Universidade do Minho
Escola de Engenharia

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Development of techniques to enhance the productivity of marine diatoms

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Dissertação de Mestrado
Mestrado em Bioengenharia

Trabalho efetuado sob a orientação da
Doutora Ana Paula Mesquita Rodrigues da Cunha Nicolau

Outubro de 2016

DECLARAÇÃO

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RESUMO

A aquacultura, um sector em franca expansão, está claramente menos desenvolvida do que a agricultura e a pecuária relativamente à formulação de dietas e das condições ecológicas para o aumento da produção. Sabe-se, no entanto, que um dos principais fatores que afetam a produtividade em aquacultura é a seleção de espécies de plâncton ótimas para prover as necessidades nutricionais dos animais a produzir, nas fases iniciais de desenvolvimento (larvas e pós-larvas). As tecnologias utilizadas para produzir dietas de plâncton para os animais em crescimento falham frequentemente em atingir os seus objetivos, tanto em qualidade como em quantidade, sendo insuficientes para satisfazer as necessidades produtivas das indústrias de aquacultura. O objetivo deste trabalho é desenvolver tecnologia industrial para produzir, nas melhores condições, as diatomáceas marinhas para a produção de várias espécies animais em aquacultura. Como já foi feito para outros microrganismos (como leveduras e bactérias), no caso das diatomáceas é certamente possível melhorar os meios de cultura e outras variáveis ecológicas (por exemplo, a utilização de atmosfera enriquecida em CO_2 e o uso de micro-suportes para adesão celular, para cultura em bio-reator). Os estudos realizados neste trabalho permitiram encontrar concentrações ótimas para os componentes dos meios de cultura. Foi igualmente identificado um suporte, a celulose bacteriana, que também aumenta a produtividade das culturas. Além disso, estudou-se a preservação de culturas de diatomáceas por longos períodos de tempo. Foi por isso testado um método de imobilização, utilizando um revestimento de látex, para averiguar a possibilidade deste permitir preservar a viabilidade celular por longos períodos de armazenamento. Este método ainda necessita de melhoramentos. As diatomáceas em estudo neste trabalho são *Navicula* sp. e *Amphora* sp., as principais espécies necessárias na produção em aquacultura de *Haliotis tuberculata*, um molusco marinho de elevado valor acrescentado.

Palavras-chave: aquacultura; diatomáceas; meio de cultura; micro suportes celulares; bio reator.

ABSTRACT

Aquaculture development is clearly behind agriculture and livestock farming, concerning manipulation of feeding and ecological conditions to enhance production. It is a fast growing sector in the food industry. One of the main factors affecting aquaculture productivity is the selection of the optimal plankton species to suit the nutritional needs of commercially targeted animals to be produced, in early stages of development (larvae and post-larvae). Moreover, the technologies used to produce plankton diets for the animals to be cultured, often fail to meet the needs of aquaculture production units, in quality, as much as in quantity. This is often the reason for failure in production goals. The objective of this work was to develop industrial technology to produce marine diatoms (essential for rearing many animal species in aquaculture), by developing culture media and also by optimizing other ecological variables (for example CO_2 enriched atmosphere and cell carrier optimization, to be used in bio-reactor culture), as was already done with other microorganisms (such as yeast and bacteria). An optimized culture medium was successfully developed. On the other hand, bacterial cellulose was found to be a suitable material to be used as cell carrier, enhancing culture productivity of the studied diatoms. A method of cell immobilization was tested, using latex coatings, to assess the possibility of preserving long-term cell viability. This preservation method still needs optimization. The diatoms chosen for the present study were *Navicula* sp. and *Amphora* sp., for they are the main plankton feed needed for the rearing of *Haliotis tuberculata,* a marine gastropod (mollusk, shellfish) highly prized in many gastronomical cultures around the world.

Keywords: aquaculture; diatoms; culture media; carrier; bio-reactor

ORGANIZATION OF THE DISSERTATION

This work is organized in Chapters concerning the different subjects addressed. In Chapter 1, basic concepts about diatoms and possible applications are presented. Also, the role of diatoms in aquaculture is explored, and their application in the production of a specific organism (mollusk, *Haliotis tuberculata*) is explained. Finally, a way of establishing the suitable diatoms for feeding this mollusk is described.

In Chapter 2, the materials used and methods tested in this work are described.

In Chapter 3, optimization of a method for bio-reactor culture of benthic diatoms is presented. This work is not yet completed.

The results of Chapter 3 created the need of finding an ideal material to use as cell carrier of benthic diatom cells. In Chapter 4, different materials were tested for *Amphora* sp. and an interesting material was found: bacterial cellulose gel. Moreover, the concentration of this material in the culture medium was successfully optimized. This subject originated a scientific paper, presented in this chapter.

In Chapter 5, a method for preserving diatom cultures for long periods of time was tested. This method consists on immobilizing diatom cells in a latex matrix (coating). This work is not yet completed.

In Chapter 6, a method for finding ideal nutrient concentrations in culture medium was applied, to enhance biomass production of *Amphora* sp. and *Navicula* sp. Ideal concentrations of silica and nitrates were found for *Navicula* sp., and biomass production was significantly enhanced. This subject led to a scientific paper, presented in this chapter. Concerning *Amphora* sp., results were not statistically valid, although the optimized culture medium found did enhance biomass production. This was however less than those found for *Navicula* sp. This subject should be further developed.

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LISTA DE ABREVIATURAS, SIGLAS E ACRÓNIMOS

ANOVA- Analysis of variance

BC- Bacterial Cellulose

BLAST- Basic Local Alignment Research Tool

CCAP- Culture Collection of Algae and Protozoa

CCD- Central Composite Design

CSLM- Confocal Scanning Laser Microscope

DSP- Diarrhoic Shellfish Poisoning

FAO- Food and Agriculture Organization for the United Nations

IPMA- Portuguese Sea and Atmosphere Institute

NCBI- National Center for Biotechnological Information

PUFA- Polyunsaturated Fatty Acids

RSM- Response Surface Methodology

SEM- Scan Electron Microscopy

CHAPTER 1- INTRODUCTION

1.1- Diatoms- Concepts and Applications

Diatoms are photosynthetic unicellular organisms that rose to prominence about 100 million years ago in the world primitive oceans. These organisms are responsible for about one fifth of the photosynthetic activity on Earth, this activity generating as much organic carbon in the seas as all the terrestrial rainforests combined, each year. The organic carbon generated by diatoms is the base for marine food webs, in coastal waters, supporting our most productive fisheries. In open-ocean, it sinks from the surface and becomes food for deep water organisms, and if it is not consumed, it settles on the sea floor and is sequestered on sediments and rocks, over geological time scales, becoming part of oil reserves (Falkowsky, 1998, Armbrust, 2009).

1.1.1-Taxonomy and Morphology

Diatoms are a group of algae, sizes between 20-200 µm (Gordon et al, 2009)with chlorophylls *a* and *c*. They can be benthic, living attached to surfaces like rocks, other algae, plants or animals, or planktonic, living free in water columns. They are widespread in the world aquatic environments. Diatoms have characteristic siliceous cell walls called frustules, and yellow-brown pigmentation. Each cell wall (frustule) is divided in two halves, called thecae, one larger than the other, that fit together like a petri dish, and are held together by a series of siliceous hoops, or girdle bands. New girdle bands are laid down during cell growth. Some diatom species have a longitudinal groove involved in motility called raphe (those who have it are said to be raphid diatoms, while those who do not are araphid). Species differ from one another in patterns of pores and other cell-wall structures, so this morphological features can be used to classify diatoms taxonomically (Armbrust, 2009). The spermatozoids of the oogamous genera are flagellated.

Nowadays, the main features used to classify diatoms are still their mode of sexual reproduction and pattern centers of symmetry (Andersen, 2004). In this manner a distinction can be made between oogamous centric diatoms with radially organized valves, from the isogamous pennate diatoms with their valves organized bilaterally and with fewer plate-like plastids (Mann, 1999).

The present classification system at order level and below is based in the morphology of cell-wall structures, and some important features used to classify diatoms at these levels are the raphe and the labiate or strutted processes through the cell wall. Three classes are now recognized: Coscinodiscophyceae (centric diatoms, Figure 1); Fragilariophyceae (araphid pennate diatoms, Figure 2); Bacillariophyceae (raphid pennate diatoms, Figure 3) (Medlin, Kaczmarska, 2004). Identification based on frustule structures is made by optical microscopy, and with SEM (Scan Electron Microscopy), when clarification is necessary to infer at species level. This classification system does not reflect phylogenetic relations between the groups of diatoms; it is however a generally accepted – however artificial – classification (Cox &Williams, 2006)

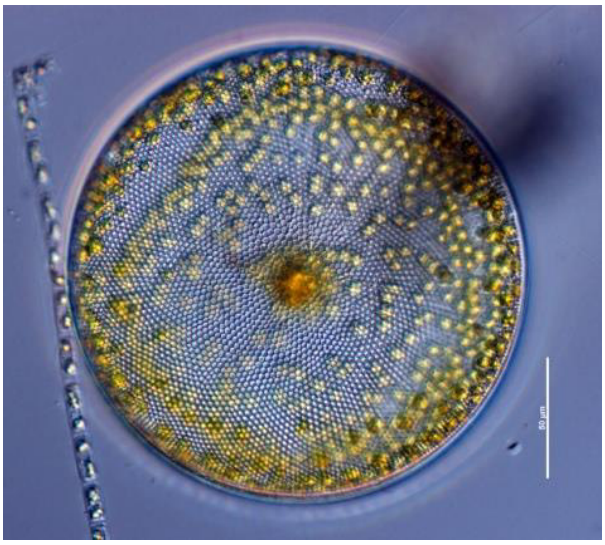


Figure 1-Example for Coscinidiscophyceae class morphology (source: Phyto'pedia). http://www.eos.ubc.ca/research/phytoplankton/diatoms/centric/coscinodiscus/c_centralis.html (29/05/2015)

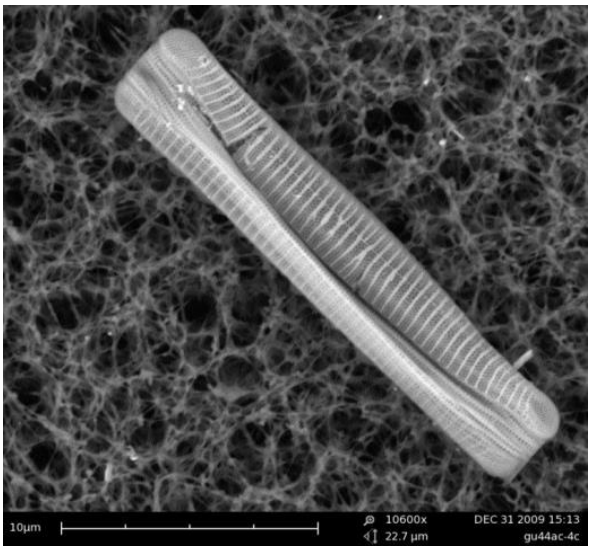


Figure 2-Example for Fragilariophyceae class morphology (source: taxateca.com).
http://www.taxateca.com/clasefragilariophyceae.html (29/05/2015)

Figure 3-Example for Bacillariophyceae class morphology (source: University of Michigan).
http://rivers.snre.umich.edu/www311/Algae_microinvert_Lab/Photos/Bacillariophyceae/ (29/05/2015)

Although these are examples of the most common morphologies inherent to each class, there are many morphologic variations even within the same classes. Figure 4 shows some of the many shapes diatoms can display.

Figure 4-Example for the variety of shapes in the diatom world (source: Wipeter, 2009).
https://en.wikipedia.org/wiki/Diatom (29/05/2015)

1.1.2- Life Cycle

There are two alternating phases in the diatom live cycle, one vegetative phase, in which the cell can be diploid for months or years, dividing mitotically and progressively decreasing in size in the process, and another phase lasting a few days, in which the cell reproduces sexually, restoring maximum size through the formation of an auxospore. Size reduction during mitosis is explained because when a cell divides in two, each daughter cell inherits one of the parental cell's thecae (from its frustule), which forms the 'top' (epitheca) of its new frustule, and then manufactures a new 'bottom' theca (hypotheca) itself during the cell cycle. The hypothecae are initiated while enclosed by the parental epithecae that preceded them, thus determining their size. The hypothecae are always smaller than the epithecae, and so the daughter cells that originated from parental hypothecae are smaller than the ones originated by parental epithecae. Over time, during the vegetative phase, cell size of the population decreases. To restore cell size within the population, diatoms develop a specialized cell, through sexual reproduction, the auxospore, sloughing off the old thecae and maturing the zygote, expanding until reaching maximum size. At this time, a new initial cell forms inside the auxospore, two to three times larger than the parental cells. These restored size cells then go back to vegetative phase and start dividing mitotically again (Chepurnov et al., 2005; Gillard, 2010). Figure 5 schematizes this process that can be said general to all diatoms.

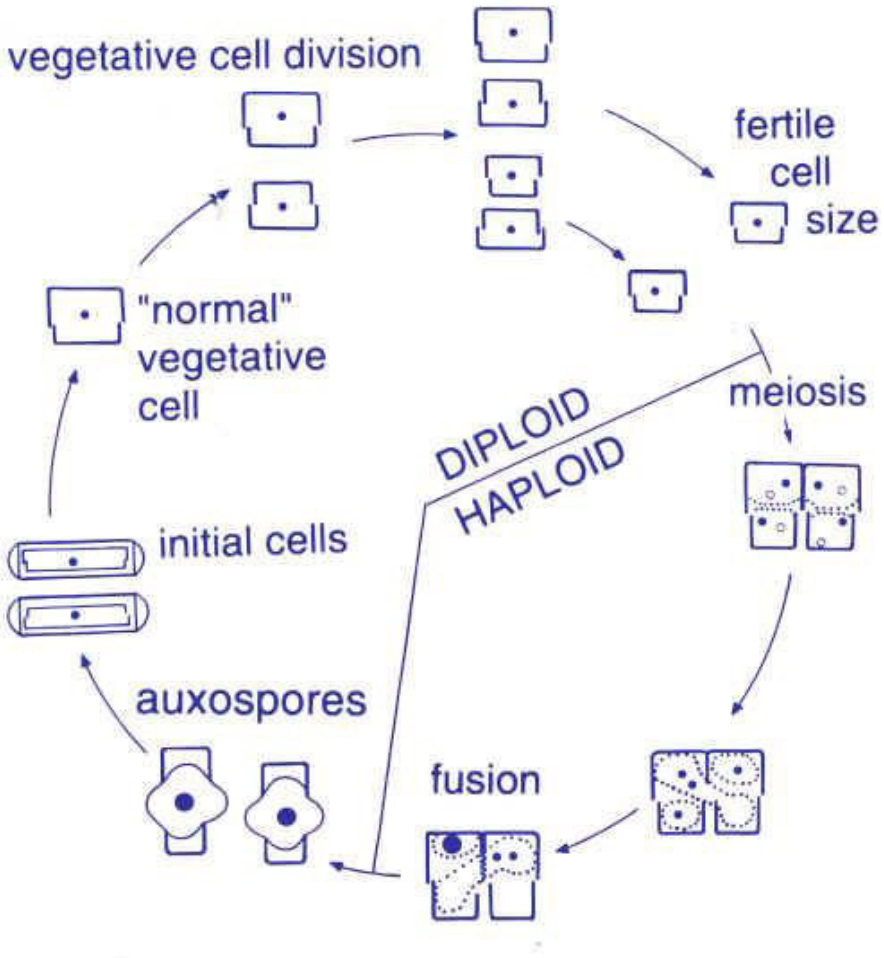


Figure 5-Diatoms life cycle (source:The Science of Biodiversity).
http://comenius.susqu.edu/biol/202/chromalveolata/heterokontae/bacillariophyta/ (30/05/2015)

1.1.3- Applications

Diatoms have several applications, for example, in cosmetic industry, due to the wide range of amino-acids they produce, pharmaceutical industry, for they produce polyunsaturated fatty acids (PUFA), producing diatomite, which is a low density, highly porous siliceous material derived from the compaction of diatom remains over geological time scales, that have various applications in the biotechnological, food and beverage, agricultural and chemical industries, for insulation, filtration, adsorption, building materials etc. (Lopez et al., 2005) Figure 6 shows various fields and application domains for diatom research.

