets of images aquired in different dates, something that was not possible in this analysis.

6 Conclusions

- From the results obtained in the bibliometric analysis it can be concluded that it has been an increasing interest in the culture of microalgae in the past 30 years to investigate the applications for bivalve feeding and aquaculture research. Exactly 49.13% of the original research articles downloaded from the Web of Science research engine database were published on the las 8 years, mostly on the fields of using the microalgae as a suitable diet to feed commercially interesting bivalves.

- Even though new types of diets are constantly investigated, most of them aiming to develop a feeding protocol that could decrease the production costs in hatcheries around the globe, the latest studies have shown that supplement bivalves with fresh microalgae source is still a better basis for nutrition over other methods, since it provides a variety of essential nutrients necessary for the proper development on the different stages of the bivalves life cycle.

- The prime candidate *Ettilia oleabundas* can be cultured successfully in small and medium scale in the malacology laboratory of the Faculty of Agrobiology, Food and Natural Resources, for long time bilvave feeding the cell count after two month of culturing reached 2.28 $x10^7$ cells/mL and a 90% of viability.

- The use of indirect methods such as image analysis to follow the development of the culture during the first phases to avoid the risk of infections is correct and reliable but some adjustments and the integration of further testings are needed to fulfill the aim of a method that can be use to assess the growth of the culture in early stages on the Malacology lab without intervening in the culture. In our case, the validation experiment found a strong correlation between the algae density and the mean hue value. Additionally, the method seems to be very sensitive to compare different values of green therefore it is necessary that the samples are a little more developed since with different colors the Hue varies too much (it needs the same range of colors to be comparable).

7 Literature

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