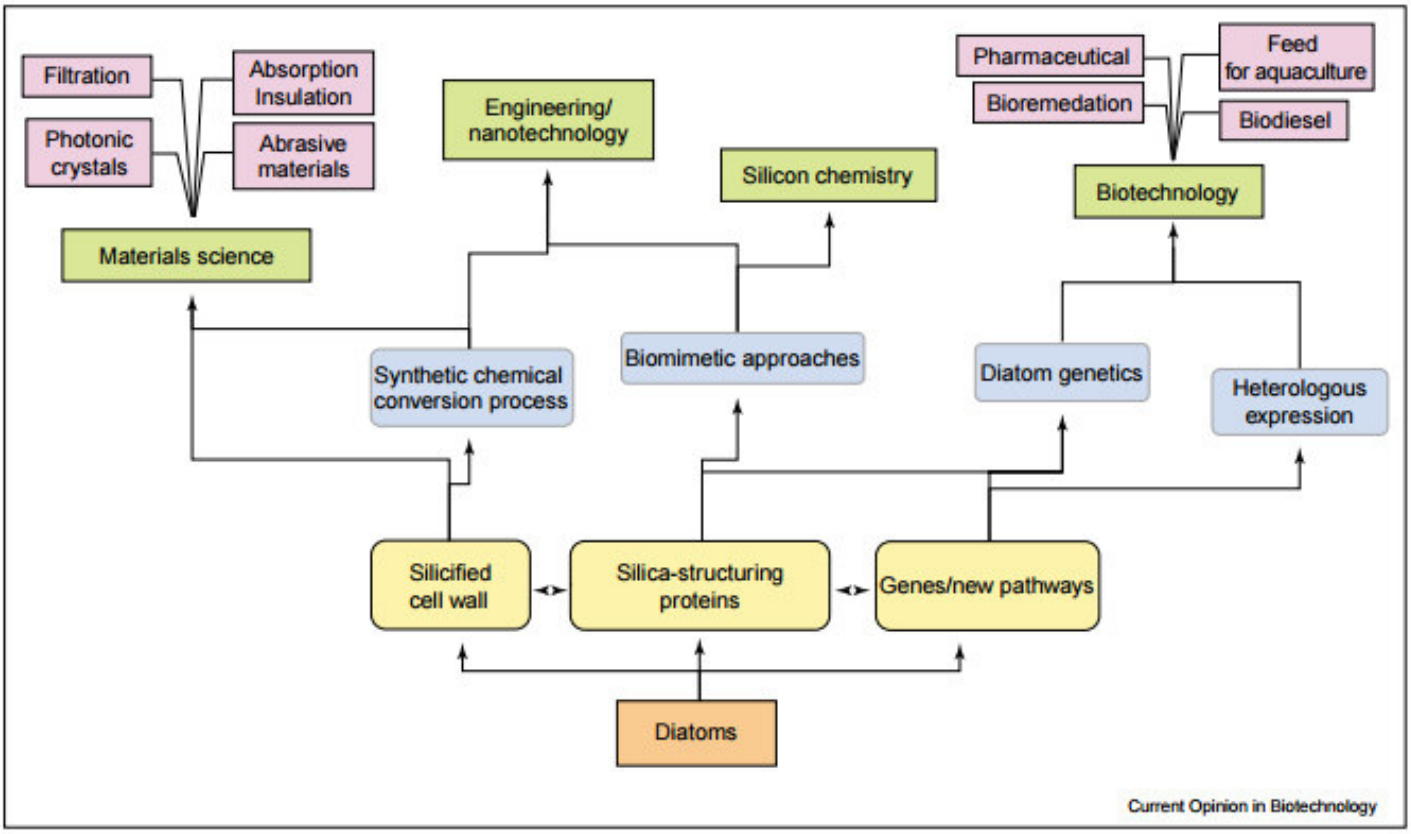


Figure 6-Fields and application domains for diatom research (Lopez et al., 2005).

Aquaculture is one of the promising biotechnological applications of diatoms: they are used to feed many animal species, especially in early stages of development, for in the larval and post larval life stages diatoms are the main source of nutrients for most marine animal species. The next chapter details the application of diatoms as aquaculture diets.

1.2- Diatoms and Aquaculture

1.2.1-Aquaculture: Present Status

Nowadays aquaculture is the fastest growing food production sector in the world (FAO, 2014). It is developing, expanding and intensifying in almost every region worldwide. The fishing captures have been diminishing for the last decades, which, considering the world growing population (and consequent growing in demand of aquatic products), calls for the

development of sustainable and efficient aquaculture techniques, to meet the market needs (Jackson et al., 2001; Subasinghe et al, 2009). Figure 7 and Table 1 present these data.

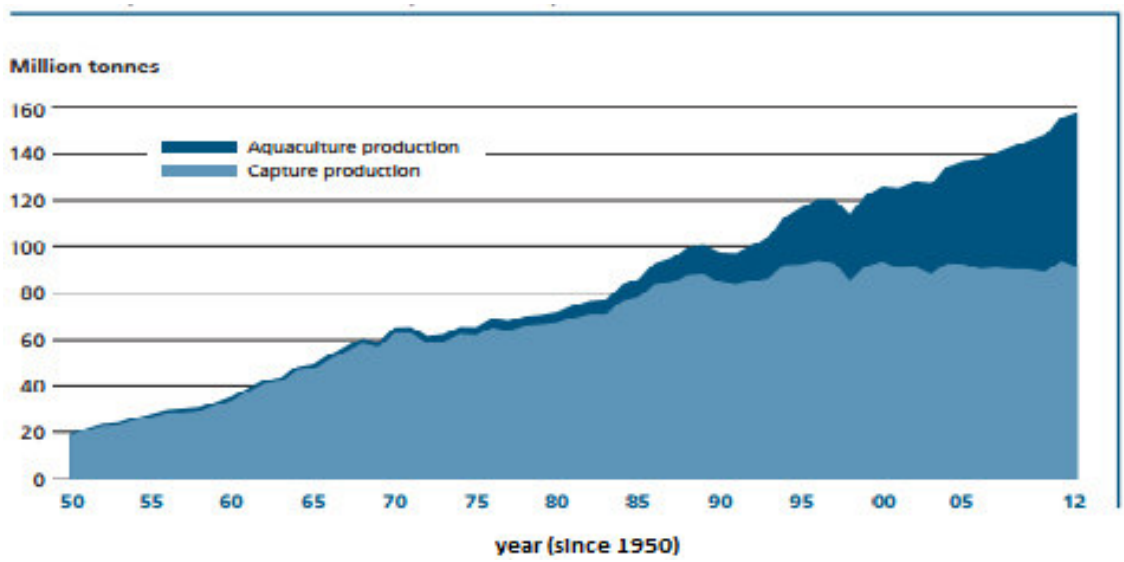


Figure 7-World capture fisheries and aquaculture production (FAO, 2014).

Table 1-Aquaculture production between 2007 and 2012 (FAO, 2014).

	2007	2008	2009	2010	2011	2012
	(Million tonnes)					
PRODUCTION						
Capture						
Inland	10.1	10.3	10.5	11.3	11.1	11.6
Marine	80.7	79.9	79.6	77.8	82.6	79.7
Total capture	**90.8**	**90.1**	**90.1**	**89.1**	**93.7**	**91.3**
Aquaculture						
Inland	29.9	32.4	34.3	36.8	38.7	41.9
Marine	20.0	20.5	21.4	22.3	23.3	24.7
Total aquaculture	**49.9**	**52.9**	**55.7**	**59.0**	**62.0**	**66.6**
TOTAL WORLD FISHERIES	**140.7**	**143.1**	**145.8**	**148.1**	**155.7**	**158.0**
UTILIZATION[1]						
Human consumption	117.3	120.9	123.7	128.2	131.2	136.2
Non-food uses	23.4	22.2	22.1	19.9	24.5	21.7
Population (*billions*)	6.7	6.8	6.8	6.9	7.0	7.1
Per capita food fish supply (*kg*)	17.6	17.9	18.1	18.5	18.7	19.2

Note: Excluding aquatic plants. Totals may not match due to rounding.
[1] Data in this section for 2012 are provisional estimates.

Aquaculture inshore production is also growing, due to the advantages of this method, namely, increased larval survival rates and less susceptibility to production losses due to bad weather, input water quality and polluting effluents treatment. Furthermore, seafood culturing in off-shore coastal farms is vulnerable to infection by plankton species that produce bio-toxins, making the products unsuitable for human consumption for indefinite periods of time. Table 2 and 3 display clearances and interdictions on the consumption of several species of shellfish caught in Portuguese waters, due to bio-toxins, on October 6th 2016.

Table 2- Consumption clearances of shellfish species by production zone, data from 6/10/2016 (IPMA- Portuguese Sea and Atmosphere Institute).
http://www.ipma.pt/resources.www/transf/biotoxinas/rb_bivalves_061016.pdf (8/10/2016)

Production Zones	**Changes since last report**
Matosinhos Coast	Bittersweet clam clearance
Peniche-Lisboa Coast	Bean clam clearance

Table 3-Shellfish species unsuitable for human consumption due to biotoxin contamination, data from 06/10/2016 by production zone and bivalve species (DSP-Diarrhetic Shellfish Poisoning) (IPMA- Portuguese Sea and Atmosphere Institute).
http://www.ipma.pt/resources.www/transf/biotoxinas/rb_bivalves_061016.pdf (8/10/2016)

Production Zones	**Bivalves**	**Motive**
Viana Coast	All species except for white clam	DSP
Matosinhos Coast	mussel	DSP
Aveiro Coastal Inlet	mussel, cockle, razor-shells	DSP
Aveiro Coast	All species except for white clam	DSP
Mondego Estuary	All species	Sample not available
Figueira da Foz- Nazaré Coast	All species except for white clam	DSP
Tejo Estuary	All species except for japanese clam and mussel	DSP
Peniche- Lisboa Coast	Al species except for razor-shells and bean clam	DSP

In inshore production units this problem can be avoided by filtering seawater, removing the phytoplankton responsible for the biotoxins production, thus making the seafood suitable for human consumption. As a result, inshore production methods optimization is being regarded as fundamental for the future of aquaculture (Muki Shpigel, 2005; Zong et al., 1996)

1.2.2-Role of diatoms in aquaculture .

One of the main constraints of inshore aquaculture, particularly of molusk production, is to select and successfully produce specific plankton, optimal for rearing each molusk species. Producing this plankton-feed is a key factor for the success of inshore aquaculture units. Some examples of marine animal species produced in aquaculture, that feed on diatoms at some development stage are:

- Pacific oyster (*Crassostrea gigas*) (Beninger et al., 2008);
- Green tiger shrimp (*Penaeus semisulcatus*) (Al-Maslamani et al., 2007);
- Sea scallops (*Placopecten magellanicus*); (Milke et al., 2004)
- Limpet (*Crepidula onyx*); (Chiu et al., 2007)
- Abalone (*Haliotis* spp.) (De Viçose et al., 2007)

These are just few of many animals of comercial value that require diatoms, to be produced in aquaculture. The goal of the present work is the optimization of culturing two diatom species, ideal for feeding the molusk "european abalone" (*Haliotis tuberculata*), a highly prized species in many countries.

1.3- Case Study- Abalone (*Haliotis tuberculata*)

Abalone is a marine gastropod (mollusk) highly prized in many gastronomical cultures around the world. It is widespread in the coastal waters of many countries, and it has been

overfished during the twentieth century, while the markets demand for this delicacy kept rising. This created a need for the optimization of aquaculture techniques to produce abalone, while preserving wild populations of the mollusk. The following pictures show the external and internal morphology of the abalone.

Figure 8-European abalone, Haliotis tuberculata (source: Encyclopaedia Britannica) http://www.britannica.com/animal/abalone/images-videos (1/06/2015)

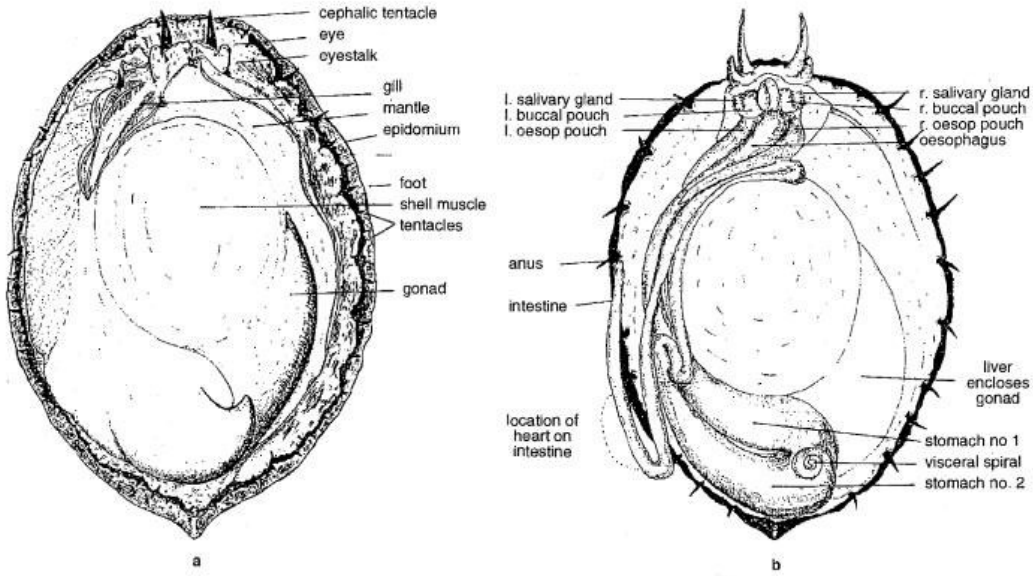
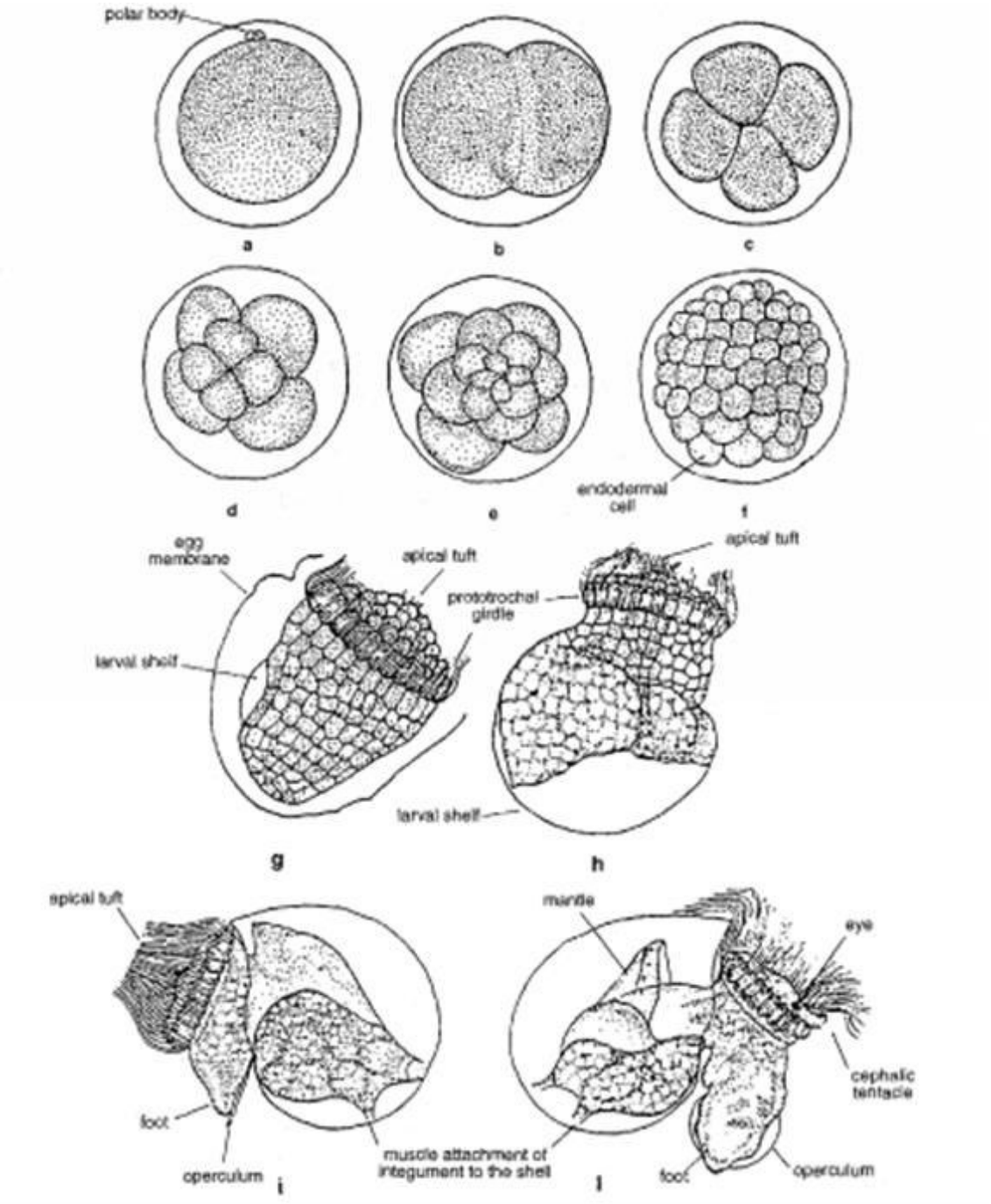


Figure 9-Abalone internal morphology (Mgaya 1995).

1.3.1- Abalone Lifecycle (Mgaya, 1995)

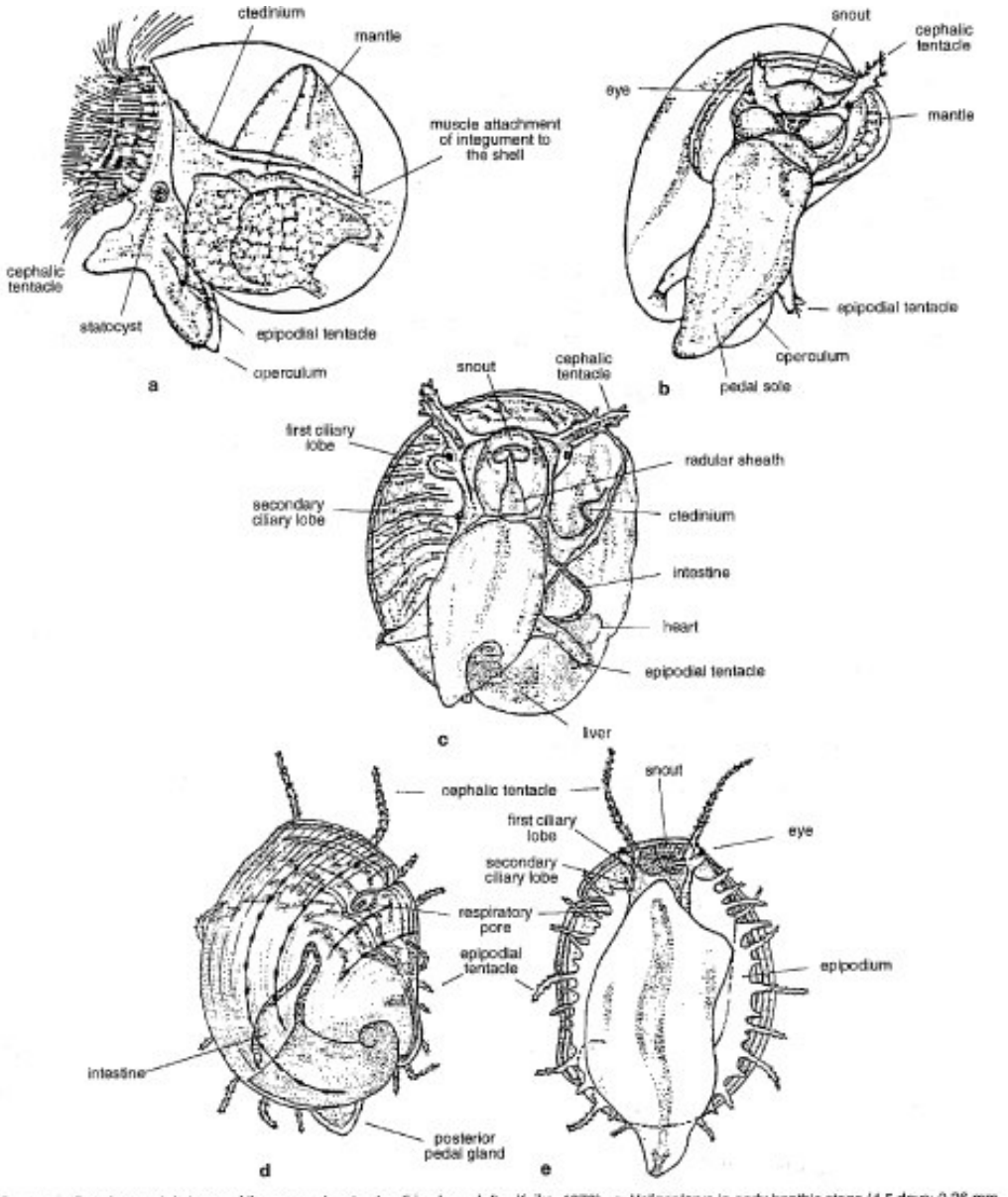
Figure 10 shows the different stages of the abalone during planktonic phase.



Developmental stages of the ormer showing embryonic and planktonic larval phase (after Kolke, 1978); **a**: Fertilized egg (0.21 mm in diameter). **b**: First cleavage (1 hr 10 min-1 hr 50 min). c: Second cleavage (1 hr 50 min). **d**: Third cleavage (animal pole view) (2 hr 20 min). **e**: Fourth cleavage (3 hr 50 min). **f**: Morula (4 hr 50 min). **g**: Trochophore just before hatching (13 hr; 0.15 x 0.20 mm). **h**: Early veliger larva (20 hr 20 min; 0.20 mm in diameter). **i**: Veliger larva after torsion (38 hr 30 min - 40 hr). **j**: Veliger larva in late planktonic stage (4.5 days; 0.26 mm in shell diameter).

Figure 10-Abalone morphology during the planktonic phase of life cycle (Mgaya 1995).

As shown in Figure 10, after hatching from eggs, abalones go through a trochophore larval stage, in which they live free in the water column (planktonic phase), using stored yolk reserves until metamorphosis. After metamorphosis, they attach to available surfaces (benthic phase), only if a settlement substratum is present, in the form of a biofilm constituted mainly by diatoms, which they graze for feeding until they reach adult morphology. Figure 11 shows the development of the abalone during the benthic phase.



Developmental stages of the ormer showing benthic phase (after Kolke, 1978). a: Veliger larva in early benthic stage (4.5 days; 0.26 mm in shell diameter). b: Creeping larva (ventral view) beginning to secrete peristomal shell (6 days; 0.27 mm in shell diameter). c: Creeping larva (ventral view), at the beginning of epipodial differentiation (16 days; 0.47 mm in shell length). d: Young ormer with the first respiratory pore (dorsal view) (40 days; 2.2 mm in shell length). e: Same as d (ventral view).

Figure 11-Abalone morphology during benthic phase of lifecycle (Mgaya 1995).

During the benthic larval phase of abalone life, feeding is exclusively on biofilm composed mainly by diatoms, after which it feeds on macroalgae abundant in coastal waters, such as *Palmaria palmata* (Figure 12), *Ulva lactuca* (Figure 13) and *Laminaria digitata* (Figure 14) (Shpigel et al., 1992). It is thus crucial to provide the specific diatom diet to hatching larvae when rearing abalone in aquaculture.

Figure 12-Palmaria palmata

(source: Seaweed Industry Association).
https://seaweedindustry.com/seaweed/type/palmaria-palmata (2/06/2015)

Figure 13-Ulva lactuca

(source:Seaweed Industry Association).
https://seaweedindustry.com/seaweed/type/ulva-lactuca (2/06/2015)

Figure 14-Laminaria digitata (source: Cape Farewell)

http://www.capefarewell.com/2011expedition/2011/07/08/seaweed-a-natural-resource-with-much-potential/ (2/06/2015)

1.4- Diatoms for aquaculture of *Haliotis tuberculata*

The selection of the best diatom species to feed a particular species of mollusk can be made by taking an environmental sample of biofilm on the natural habitat of the mollusk, isolate the several microalgae species present, and make trials to observe which alga species produce the fastest growth in the populations of the animal larvae (Zhang et al., 2010).

Mollusk larvae diets are often composed by several algae species, most of them diatoms (Chiu et al., 2007; Milke et al., 2004; Slattery, 1992).

Because diatoms siliceous cell wall shapes are characteristic of each species, one way of selecting the best diatoms to feed the abalone *Haliotis tuberculata* is to prepare a mixed culture of several benthic diatom species and add abalone larvae to the biofilm formed by the algae culture. The larvae are going to graze on the biofilm, and later the larvae guts can be dissected and inspected for the presence of frustules of ingested diatoms with SEM (Scanning Electron Microscope) (Siqueiros-beltrones et al., 2005). In this way, diatom species ingested by the larvae, can be identified by the shapes of the frustules, particular to each species. Observing the biofilm in SEM too, a relation between the amount of each species in the larvae guts and in the biofilm can be determined. This relation can tell us which diatom species are preferred by the larvae, and then select these diatoms to be cultured and fed them to the larvae (Ko & Hur, 2011). Figure 15 shows different diatom species in the gut content of abalone larvae, observed in SEM.

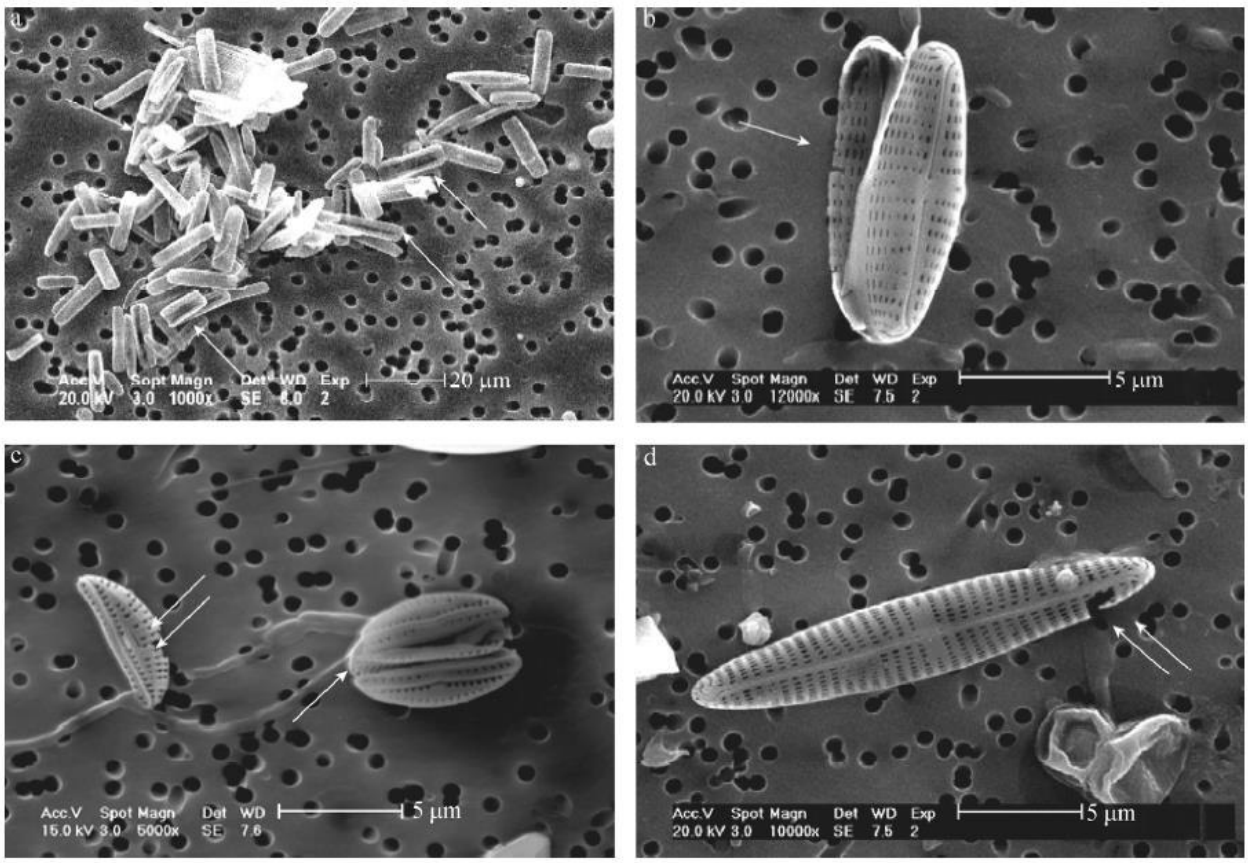


Figure 15-Diatom frustules in the gut content of abalone larvae (Zhang et al. 2010).

The techniques referred above allowed us to know that the ideal diatom species to feed *Haliotis tuberculata* larvae in aquaculture units are *Navicula* sp. and *Amphora* sp.

CHAPTER 2- MATERIALS AND METHODS

2.1- Obtaining the diatoms *Navicula* sp. and *Amphora* sp.

The strains were obtained from CCAP (Culture Collection of Algae and Protozoa). They were originally collected in Crinan Harbour, near Oban, Scotland, UK 56.4200N 5.5200W (v. approx.) and isolated by Andersen in 2006. Cultures came with bacteria, from the environmental samples used to isolate diatom species. Several trials were made to turn the cultures axenic but they were all unsuccessful, so the work was carried on with these bacteria.

2.2- Cultivation of *Navicula* sp. and *Amphora* sp.

Culture medium

F2 medium (Guillard & Rhyther, 1975) was used to maintain all the cultures of this assay (formulation is available in Annex 1 and is the one suggested by CCAP. Natural seawater was collected during the high tide in the coordinates 41°29'06.6"N 8°46'51.5"W, filtered (0.2 µm pore size) and sterilized in an autoclave.

Light, temperature and stirring

Stock-cultures were incubated in T-flasks inside a cabinet with air-conditioner (Panasonic Inverter Econavi) keeping temperature near 20 °C at all times. Light cycle was controlled by a timer (CYLLUX ELECTRIC), 12 h/12 h light/dark. The T-flasks (Orange Scientific- or flask 150 cm^2) were gently stirred at 50 rpm on a stirring plate (ELMI Type DOS-20 L), as showed in Figure 16.

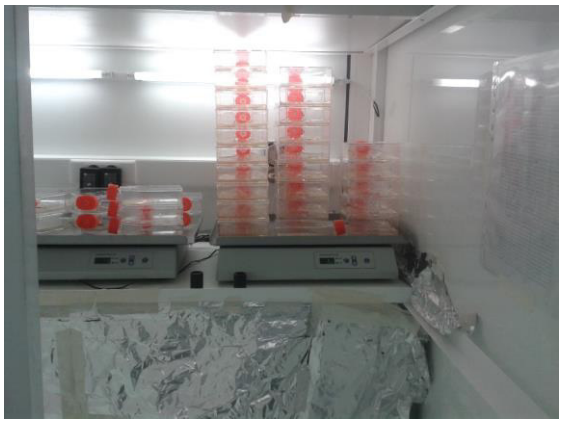


Figure 16-Cabinet containing cultures in the conditions described above.

Culture maintenance and monitoring

New cultures were made every two weeks to maintain culture viability constant. Cultures are observed at the microscope every week to assure culture viability.

2.3- Culture and preservation methods tested and optimized

2.3.1- Analytical Methods

This work focused on monitoring the cell concentration of diatom cultures exposed to different environmental variables and find the ones that traduce in fastest population growth. Furthermore a new preservation method was be tested to secure the viability of refrigerated diatom vials, used for cell propagation.

The methods used for monitoring cell concentration were flow cytometer analysis and dry weight measurements. Results were initially compared to cell counts done in a Neubauer chamber, observed by optical microscopy. Also, in assays using culture medium containing cell carriers, spectrefluorimetry readings were used to monitor diatom concentration in cultures.

In flow cytometry, a known volume of cell solution is pumped in a single fluid stream through the analysis point (which is a zone of intense illumination, where the light scattering and fluorescence of particles are detected) at speeds of more than 1000 cells per second, and each passing particle is detected by photomultiplier tubes. This way it is possible to detect diatom culture cell concentration and viability, because the chlorophyll in diatoms chloroplasts appears red when exposed to ultraviolet light. The sensors in the cytometer detect the amount

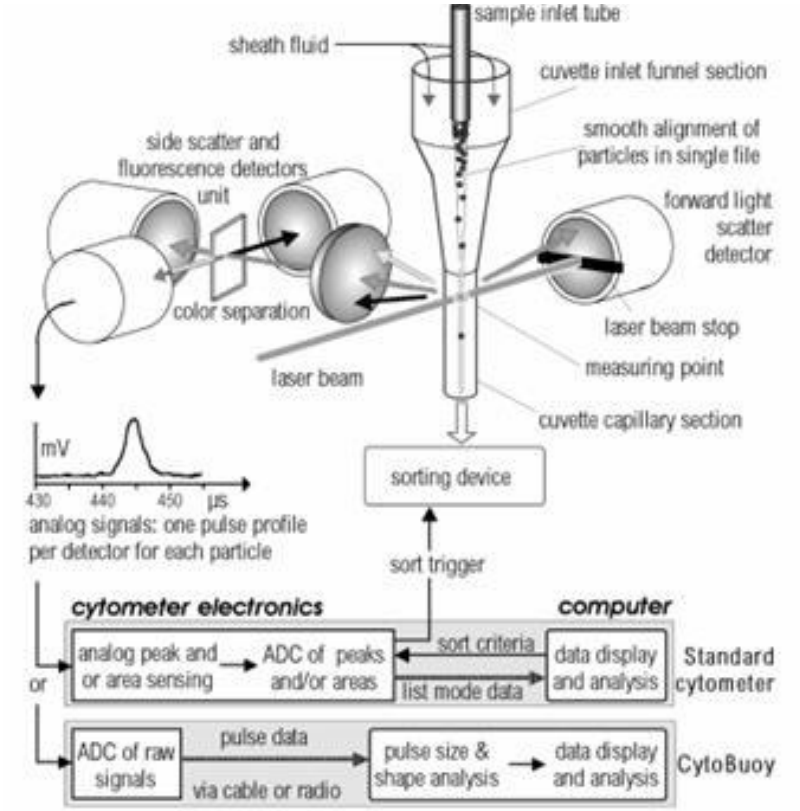
of cells that turn red when exposed to the ultraviolet light, and give information about the amount of cells, and the cell viability percent on a sample. Furthermore it is possible to monitor the growth of bacteria associated with the diatoms, using a dye (Syto 9, Thermofisher Scientific) that discriminates the bacteria, making it possible to monitor the diatom/bacteria ratio in cultures.

For this work a EC800 Flow Cytometry Analyser (Sony Biotechnology Inc., Champaign, IL, USA) equipped with an argon-blue laser (488 nm) was used (Figure 18).

For fluorimetry readings, a Spectroflurimeter (HORIBA AQUALOG 800) and a quartz cell (QS High Precision cell, Art. Nº 140-F-10-40, Hellma Analytics) were used.

Figure 17-Flow cytometer used in this work.

Figure 18 shows the schematic drawing of a flow cytometer operating principle and signal processing.



– Schematic drawing of the flow cytometer operating principle and signal processing. The sample is injected in a sheath fluid that funnels it into a thread, so thin that the particles are well separated, intersecting a focussed laser beam one by one at high flow speed. Here, each particle emits a short (typically a few microseconds) flux of photons by scattering or fluorescing. Photodetectors convert those into electrical pulses. Small particles give similar, gaussian shaped pulses, following the distribution of light in the laser focus. From these signals (top electronics panel), either the pulse maximum or the pulse area is sensed with analog electronics, subsequently digitized and stored on computer disc in so-called list mode data files. In these files each detected particle is listed with its indivíual pulse values, one for each detector. The directly available listmode data per cell may also be used to control a sorting module for the real-time physical selection (flow sorting) of particles downstream of the laser focus. The shape and length of big diatoms and filaments dominate their detector pulses, impairing analog electronic processing. This requires direct digitization of the detector output (lower panel) to obtain correct pulse area (integral) values and to allow any other type of pulse analysis in principle, as applied in the CytoBuoy instrument (Dubelaar *et al*., 1999, Dubelaar and Gerritzen, this volume).

Figure 18- Flow cytometer operating principle and signal processing (Bauer et al. 1980; Dubelaar et al. 1999)

Figure 19 shows the spectrofluorimeter used in this work

Figure 19-Spectrofluorimeter used in this work.

A Confocal Scanning Laser Microscope (Olympus BX61, Model FluoView 1000, Olympus Iberia S.A.U. Portugal) and an epifluorescence microscope (Olympus BX51, Olympus Iberia S.A.U. Portugal) were used to observe the cultures.

Figure 20-Confocal Scanning Laser Microscope used in this work.

Figure 21--Epifluorescence Microscope used in this work.

2.3.2- Bio-Reactor Cell Culture

Cell culture in bio-reactor is indispensable in industrial algae production, for it enables high biomass yields, due to high culture volumes and wide manipulation of environmental variables. Existing methods for diatom culture in bio-reactor are still underdeveloped, therefore an investigation was carried out to address this issue. Diatoms are photosynthetic organisms, therefore they consume carbon dioxide. As a result, culturing diatom cells in a *gas-lift* bio-

reactor would make possible to provide high carbon dioxide concentrations to the cells, and that might have a positive effect on cultures cell concentration increase (Wu et al., 2010). Several experiments were carried out, testing different techniques, to ascertain an efficient method of culturing diatoms in bio-reactor. This subject will be addressed in Chapter 3.

2.3.3- Cell carrier optimization

The diatoms on study are benthic, meaning they grow attached to surfaces. Thus, it can be hypothesized that cell culture performance in a bioreactor might be enhanced by providing extra surface area to carry the cells (Branco et al., 2007). Several materials were tested, using culture flasks. This subject will be addressed in Chapter 4.

2.3.4- Cell preservation in latex coatings

Cell immobilization can increase viability of preserved microbial cultures.

An immobilization technique was tested, already in use for other microorganisms (Bernal et al., 2014). This method consists on using latex coatings to immobilize microorganisms. The viability of diatom preserved cultures with this method was assessed periodically.

Using this method, Gosse et al. observed that immobilized bacterial cells were more reactive than an equivalent number of suspended or settled cells and remained active after hydrated storage for greater than 3 months in the dark and 1 year when stored at -80 ºC (Gosse et al., 2007). For these reasons it is possible that the immobilization method described in Chapter 5 can preserve diatom cells for longer periods of time than the previously existing methods.

2.3.5- Culture media optimization

Because diatoms siliceous cell wall comprises a large part of the cell volume, trials were conducted to see if higher concentrations of silica in the culture medium would result in faster population growth (Thamatrakoln & Hildebrand, 2008).

Culture media with growing concentrations of silica, starting from the standard f2 medium concentration (control, see Annex 1) were formulated, and the effects on cell concentration were compared with the ones obtained in control cultures.

Diatoms also need a source of nitrogen, for protein synthesis, which is provided in the f2 culture medium, under the form of $NaNO_3$, so the study proceed, as described above for the

optimization of silica concentration in the culture medium (Daume et al., 2003). This subject will be addressed in Chapter 6.

CHAPTER 3- BIO-REACTOR CELL CULTURE

3.1- Bio-reactor culture using 4.5 L bio-reactor and dosing pump

Preliminary tests were performed using a bio-reactor displayed in Figures 22, 23 and 24. Red arrows represent water going out of the reactor, yellow arrows represent water going inside the reactor, and green arrows represent CO_2 going inside the reactor (when activated).

In this chapter, *Amphora* sp. diatoms were used.

At first, no extra CO_2 was provided to the reactor. Water was recirculated using a dosing pump (Plasteral S.A- Type HD MA), displayed in Figure 25, leaving the bio-reactor through tube 1, and being pumped inside again through tube 2 and tube 3. So, the mixture of the cell solution must be promoted, making conditions homogenous to all the cells in the reactor. Air exchanges were made through an entrance covered in gauze on the top of the reactor. Daily samples were observed in epifluorescence microscopy. This trial lasted 4 days.

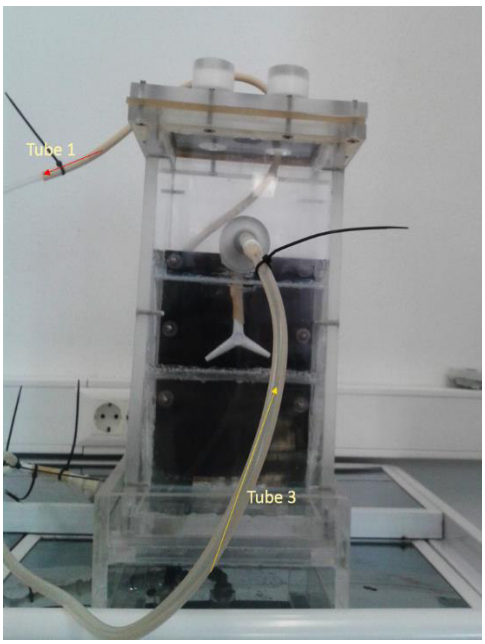


Figure 22-Bio-reactor (4.5 L) used in this work (front view).

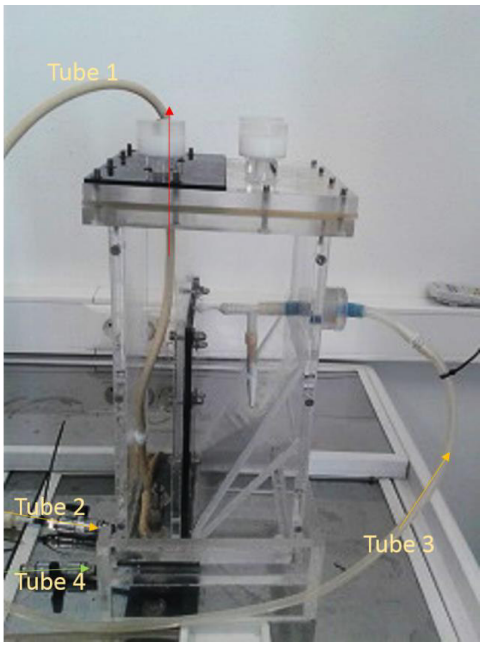


Figure 23-Bio-reactor (4.5 L) used in this work (side view).

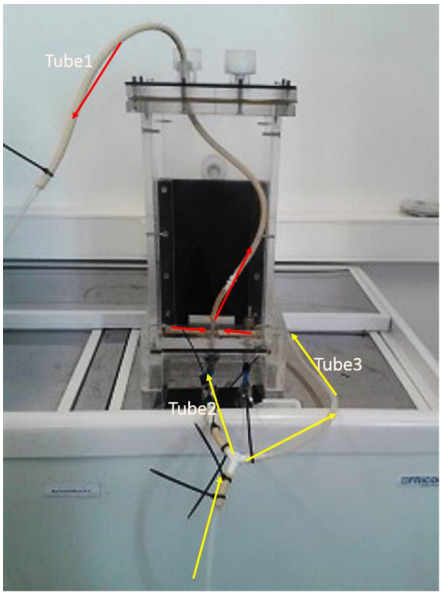


Figure 24-Bio-reactor (4.5 L) used in this work (back view).

Figure 25-Peristaltic pump used in this work.

Diatom cells viability decreased over 3 days until it was close to zero, along with continuous increase in bacteria concentration. Microscopic observation showed that the diatoms frustules were shattered, probably crushed by the dosing pump's piston, for they are made of silica (glass), and are consequently fragile to mechanical stress. Although there is no information on the bacteria present, this could indicate that, maybe, they are heterotrophic and feed on dead diatoms. Furthermore, this trial made us realize the reactor's design was prone to cell accumulation on corners and on the ramp. A steeper ramp was installed.

The dosing pump was replaced by a peristaltic pump to test if this would relieve the cells from the mechanical stress.

3.2- Bio-reactor culture using 4,5 L bio-reactor and peristaltic pump

Following the results described previously, a test took place using the reactor with a steeper ramp, and replaced the previously used dosing pump with a peristaltic pump (Watson Marlow 323 U.S.A. Figure 26), to check if this new pump would subject the cells to less

mechanical stress, and so preserve their viability. Samples were observed daily in epifluorescence microscopy. This trial lasted 4 days.

Microscopic observations showed that the peristaltic pump also crushed the cells and so it would have to be replaced. Furthermore, the new steeper ramp installed did not solve the problem of cell deposition.

Figure 26-Persistaltic pump used in this work.

It was concluded that this reactor design is not appropriate for culturing cells that are prone to settling, and should be more efficient to grow lighter cells with more buoyancy, so the cells are more easily kept in circulation. Also, it was clear that the types of pumps used expose the cells to excessive mechanical stress, and a different approach should be used. Moreover, the substantial volume of this reactor resulted in high costs in culture media preparation, for the many trials required to optimize the reactor configuration.

3.3- Bio-reactor culture using 600 mL bio-reactor and air compressor

This new approach used smaller bio-reactors (Figure 27) along with the use of an air compressor (Figure 28) connected to perforated tubes, through which filtered air bubbles entered the reactor, mixing the culture inside.

Figure 27-Bio-reactors (600 mL) used in this work

Figure 28-Air compressor used in this work

These perforated tubes were assembled in a spiral shape and kept on the bottom of the reactors, so that the most of the bottom surface of the reactor was covered, in such manner that the air bubbles produced would re-suspend cells that settled there.

The 600 mL bio-reactor and air compressor were assembled, connected by the perforated (spiral shaped) tubes devised, and a test was performed to check if this new culture mixing method would subject the cells to less mechanical stress, while keeping the culture homogenous. The air compressor was connected to an electric timer, programed to be turned on automatically, for 15 min at a time, every 15 min of the day and night. Samples were observed daily in epifluorescence microscopy, and analyzed in flow cytometry every two days, to monitor diatom and bacterial growth. This trial lasted 5 days.

Microscopic observations showed that the new method for mixing the culture preserved diatom cell integrity in the first 3 days, for the air bubbles produced by the air compressor submit the cells to less mechanical stress than the previously tested equipment. Also, this approach successfully kept the culture homogenous, keeping the cells from settling on the bottom of the reactor. However, cytometry readings showed that bacterial growth exceeded diatom growth, which decreased over time. By day 5, microscopic observation showed that diatom cells were completely covered in bacteria. This could derive from the possibility of the bacteria in culture being aerobic, and the oxygen inside the air bubbles (provided by the air compressor) enhanced their growth, resulting in their proliferation over the diatoms (which require CO_2 for their metabolism, during the day time of the light cycle). It is possible that the excess of bacteria covering the diatoms prevented light from reaching the cells, thus hampering their growth.

Possibly, bacterial overgrowth could be prevented by providing CO_2 to the culture, and simultaneously enhance diatom growth. This hypothesis will be tested in section 3.4.

This type of bio-reactor, with the described configuration (using an air compressor to promote the mixture inside), can be a viable option to culture diatoms, for it does not damage the cells through mechanical stress. This approach will be explored throughout this chapter.

3.4- Bio-reactor culture using 600 mL bio-reactor and air compressor, with CO_2 sparging from a 70 L bottle

3.4.1- An equipment configuration the same as in 3.3 was used.

The objective of this assay was to test the hypothesis of enhancing diatom productivity, while preventing bacterial overgrowth, by providing extra CO_2 to the cells in culture. This was tried by sparging this gas for periods of 15 min periods every hour, 6 times a day during the daytime of the light cycle. The CO_2 sparging was turned on and off automatically by an electronic valve (ACL Italy, type 20E), connected to an electric timer. The gas stream was kept at the lowest possible by the available flowmeter (ABB PurgeMaster, Workington Uk; minimum flow≈3 L/min). Also, a pH meter (Hanna Instruments, pH 209) was used to monitor changes in pH inside the reactor. Samples were observed daily in epifluorescence microscopy and analyzed in flow cytometry, to monitor diatom and bacterial growth.

This trial lasted 4 days.

When the CO_2 sparging was started, an abrupt lowering in pH values was observed (from 7.5 to 5.6 in less than 2 min). Diatom and bacterial concentrations in culture lowered since the tests have started, until they reached zero at day 3. Acceptable pH values for diatom cells are between 7 and 8.5.

The extra CO_2 caused an abrupt pH decrease, which was lethal for the microorganisms in culture. It is possible that we the abrupt decrease in pH could be prevented by adding a buffer solution to the culture medium. This approach will be tested in following sections.

3.4.2- Testing the buffer effect of NaOH (10M) to prevent abrupt decrease in pH.

An equipment configuration the same as in 3.4 was used, but

NaOH (10 M) was progressively added to the culture, when CO_2 sparging was activated.

This trial lasted 3 days.

The adding of NaOH did prevent the abrupt decrease in pH, however it generated white clots immediately when added. Diatom and bacterial concentration in culture decreased over time, possibly because the formation of clots in the culture damaged the cells.

This buffer solution is not a viable solution for the targeted problem.

3.4.3- Testing the buffer effect of Na_2CO_3 (30 %) to prevent abrupt decrease in pH.

An equipment configuration the same as in 3.4 was used, but

Na_2CO_3 (30 %) was progressively added to the culture, when CO_2 sparging was activated.

This trial lasted 2 days.

Progressively higher amounts (5 mL, 10 mL, 20 mL, 30 mL, 40 mL) of Na_2CO_3 (30 %) were added to the culture, upon the start of CO_2 sparging. This resulted in an increase in pH which compensated the decrease caused by CO_2 sparging. However, the amounts of buffer required to achieve this compensation were too high for the available culture volume, and would end up diluting the culture (considering the buffer adding was made 6 times a day, even if it was tried to do it fewer times a day, after one week of culturing it would be too high). Furthermore, diatom growth was not increased, compared to control culture values (flask culture).

This buffer solution is not a viable option to solve the targeted problem.

3.4.4- Testing the buffer effect of K_2CO_3 (5.25 g/L) to prevent abrupt decrease in pH.

K_2CO_3 (5.25 g/L) was progressively added to the culture, when CO_2 sparging was activated.

This trial lasted for 2 days.

The same results as in 3.4.3 were obtained.

This buffer solution is not a viable solution for the targeted problem.

3.4.5- Testing the buffer effect of Na_2HPO_4 (28.3 g/L) + KH_2PO_4 (13.6 g/L) to prevent abrupt decrease in pH.

A buffer solution composed by Na_2HPO_4 and KH_2PO_4, was progressively added to the culture, when CO_2 sparging was activated.

This trial lasted 2 days.

The same results as in 3.4.3 and in 3.4.4 were obtained.

This buffer solution is not a viable option to solve the targeted problem.

After the tests described in sections 3.4.1, 3.4.2 and 3.4.3, it was observed that sparging CO_2 from a bottle with the available equipment resulted in too abrupt pH decreases, which could not be compensated by the tested methods. For this reason, an alternative method for providing CO_2 to the cultures was investigated, as described in section 3.5.

3.5- Providing CO_2 to the cultures through a chemical reaction

An alternative method for providing CO_2 to the culture was tested, which consisted of generating CO_2 inside the reactor through a chemical reaction. By adding equal amounts of $NaHCO_3$ (1 M) and HCl (1 M) to the culture, an equal amount of CO_2 was generated localy, through the reaction: $NaHCO_{3\,(aq.)} + HCl_{\,(aq.)} \rightarrow CO2_{\,(g)} + H_2O_{\,(l)} + NaCl_{\,(aq.)}$

An equipment configuration the same as in section 3.3 was used. This time, no CO_2 was provided through sparging; instead, 2 mL of $NaHCO_3$ were added to the culture, followed by 0.5 mL $NaHCO_3$ every hour, until reaching 2 mL $NaHCO_3$. This proceeding was performed during day period of light-cycle, every day, except on days 5 and 6. The pH values were monitored periodically. Triplicate samples were analyzed daily in flow-cytometry to assess diatom and bacteria concentration in culture.

This assay lasted 11 days.

Diatom concentration in culture increased over time, while bacteria concentration kept stable (results presented in Figure 29). The doubling time obtained for diatoms (dt= 1.03 days) was lower than mean values obtained in flask culturing (dt control≈ 1.6 days). The pH values were kept between 7.0 and 8.5 during the adding of $NaHCO_3$ and HCl, with the proceeding described above. During the assay, a biofilm started to colonize the reactor's walls, which was visible with the naked eye. The biofilm got thicker trough the assay. In the last day of the assay, a sample of the biofilm was taken and observed in microscopy, which showed it was composed of diatoms and associated bacteria.

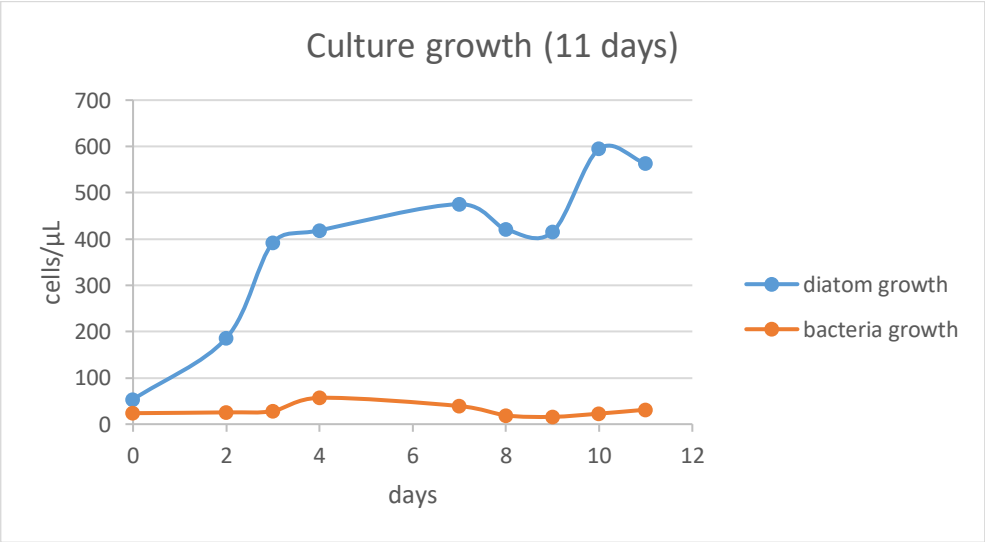


Figure 29-Diatom and bacteria growth monitoring.

This method was successful in controlling bacterial overgrowth and it did not lower pH to lethal values for diatoms. It is not clear why bacterial growth was not enhanced by aeration, as it had occurred in section 3.3. It is possible that the CO_2 generated in the chemical reaction between $NaHCO_3$ and HCl enhanced diatom growth (because these are photosynthetic organisms), and suppressed bacterial growth (because these are aerobic organisms), therefore avoiding bacterial proliferation. Also, it is possible that the pH lowerings caused by the adding of HCl affected bacterial growth negatively.

The appearance of a biofilm coating the walls of the bio-reactor was expectable, since we were working with benthic diatoms (organisms that grow attached to surfaces). We may speculate that a suitable material to be used as cell carrier, could allow to take full advantage of the reactor volume. This matter will be further developed in Chapter 4.

CHAPTER 4- FINDING A SUITABLE MATERIAL TO USE AS CELL CARRIER FOR THE CULTURE OF BENTHIC DIATOMS

4.1- Testing microcrystalline cellulose (Avicel PH-101 from Sigma-Aldrich) particles as cell carriers

An equipment configuration the same as in 3.3 was used and the proceeding described in 3.5 was performed (to keep the culture homogenous and prevent bacterial overgrowth).

In this assay, microcrystalline cellulose 0.1 % (m/V) was added to the standard culture medium. Samples were observed in microscopy and analyzed in flow cytometry, daily.

This assay lasted 9 days.

Microscopic observations showed that diatoms did not colonize the microcrystalline cellulose particles significantly. Flow cytometry readings showed that diatom growth (was not higher than one obtained in section 3.5. Furthermore, a bacterial growth higher than the one obtained in section 3.5 was also observed, which could be related to the interference of the microcrystalline cellulose particles, made visible at the same wavelength of the bacterial fluorescence. Indeed these bacteria were stained in green from Syto9 dye. Moreover, the naked eye could identify microcrystalline cellulose particles settled at the bio-reactor bottom, seconds after aeration was turned off. This suggested that the particles have low buoyancy for the desired effect.

It can be concluded the tested material is not ideal for carrying benthic diatom cells, because diatoms do not spontaneously colonize the carrier particles, and for its low buoyancy, which might lead to an underutilization of the bio-reactor.

After these results it was decided to test different materials using flask culture, to save resources and better manage the variables involved.

4.2- Testing different materials to be used as cell carriers, through experimentation in flask culture

Before starting to test different materials, a different method to monitor diatom growth had to be developed, for the subject materials are composed of particles too big to be analyzed in flow cytometry. For this reason, a correspondence between flow cytometry measurements and other analytic methods was accomplished. These tests lasted 14 days.

The first method tested was to establish a correspondence between flow cytometry values, and absorbance values of a chlorophyll extract from a sample, read in spectrophotometry. Extraction was made using acetone 80 % as solvent. Chlorophyll content of a sample should be proportional to the amount of cells present (Nestler et al., 2012). This approach did not work, because no valid correlation was found (p›0.005 ; R^2=0.699).

The second method tested was to establish a correspondence between flow cytometry values, and dry weight and ashes weight of a filtered sample (fiber glass filter 47 mm). A sample (20 mL) was filtered, and then standard protocol to evaluate organic and inorganic matter content was performed. It was concluded that the volume of sample required to have a reliable correlation (between flow cytometry values, dry weight and ashes weight) was too high (approximately 80 mL) and would lead to a waste of cells, that would affect productivity negatively.

The third method tested, was to establish a correspondence between flow cytometry values, and spectrofluorimetry readings of a sample. The full specter of a sample was first performed, being the highest peaks observed at 456 nm (excitation) and 681 nm (emission), so these wavelengths were used throughout the assay. Values obtained for emissivity at 681 nm were used to establish a correlation with flow cytometry values. The same sample was then analyzed in flow cytometry. This proceeding was used to devise a linear regression, which related flow cytometry and spectrofluorimetry values (R^2=0.907), thus allowing us to calculate cell concentration of samples containing cells carriers, which could not be analyzed in flow cytometry.

Several materials were tested as cell carriers, and an ideal material was found. This investigation is described in the following scientific paper, which was submitted to *Aquaculture* journal (presented in the next page).

Bacterial cellulose gel increases growth productivity of marine diatom *Amphora* sp.

David K. Mota, Nicolina Dias, Ana Nicolau, José Teixeira, Manuel Mota

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Abstract

Benthic diatoms are used in aquaculture as larvae feed of many commercial valued seafood, such as abalone, and high efficiency cell culture methods in bioreactors are needed to meet the increasing production demand of primary feed. As they grow better attached to surfaces, finding an ideal material for cell carrying is essential. After tests with several materials, bacterial cellulose gel was found to be ideal for carrying benthic diatom cells, because of its buoyancy, fiber size, cell affinity and biodegradability. Different concentrations of bacterial cellulose were tested and an optimal concentration was found. Using this new material avoids cell deposition at the bottom of bioreactors and the surface area available for cell attachment is increased, thus allowing to double biomass production.

Research highlights

A suitable, biodegradable material for benthic diatom cell carrying was found (Bacterial Cellulose gel), enhancing productivity over 2-fold, under the optimized conditions described.

Keywords: aquaculture; benthic diatoms; cell carrier; bacterial cellulose

1-Introduction

Diatoms of the *Amphora* species are commonly used as feed for the larvae of marine gastropods (Chiu et al., 2007), particularly the abalone *Haliotis tuberculata* (Shpigel et al., 1992). After hatching from planktonic eggs, abalone larvae go through a pelagic life stage during which dispersion and colonization of new habitats take place (De Viçose et al., 2007; Pechenik, 1999). Chemical signals produced by diatoms living on biofilms stimulate larvae settling and metamorphosis to develop morphological features which allow them to graze on diatom biofilms (post larvae benthic life stage) until the juvenile stage is reached (Morse & Morse, 1984; Slattery, 1992). From then on, abalone juveniles and adults feed on macro-algae of several species (Setyono, 2005). Producing enough diatoms to feed abalone larvae until they reach the juvenile phase is crucial for successful aquaculture production of this seafood species

(Takami, 2003). *Amphora* diatom species are benthic, meaning they grow attached to surfaces. It can be hypothesized that cell culture performance in a bioreactor may be enhanced by providing extra surface area to carry the cells (Branco et al., 2007). In this work, several materials were tested to appraise their viability as benthic diatoms cell carriers.

Cellulose biosynthesis by the bacteria *Acetobacter xylinum*, currently named *Gluconacetobacter xylinus* (Yamadaet al., 1997), was first described in 1886 by Brown (Brown, 1886), who isolated this microorganism and identified the polymer it produces as cellulose. This process was thoroughly studied since (Brown et al., 1976) for the potential of this material mechanical properties, such as mechanical strength, hydrophilicity, chirality, biodegradability, high water content, high degree of crystallinity and polymerization, and high purity (Klemm et al., 2005). Bacterial Cellulose (BC) has been used successfully as carrier for the immobilization of yeast (Yao et al., 2011) and bacteria cells (Rezaee et al., 2008). The goal of the present work was to appraise the possibility of increasing *Amphora* sp. culture productivity by using BC gel under optimized conditions.

2-Materials and Methods

2.1- Preliminary tests were performed to select a suitable material to be used as cell carrier. The following materials were tested:

2.1.1- Microcrystalline cellulose (Avicel PH-101 from Sigma-Aldrich) in a concentration of 1 g/L.

2.1.2- Glass fiber tatters (Motip, 300 g/m^2). In this work, tatters sized approximately 12 mm^3 were mixed in the standard culture medium; it was used in a concentration of 10 g/L.

2.1.3- Bacterial cellulose paste (Satisfibre, Braga, Portugal) was used in a concentration of 10 g/L.

2.1.4- Bacterial cellulose gel (Satisfibre, Braga, Portugal). This material has the same composition as BC paste, in gel form. Concentrations of 10, 20 and 50 g/L were tested.

2.2- Cell culture

Amphora sp. diatom cultures were obtained from the Culture Collection of Algae and Protozoa, Scottish Marine Institute, Scotland, United Kingdom. Cultures usually contain bacteria, coming from environmental samples used to isolate diatom strains, whose concentration can be measured by flow cytometry. Orange Scientific 175 cm^3 tissue culture flasks (sterile), stirred at 40 rpm on an orbital shaker (Sky Line shaker DOS-20L), were used to incubate all cultures.

Lighting was provided by two lamps (Philips Master t5 14W/840), timed 12h/12h light/dark periods by an electric timer (CYLLUX ELECTRIC). The cultures were kept inside a thermalized cabinet (T≅20°C) with air conditioner (Panasonic Inverter ECONAVI).

2.3- Culture medium

F2 medium (Guillard & Rhyther, 1962) was used. Natural seawater was collected during the high tide in the coordinates 41°29'06.6"N 8°46'51.5"W, filtered (0.2 µm pore size) and sterilized in an autoclave.

2.4-Cell density assessment

A calibration curve was made to measure cell density in the Spectrofluorimeter (HORIBA AQUALOG 800, Horiba ABX S.A.S. Portugal Branch, Amadora, Portugal) relating its readings with those of a EC800 Flow Cytometry Analyser (Sony Biotechnology Inc., Champaign, IL, USA). Spectrofluorimetry was used instead of flow cytometry in this experiment because cell carrier particles potentially damage the flow cytometer pipes. For this reason, cell concentration was calculated from the spectrofluorimetry readings, using a linear regression, previously devised, which related spectrofluorimetry and cytometry readings (R^2=0.907). The full specter of a culture sample was first performed, being the highest peaks observed at 456 nm (excitation) and 681 nm (emission). These wavelengths were used throughout the assay. A control was used containing culture medium, each with the different concentrations of BC, to take in consideration its contribution to the emissivity of the samples. Cytometry and fluorimetry readings were used to assess culture growth over 8 days, until plateau phase was reached.

2.5- Culture preparation

Several sub-cultures were prepared, one week in advance, so that when the assay started, these were in exponential growth phase. Using cytometry readings, cell concentration was adjusted until readings were between 200 and 400 counts/µL.

2.6- Cytometry protocol

Cell concentration of Amphora sp. in cell cultures was determined by flow cytometry with an EC800 Flow Cytometry Analyser (Sony Biotechnology Inc., Champaign, IL, USA) equipped with an argon-blue laser (488 nm). Autofluorescence was used as a discriminating characteristic

to detect the diatom, and the signal was detected on channel FL3 (BP 665/30 nm). A flow rate of 40 µL/min, with a sample volume of 50 µL and a maximum of 15000 counts/sample were established as setting parameters for acquisition. The mean value ± standard deviation was obtained. Analysis of data was performed on the EC800 software version 1.3.6. (Sony Biotechnology Inc., Champaign, IL, USA). Diatom cultures form cell clumps, so it is necessary to disperse diatom aggregates, to prevent cytometry readings biasing. To do so, Eppendorf tube containing sample was vortexed (15 seconds), sonicated in ultrasonic bath (30 seconds), vortexed again and read immediately, to prevent cells from settling. This method was performed for each 0.6 mL sample (triplicates).

2.7- Fluorimetry protocol

The sample in an Eppendorf tube was vortexed and 0.45 mL were transferred to the fluorimetry cuvette. The same was done with triplicates. Then cuvette was inserted in the spectrofluorimeter and the reading was made. Between each reading the cuvette was moved up and down to re-suspend the cells, and inserted in the spectrofluorimeter to take another reading. Three readings were made for each sample and the average value was calculated. This method was carried on in triplicates.

2.8- Microscopic observations

Samples from preliminary tests with different materials (microcrystalline cellulose, fiber glass tatters, bacterial cellulose paste) were observed in epifluorescence microscopy (using an Olympus BX51 microscope) coupled with a DP71 digital camera and three sets of filters (DAPI – 360-370/420; FITC – 470-490/516; and TRITC – 530-550/590) (Olympus Portugal SA, Porto, Portugal). Samples containing microcrystalline cellulose were dyed blue with calcofluor. All images were acquired using the Olympus CellSens software. Samples containing BC gel were observed using a Confocal Scanning Laser Microscope (Olympus BX61, Model FluoView 1000). Calcofluor was used for detection of cellulose fibers (laser excitation line 405nm and emissions filters BA 430–470); Syto9 was used for detection of bacteria (laser excitation line 488 nm and emissions filters BA 505–540 nm) and auto-fluorescence (laser excitation line 559 nm and emissions filters BA 575–675 nm) for *Amphora* sp. cells. Images were acquired with 1024 x 1024 resolutions using the program FV10-ASW 4.2.1.20 (Olympus). Selected images were presented either as single confocal optical sections or maximum intensity type constructions.

3- Results

The following results were obtained for each material tested as cell carrier:

3.1- Microcrystalline cellulose. Microscopic observation (on day 5) showed that diatoms do not have high affinity to this material, growing unattached around the particles (Figure 1, AI). Also, experimentation showed that it has poor buoyancy and sinks to the bottom of the culture medium;

3.2- Glass fiber tatters. Microscopic observation (on day 5) shows that diatom cells have high affinity to this material, spontaneously attaching to the glass fibers (Figure 1, AII). However, this material is not biodegradable and its buoyancy keeps the tatters submerged immediately under the surface of the culture medium;

3.3- Bacterial cellulose paste (BC paste). This material proved to have excellent buoyancy, remaining suspended in the culture medium for long periods (more than 2 hours) even without stirring. Also, diatoms spontaneously grow interspersed in the cellulose fibers after 1 day growth (Figure 1, AIII). It is a biodegradable material but the large size of the fibers makes the suspension inhomogeneous, disturbing fluorimetry measurements.

3.4- Bacterial cellulose gel (BC gel). Having the same composition as the BC paste, but with smaller fibers, it produces a homogeneous suspension where the cells grow, trapped between the cellulose fibers (Figures 1, BI, BII, BIII, BIV). This material has all the advantages of the previously used BC paste. These suspensions elicit more reliable fluorimetry readings, due to the more homogeneous cell distribution in the culture medium, when compared to the obtained with BC paste.

Because BC gel proved to have the desired characteristics to be used as diatom cell carrier, its concentration in culture medium was optimized.

The presence of BC gel in the culture medium enhanced cultures specific growth rate (μ, days-1) and reduced doubling time (dt, days) when comparing to the control. Moreover, BC gel enhances biomass production significantly, which was more than 2-fold higher than in the control, in the flasks containing 50 g/L BC gel concentration. Results are presented in Table 1. Culture growth is presented in Figure 2. Variations on BC gel concentration in the culture medium resulted in different growth rates. Higher BC gel concentration resulted in faster initial growth and higher maximum cell concentration. Results are presented in Table 2. Above 50 g/L, no improvement was observed (results not shown).

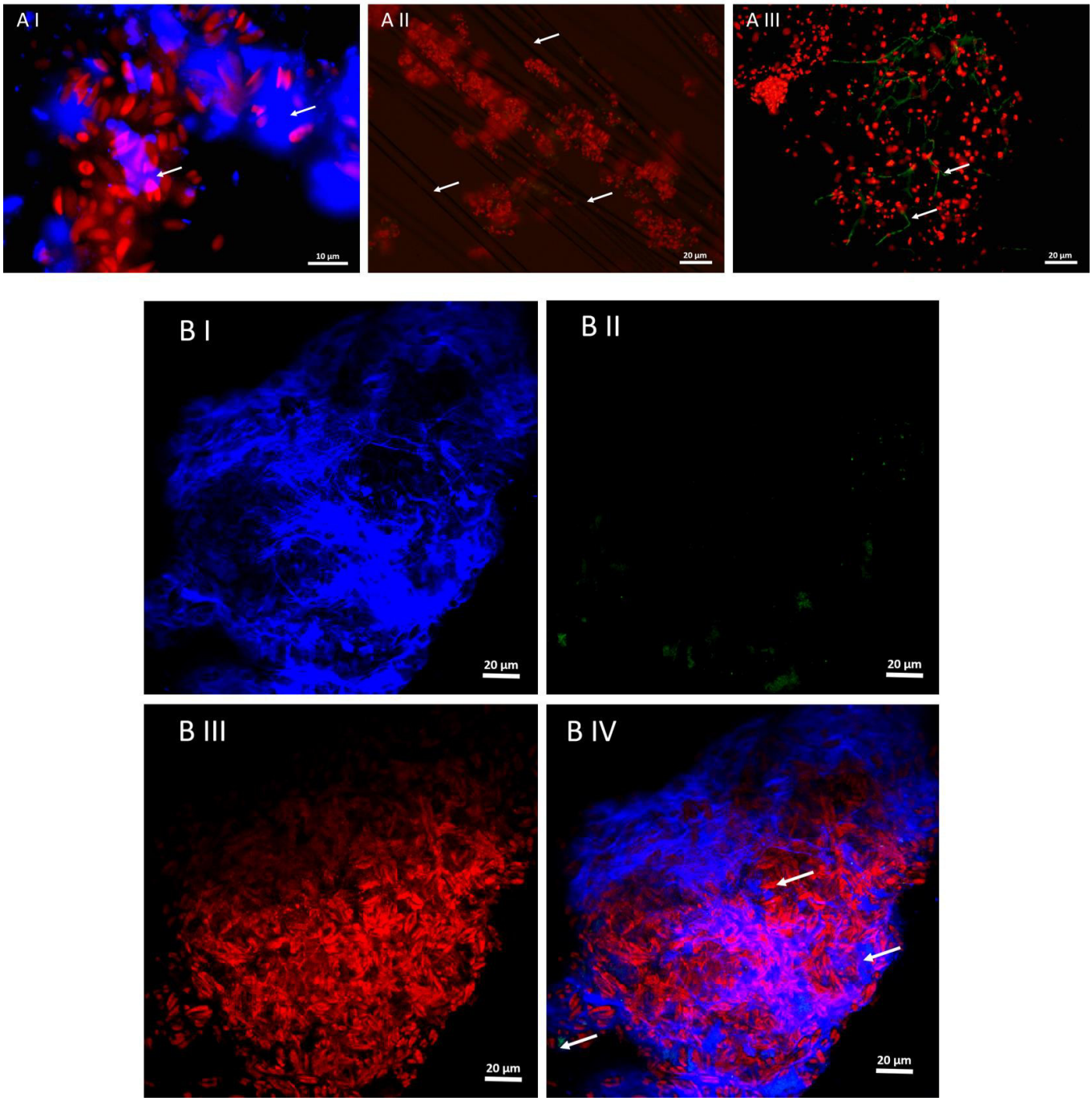

Figure 1-Amphora sp. diatoms growth on different materials. The scale bars represent the magnification. (A) Epifluorescence images of (arrows indicate the material): (AI) Diatom cells (red from auto-fluorescence) in culture medium containing microcrystalline cellulose (dyed blue with calcofluor) (AII) Diatom cells (red from auto-fluorescence) in culture medium containing glass fiber tatters (AIII) Diatom cells (red from auto-fluorescence) in culture medium containing BC paste (green from auto-fluorescence). (B) CLSM images of: (BI) BC gel particles (dyed in blue with calcofluor) in the blue channel (BII) Bacteria (dyed green with SYTO9) in the green channel (BIII) Diatom cells (red from auto-fluorescence) in the red channel (IV) Overlapping of the blue (right arrow), green (left arrow) and red (central arrow) channels.

Table 1-Performance of different BC gel concentrations.

BC gel concentration in culture medium (g/L)	μ (days-1)	dt (days)	Increase in μ compared to Control (%)	Increase in maximum biomass (cells/μL) T5-T0	Increase in maximum biomass compared to Control (%)
0 (Control)	0,276	2,5114	-	704	-
10	0,3175	2,18314	+15,0	1324	+88,1
20	0,3155	2,19698	+14,3	1325	+88,2
50	0,3378	2,05195	+22,4	1456	+106,8

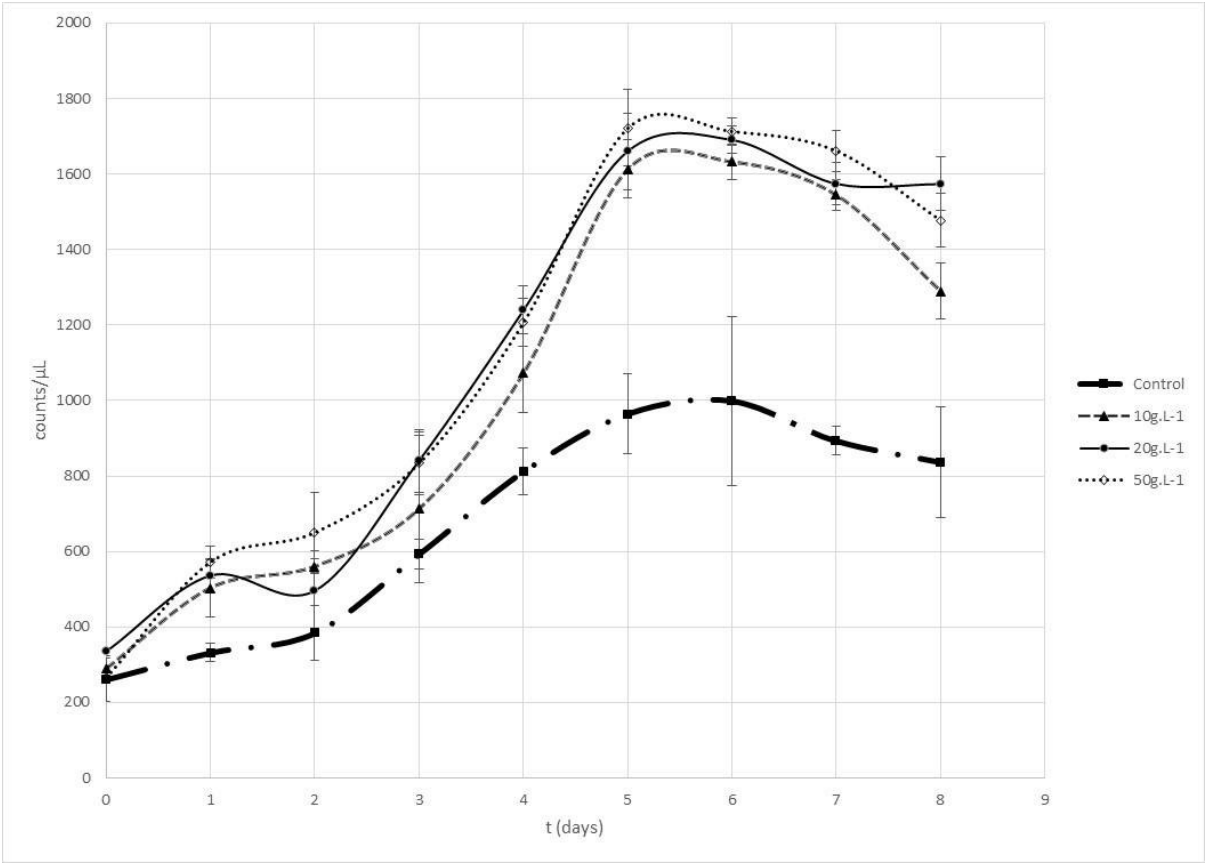

Figure 2-Cultures growing (8days) on media containing increasing BC gel concentrations.

Table 2-Cell concentration of different culture conditions over time (cells/µL).

T (days)	BC gel concentration in culture medium (g/L)			
	0 (Control)	10	20	50
0	260,7679	288,9102	334,9069	265,9969
1	331,9923	502,858	536,1674	572,3587
2	384,2989	559,7717	496,1428	650,3935
3	593,3141	712,9379	840,5646	833,7979
4	811,8601	1072,392	1238,893	1206,551
5	964,7981	1613,239	1660,239	1721,769
6	999,0143	1632,597	1690,32	1712,879
7	893,3069	1544,519	1574,282	1661,263
8	836,5021	1289,324	1573,43	1477,257

4-Discussion

Poor buoyancy and low cell affinity of microcrystalline cellulose lead to an underutilization of the available culture volume. This type of material has been used successfully to immobilize yeast cells (Sakurai et al., 2000), so it is possible that different blends of microcrystalline cellulose would have better performance.

Microalgae have been successfully immobilized before in fiber glass matrices (Lauravichene et al., 2016). However, glass fiber buoyancy is not ideal for taking advantage of the culture volume, for the tatters concentrate immediately under the surface, leaving the rest of the reactor's volume underused. Furthermore, the size of the tatters used makes fluorimetry readings unreliable, for it makes it impossible to obtain a homogenous sample. This problem extends to cytometry and traditional cell counting methods. Because of the nature of this material, trying to make smaller tatters through trituration or shredding would be dangerous, because these fibers can be inhaled and damage respiratory system. Also, this being a non-biodegradable material can complicate waste management, on an industrial level. After the larval phase, juvenile abalones feed on macro-algae, so juveniles have to be transferred to different culture tanks. Because abalone larvae will spontaneously attach to the diatom film, fiber glass tatters can hamper this transfer, for the tatters are bigger than larvae. The use of smaller, biodegradable carriers enables the separation of larvae from carriers by filtration, and used carriers can be disposed in common waste containers.

BC has been used as yeast cell carriers with good results (Kirdponpattara & Phisalaphong, 2013). The buoyancy of BC paste enables it to spread through all the culture medium available in a flask or bio-reactor, thus taking full advantage of the available culture volume. The only flaw are biased fluorimetry readings due to the inhomogeneous suspensions produced. It is possible that grinding BC paste dissolved in culture medium would produce more homogenous suspensions. However, the use of BC gel overcame this constraint. BC gel concentrations higher than 50 g/L were tested in separate trials, but the growth rates achieved were not higher. This may be due to high BC concentrations blocking light from reaching the cells.

5-Conclusion

BC gel is an ideal material to be used as cell carrier for the growth of *Amphora* sp., since the main goal of diatom production for marine larvae feed is to maximize the biomass primary production. It is likely that this support might also be useful for the enhanced growth of other kinds of benthic diatoms. This method may significantly contribute to ensure that aquaculture production has enough larva feed for a wide range of seafood. The buoyancy of BC gel allows its use in bio-reactor cell culture, to take full advantage of the reactor available volume for cell attachment. Moreover, the homogenous suspensions produced in culture medium allow easy and reliable assessments of culture growth, through fluorimetry. Finally, its biodegradability and particle size facilitate downstream aquaculture processes and waste management.

6- Acknowledgements

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CHAPTER 5- DIATOM CULTURE PRESERVATION THROUGH IMMOBILIZATION IN LATEX COATINGS

Preservation of diatoms cultures would facilitate technician's work on culture collections, for the most common method used now is preservation through refrigeration (4 ºC), which is not very efficient preserving cultures viability. Also, this method do not preserve cultures for long periods of time (Montaini et. al, 1995). This creates the necessity of, periodically reactivating and re-cultivating preserved cultures, to perform preservation techniques again, and this way keep viable specimens permanently.

In aquaculture industries, high volumes of plankton (frequently composed of diatoms) are required to meet production needs. It would be useful if an efficient preservation technique was developed, enabling the storage of high biomass amounts, thus preventing shortages that would affect productivity.

It is clear that more efficient preservation methods should be developed, so in this work, an attempt was made to develop a preservation method, consisting of immobilizing diatom cultures in a latex matrix, as it was used before with other microorganisms (Figure 1).

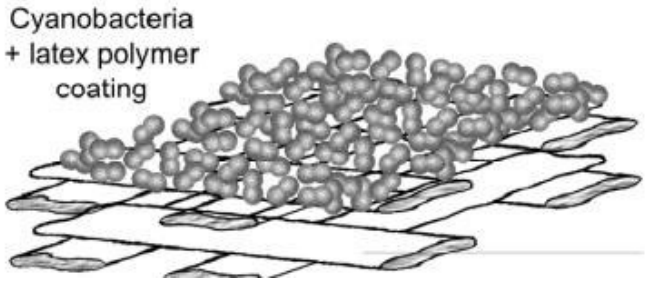

Figure 1-Cyanobacteria immobilized in latex coating (Bernal et al., 2014)

A culture (800 mL) was analyzed in flow cytometry to assess cell concentration, and then divided into twenty equal parts. Ten control cultures were centrifuged, supernatant discarded, and pellet containing cells was re-suspended in 3mL of a sterile saline solution (NaCl 30% m/V).

Ten cultures were treated following the protocol described in Annex 2.

All cultures were stored in the dark at 4 ºC.

After 3 months storage, one control culture was reactivated, by inoculation in fresh culture medium and incubation at standard conditions. After 4 days, the culture was observed in microscopy and diatom concentration was assessed through flow cytometry. Culture's viability had decreased 50 %, and the live cells appeared to be in good morphological conditions. One immobilized culture (in coating) was also reactivated, by inserting the coating into a culture flask containing fresh culture medium, incubated at standard conditions. It would be expected that the diatoms inside the latex matrix would come out of the coating to access the nutrients in the fresh culture medium, however, after 4 days incubation, microscopic and cytometry analysis showed that no diatoms were present in the culture medium. This can be due to the latex matrix produced having pores too small for diatoms to come out of the coating. The coating was cut with a scalpel, scraped with a cell-scraper and its content observed in microscopy, confirming that there were live diatoms inside the coating. This suggests that it might be possible to formulate a variation of the latex matrix used, with larger pores, which would let diatom cells out, at the time of reactivation. The pores in the latex matrix are produced by adding sucrose to a latex solution, which solidifies creating inclusions inside the matrix. Later, the sucrose is dissolved, at the time of culture reactivation, creating pores in the sites of inclusions. The size of the pores is determined by sucrose concentration, so it should be possible to create larger pores by enhancing it, thus allowing diatoms to escape from immobilization, when the coating is immersed in culture medium. This is a promising approach that should be investigated, however, it would consume time, which was needed to investigate culture medium optimization, since this might lead to an increase in productivity, greater that the one possibly achieved by preservation. For this reason, development of preservation methods was suspended, and the work on culture medium optimization was started, which would lead to successful results.

CHAPTER 6- CULTURE MEDIUM OPTIMIZATION

As it was referred in the Introduction, productivity of diatom culture can be enhanced through manipulation of culture media formulations. Particularly on diatoms, this could be achieved by optimizing silica and nitrates concentrations in culture medium.

Silica is the main constituent of diatoms cell wall, and nitrates are used in protein synthesis, so it is likely that diatoms growth can be enhanced by providing optimized concentrations of these nutrients to the cells, hence boosting productivity.

This was tested by formulating culture media containing different combinations of concentrations for these nutrients. The combinations tested were determined using Response surface methodology (RSM), which is a valid strategy to accurately predict the complex influences of multiple variables in a predetermined response, by the development of a statistical model fitting the experimental data (Box & Draper, 1987, Reid, 2003). This approach was used to design the experiments of media optimization, and to interpret the derived data. This investigation originated a scientific paper, in which the referred procedure in further described (presented next).

This method was first applied to *Amphora* sp. diatoms, and the optimized medium discovered did increased biomass production by 1.65-fold, compared to control values, however, these results were not statistically significant ($p \rangle 0.05$).

Applying this method to *Navicula* sp. diatoms increased biomass production 3.5-fold, with statistically valid results, so a scientific paper was written on the subject, and submitted to *Algal Research* journal (presented in the next page).

Culture medium optimization to grow marine diatom *Navicula* sp.

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Abstract

Diatoms of the *Navicula* species are widely used as larvae feed in aquaculture for the production of many valued seafood species. Enhancing diatoms biomass is crucial to meet the ever-growing production needs in this industry. Culture medium composition affects culture growth and its optimal formulation varies with the diatom species. Based on the standard culture medium for marine diatoms optimal concentrations of nitrate and silica were found for *Navicula* sp. Different concentrations of these nutrients were tested using a design of experiments that enabled to find out a response surface, which allowed the construction of a second order model for *Navicula* growth. The optimized formulation led to a 2-fold increase of the obtained biomass-for *Navicula* sp.

Keywords: *Navicula* sp., response surface methodology, biomass optimization, nitrate, silica, diatom

1. Introduction

Diatoms of the *Navicula* species are commonly cultured at industrial scale in aquaculture production units, to be used as primary food for larvae of many commercially valued seafood species, such as *Penaeus monodon* (Asian tiger shrimp), *Crepidula onyx* (limpet), *Venerupis philippinarum* (clam) and *Haliotis* sp. (abalone), among others (Kanaya et al., 2005, Chiu et al., 2007, Khatoon et al. 2009, Keun & Bum, 2011). As aquaculture production is steadily increasing since the second half of the 20th century, techniques to enhance the productivity of these diatoms are needed (Admiraal & Peletier, 1979, Csavina et al., 2011, Nguyen-Deroche et al., 2012, FAO, 2014). *Navicula* sp. is among the most used diatoms to feed the post-larvae of abalone, sea snails of the *Haliotis* genus which are highly prized in gourmet cuisine (Cox, 1962, Shpigel et al., 1996, Daume et al., 2003, Ko & Hur, 2011). The objective of this work is to appraise the possibility of enhancing the biomass productivity of *Navicula* sp. cultures by

optimizing the concentration of two main nutrients, present in the standard culture medium for marine diatoms (Guillard's f/2+Si culture medium), namely,nitrate and silica. These essential nutrients are usually present in the culture medium of marine diatoms under the form of sodium nitrate ($NaNO_3$) and sodium metasilicate ($Na_2SiO_3.9H_2O$). Nitrates are required for the synthesis of amino acids and silica is a crucial component of the frustule (siliceous cell wall). Response surface methodology (RSM) is a valid strategy to accurately predict the complex influences of multiple variables in a predetermined response, by the development of a statistical model fitting the experimental data (Box & Draper, 1987, Reid, 2013). RSM allows an important reduction of experimental time and costs through the application of design of experiments (DoE), making it a widely used approach to optimize processes in several industries, such as, biotechnological, pharmaceutical, chemical, automotive and aerospace (Myers et al., 2016). DoE is used to screen the effect of pinpointed variables that are considered to have an important influence on the response. In this work, a central composite design (CCD) was used to determine the optimal value of nitrate and silica in the culture medium of *Navicula* sp., with the objective of maximizing its biomass yield (Box & Wilson, 1992, Montgomery, 1997)

2. Materials and Methods

2.1 Culture preparation and maintenance

The optimal concentration of nitrate and silica was evaluated at different levels of these two essential nutrients in the standard culture medium for marine diatoms (Guillard's f/2+Si). Seawater to prepare culture media was collected during the high tide in 41°29'06.6"N 8°46'51.5"W. After its collection, seawater was filtered (0.2 µm pore size) and sterilized in an autoclave (121 °C for 15 minutes at 1.2 Bar). The standard f/2+Si recipe, described by Guillard (Guillard, 1975) comprised the following components per litre: 75 mg sodium nitrate (Panreac), 5.65 mg sodium di-hydrogen phosphate dihydrate (Panreac), 3.15 mg iron chloride hexahydrate (Fluka), 10 µg copper (II) sulphate pentahydrate (Merck), 4.16 mg ethylenediaminetetraacetic acid disodium salt (Panreac), 22 µg zinc sulphate heptahydrate (Fluka), 180 µg manganese (II) chloride tetrahydrate (Fluka), 10 µg cobalt (II) chloride hexahydrate (Fluka), 6 µg sodium molybdate dihydrate (Merck), 30 mg sodium metasilicate monahydrate (Sigma), 0.5 µL cyanocobalamin (Sigma-Aldrich), 100 µg thiamine hydrochloride (AppliChem), 0.5 µg biotin pure (AppliChem). A precision scale (KERN ABJ-

NM/ABS-N) was used to weigh all compounds used. Several sub-cultures were prepared, one week prior to their use as inocula, so that when the assay began, *Navicula* sp. was at the exponential growth phase. All the experimental runs were inoculated with approximately 300 cells/µL, in 10 mL of culture medium, poured in a 25 cm^3 vent cap tissue culture flasks (Orange Scientific). All cultures were incubated at orbital shakers (Sky Line) set to 60 rpm, in a thermalized cabinet by an air conditioner (Panasonic), where the temperature was maintained at 20°C. Lighting was provided by two lamps (Philips Master t5 14W/840), in 12h/12h light/dark periods controlled by an electric timer (CYLLUX ELECTRIC). The incubation period lasted 3 days.

2.2 Sample collection, cytometry and fluorimetry readings

Navicula sp. cells form clusters and these diatoms tend to adhere to the flask walls due to their benthic nature. Therefore an effective detachment and cell homogenization process is essential (Targett et al., 1983). All runs and samples prior to their collection were submitted to 30 seconds of cavitation in a ultra-sonic bath (Bandelin Electronic U≅230V, V≅50/60Hz, I≅1,4A, P≅140/560W, f:35kHz), and a sterile cell scraper(Sigma) was used when required. The cultures were permanently handled under aseptic conditions using a vertical flow chamber (Nüve LN090). The diatom cell concentration was measured using a flow cytometer analyser, equipped with an argon-blue LASER (488 nm) (EC800, Sony Biotechnology Inc.). A spectroflurimeter (HORIBA AQUALOG 800) and a quartz cell (QS High Precision cell, Art. Nº 140-F-10-40, Hellma Analytics) were used to assess the cultures emissivity (proportional to diatom cell concentration, through a regression line previously devised, relating cytometry and fluorimetry readings (data not shown)). The wavelengths used throughout the assay were 426 nm (excitation) and 681 nm (emission). These wavelengths correspond to the highest peaks usually observed in full spectra of a sample. A vortex mixer (Stuart Scientific SA3) was used to stir all samples before each reading. Cell concentration of all culture flasks was assessed at inoculation (T_0) and after three days of incubation (T_3) by fluorimetry. Two 0.3 mL samples were analysed. For the analysis by flow cytometry, auto fluorescence was used as a discriminating characteristic of the diatoms, and its signal was detected on channel FL3 (BP 665/30 nm). A flow rate of 40 µL/min, with a sample volume of 50 µL and a maximum of 15000 counts/sample were established as setting parameters for acquisition. The mean value ± standard deviation was obtained. Analysis of data was performed on the EC800 software version 1.3.6 (Sony Biotechnology Inc.). Prior to each flow cytometer run, to prevent cytometry

readings biasing due to diatom clustering, the Eppendorf tube containing the sample was vortexed (15 seconds), submerged in an ultrasonic bath (30 seconds), vortexed again and immediately read, to prevent cells from settling.

2.3 Statistical analysis

2.3.1-Response Surface methodology – Central Composite Design

Prior to the establishment of the range of nitrate and silica values to be used in a response surface methodology (RSM), a screening was performed using 0.5, 1, 2, 5 and 10 fold higher concentrations of nitrate and silica than of the standard culture medium. The Design Expert 7 (Stat-Ease Inc.) software was used to establish the experimental design and to perform the statistical analysis of the model. CCD (Central Composite Design) allows the construction of a second-order model, since it consists in a first-order design augmented by axial points which confer rotatability and expand the design's analysis range (Montgomery, 1997, Myers et al., 2016). This design possess 2 variables, x_1: nitrate and x_2: silica, thus the axial points (represented by the α character) comprise the coded factor value of 1.41 (Myers et al., 2016). The model response was determined as *Navicula* sp. biomass production estimated by the ratio between the cell concentration (cell/µL) obtained after 3 days of incubation and the initial cell concentration. The experimental design involved 21 runs comprising 5 replicates of the centre point, 2 replicates of each factorial level and 2 replicates of the axial points. A quadratic equation model (Eq. 1) was used to evaluate the biomass concentration ratio:

$$Y_i = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij}\, x_i x_j \qquad \text{Eq.1}$$

where, Yi is the response, β0 is a constant, βi corresponds to the linear coefficient, βii to the quadratic coefficient, βij the interactive coefficient and xi represents the variable. The response was fitted through a polynomial analysis, and the design's statistical significance value was $p < 0.05$. Table 1 displays the nitrate and silica concentrations and respective codded factors used in the CCD. The obtained model's performance may be evaluated by several parameters, such as the predicted sum of squares (PRESS), coefficient of determination (R2), absolute average deviation (ADD), adjusted coefficient of determination (R2adj.), adequate precision (AP), and coefficient of variation (CV).

The stationary point of the model was determined by using equation 2 (Myers et al., 2016):

$$x_s = -\frac{1}{2}\widehat{B}^{-1}b \qquad \text{Eq. 2}$$

Where x_s is the stationary pointy, b and $\widehat{B}$ correspond to the linear and second-order coefficients respectively.

Table 1 – Experimental variables, levels and concentrations used in the central composite design.

Variable	**Parameter value**				
Coded	-α	-1	0	+1	+α
x_1 : Nitrate (g/L)	0.30	0.50	1	1.5	1.71
x_2 : Silica (g/L)	0.03	0.11	0.32	0.52	0.60

2.3.2- Model confirmation

Using the same culture conditions as described previously, a confirmation of the optimized culture medium recipe was performed, by simultaneously incubating 5 flasks with the improved medium and 5 flasks with the standard f/2+Si medium. The statistical analysis of the confirmation consisted in a t-test with a 95 % confidence interval, performed using software GraphPad Prism 5 (Graph Pad).

3. Results and discussion

A CCD was used to optimize the standard marine diatoms culture medium by tuning the nitrate and silica concentrations to promote a higher *Navicula* sp. biomass output. Table 2 displays the design matrix, the experimentally obtained cell concentration ratio (t3/t0) and its predicted value by the model.

Table 2 – Central composite design matrix, comprising the experimental runs, variables and obtained response values and respective predicted values.

	Variable coded factor (numeric factor g/L)		**Response (Cell ratio)**	
Run	**x_1 : Nitrate**	**x_2 : Silica**	**Observed**	**Predicted**
1	0 (1.00)	0 (0.32)	2.17	2.40
2	+1 (1.50)	-1 (0.11)	1.90	1.68
3	0 (1.00)	+α (0.60)	2.64	1.77
4	-α (0.30)	0 (0.32)	1.90	1.91
5	0 (1.00)	+α (0.60)	1.82	1.77
6	+α (1.71)	0 (0.32)	1.89	2.07

7	0 (1.00)	-α (0.03)	1.14	1.40
8	0 (1.00)	-α (0.03)	1.28	1.40
9	0 (1.00)	0 (0.32)	2.25	2.40
10	+1 (1.50)	+1 (0.52)	1.86	2.00
11	-α (0.30)	0 (0.32)	1.81	1.91
12	-1 (0.50)	-1 (0.11)	1.85	1.62
13	0 (1.00)	0 (0.32)	2.67	2.40
14	0 (1.00)	0 (0.32)	2.71	2.40
15	-1 (0.50)	-1 (0.11)	1.66	1.62
16	+1 (1.50)	-1 (0.11)	1.83	1.68
17	+1 (1.50)	+1 (0.52)	2.22	2.00
18	+α (1.71)	0 (0.32)	2.02	2.07
19	-1 (0.50)	+1 (0.52)	1.97	1.82
20	0 (1.00)	0 (0.32)	2.18	2.40
21	-1 (0.50)	+1 (0.52)	1.63	1.82

Run 3 was considered as an outlier and it was discarded from all model analysis. Statistical multiple regression analysis of the experimentally obtained biomass ratio (t_3/t_0) values shown in Table 2 allowed the achievement of a second-order polynomial equation which represents the model in actual factors (Eq. 3):

$$Biomass\ ratio\ = 0.35\ + 1.68\ x_1 + 6.64\ x_2 + 0.32 x_1 x_2 - 0.083 x_1^2 - 10.01 x_2^2 \quad \text{Eq. 3}$$

The model's analysis of variance (ANOVA) results (Table 3) indicate that its representation of the experimentally collected data is highly significant ($p < 0.001$), thereby proving its validity. Nitrate concentration affects biomass response, considering the significance of its quadratic term (x12 $p < 0.01$). On the other hand, silica concentration has a key role in the cell concentration ratio, as both linear and quadratic terms of silica concentration have a significant impact on the response (x2 - $p < 0.05$ and x22 - $p < 0.0001$, respectively). Diatom proliferation requires the generation of new frustules, which are mainly composed of silica, which corroborates this results. Additionally, there is an indication of absence of interaction between

the two studied variables, due to the non-significant p value of the interaction term (x1x2) ($p > 0.005$).

Table 3 – Central Composite Design (CCD) analysis of variance (ANOVA) parameters of the quadratic model for the cell concentration ratio production. "x_1" corresponds to nitrate, "x_2" to silica and "*" indicates the significant model terms.

	Sum of squares	Degree of freedom	Mean square	F value	*p* value
Model	2.07	5	0.41	8.72	0.0006*
x_1	0.06	1	0.06	1.27	0.2788
x_2	0.23	1	0.23	4.92	0.0436*
x_1x_2	0.01	1	0.01	0.17	0.6839
x_1^2	0.43	1	0.43	9.06	0.0094*
x_2^2	1.51	1	1.51	31.79	< 0.0001*
Residual	0.66	14	0.05	-	-
Lack of fit	0.21	3	0.07	1.69	0.2274
Pure error	0.45	11	0.04	-	-
Total	2.73	19	-	-	-

The tests to verify the models adequacy can be assessed by the lack of fit (Table 3), as well as important statistical evaluation factors displayed in Table 4. The model PRESS value, a value that measures how the model fits each experimental point is 1.28. The R^2 is calculated from the PRESS value (Baş &Boyaci, 2007) . Usually, the obtained R^2 value should be close to unity to present a good agreement between the predicted data and the experimental results. However, in the case of second-order models, a high value of R^2 does not inevitably imply a good adequacy of the model (Myers et al., 2016). AAD is an additional test that may enlighten the fitness of the model, which must be as low as possible (Baş & Boyaci, 2007). This model presents an AAD lower than 10 %. The R^2adj. consists on a R^2 more adequate in comparing models with independent variables, and it differs from the R^2 due to the existence of non-significant parameters, namely the interaction (x_1x_2) and the linear nitrate (x_1) terms (Myers et al., 2016). AP comprises the value of the distance between the predicted response relatively to its associated error (signal to noise ratio), and is considered as a certified signal if it is higher than 4. This model's AP is more than twofold above the adequacy threshold (Barbosa et al., 2016).

The precision and repeatability of the experimental design is expressed by the CV value, which in this model is 11.24 % (Beg et al., 2003, Barbosa et al., 2016).

Table 4 – Model adequacy values, the standard deviation (SD), prediction error sum of squares (PRESS), coefficient of determination (R^2), absolute average deviation (ADD), adjusted coefficient of determination (R^2adj.), adequate precision (AP) and coefficient of variation (CV).

SD	0.22
PRESS	1.28
R^2	0.76
AAD (%)	9.52
R^2adj.	0.67
AP	8.38
CV (%)	11.24

The model's graphical representation is shown in Figure 1, where the peak is near the centre of the experimental design space, thus the optimum biomass ratio is clearly within the design borders. The stationary point was calculated using Eq.2 and resulted in the values of x_1 = 1.07 and x_2 = 0.32.

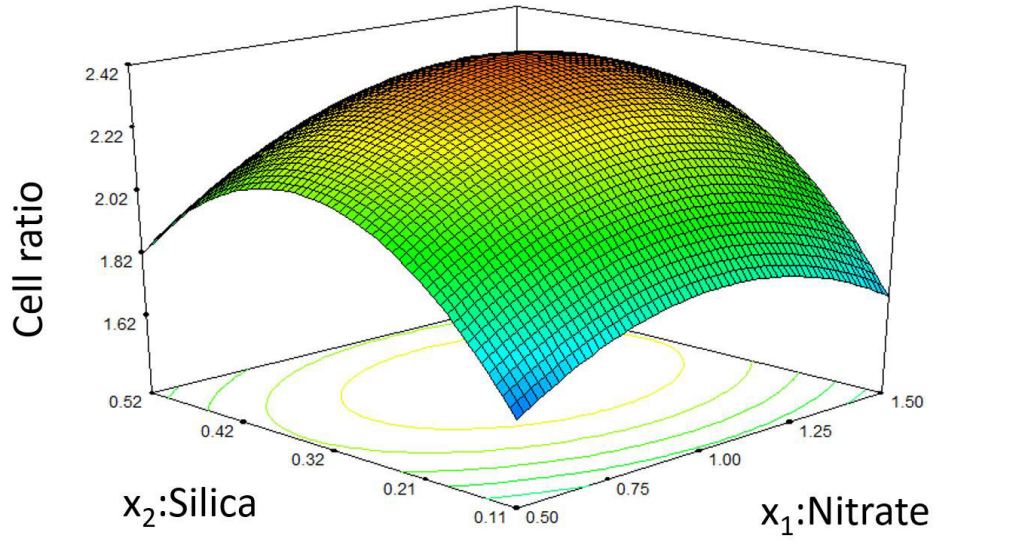

Figure 1 – *Navicula* sp. cell concentration ratio model response graph.

Following the construction of a valid model, the nitrate and silica concentration values to be used in the culture medium formulation, to achieve a maximum cell ratio of *Navicula* sp., were estimated. The optimum f/2+Si formulation for *Navicula* sp. is 1.07 g/L of nitrate (x_1) and 0.32 g/L (x_2) of silica. These values correspond to an increase of 14.3 fold and 10.7 fold on the

concentration of nitrate and silica respectively, in comparison to the standard values. Moreover, the optimal values are equivalent to the results obtained for the stationary point, meaning that it represents the maximum value.

The optimized formulation of the f/2+Si medium was then assayed on *Navicula* sp. cultures and compared to cultures incubated in standard f/2+Si (control). Figure 2 displays the cell concentration ratio obtained by the *Navicula* sp. cultured in f/2+Si and in the optimized formulation. The cell ratio obtained by the optimized medium was 3.21 ± 0.57, which represents a significant improvement when compared to the 1.72 ± 0.21 obtained in the control medium ($p < 0.001$). It represents a 1.87-fold increase. The maximum predicted cell ratio in the second order model was of 2.4, which may be considered as in reasonable agreement with the experimentally obtained, since its lower 95 % confidence limit of the mean value is 2.50.

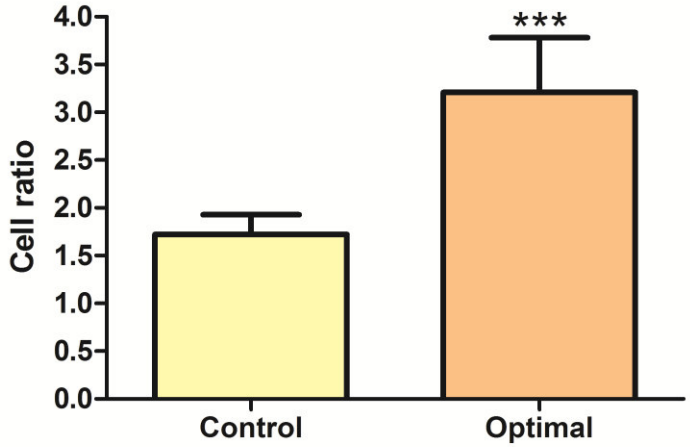

Figure 2 – Obtained cell concentration ratio when incubating *Navicula* sp. in standard f/2+Si culture medium and in the optimized f/2+Si using the central composite design (CCD). The "***" represent a statistical significant difference ($p < 0.001$) by using t-test (n = 5). The results are presented as average ± standard deviation.

4. Conclusion

The culture medium f/2+Si is the standard medium for the culture of marine diatoms but it may be optimized to further improve the culture of a group or even a single diatom species. A RSM was employed to enhance the cell concentration of *Navicula* sp., an important feed stock for economical relevant sea snails. Nitrate and silica concentrations were the optimized variables. Silica concentration displayed an essential importance for the culture of these diatoms, reflected on the optimized medium formulation with an increase of approximately 11-fold on its value. Nitrate did not present such a high importance, yet its optimized concentration requires an increase of approximately 14.3-fold when compared to its standard formulation. The optimized

Navicula sp. f/2+Si medium contains 1.07 g/L of nitrate and 0.32 g/L of silica and significantly almost doubled the obtained cell concentration ratio.

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CONCLUSION

From the present work it can be concluded that bio-reactor diatom culture may enhance biomass productivity, under optimized conditions. By providing extra adhesion surface to cells through the adding of bacterial cellulose gel (BC gel) to the culture medium (in a concentration of 50 g/L), biomass production is enhanced. This was tested in flask culture of *Amphora* sp. and is probably adequate for the culture of other benthic diatoms. Moreover, productivity can be boosted through culture medium optimization, as was described for *Navicula* sp. Combining these conditions, and applying them to air-lift bio-reactor culture would probably result in an efficient method for culturing benthic diatoms, on an industrial scale. This method should be further investigated in future works. Also, the relation between the diatoms and the bacteria present in cultures should be further investigated, for their presence can be interfering in diatom growth. A parallel investigation took place to understand this relation. It was observed that bacterial overgrowth lowered diatom growth, and that this overgrowth could be reduced by manipulating pH values in culture medium. Moreover, these bacteria were isolated by differential centrifugation and its DNA was sequenced. Results were analyzed in NCBI BLAST and these bacteria were found to have a 74 % similarity with *Phyllobacterium* sp. These bacteria were cultivated in standard f2+Si medium (inorganic) and no growth was observed, which means the bacteria are heterotrophic, probably feeding on diatoms metabolites, or/and on decomposing diatoms. Microscopic observations showed that bacteria colonize diatom frustules, so it is possible that they are keeping light from reaching diatoms photosynthetic system, thus having a negative impact on their growth. Understanding the relation between these bacteria and the diatoms is important, for it can be used to further enhance diatom growth. This investigation should be further developed.

The preservation technique tested was not successful on diatom cultures, because it was impossible to detach the preserved cells from the immobilization matrix (latex coating) devised. However, the cells were preserved inside the latex matrix, so it is possible that this method would be successful, if a different latex formulation was used, or if a new method for detaching cells from the coating was investigated. This approach might lead to positive results, so this should be further investigated in future works.

This work might be an important contribution to ensure aquaculture units have enough plankton feed to meet production needs.

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ANNEX I – GUILLARD'S F2+SI CULTURE MEDIUM FOR MARINE DIATOMS

- Filter natural seawater (pore size 0.2 µm).

- Prepare the stock solutions described below (compounds from different suppliers may be used).

- **Stock solutions (per litre):**

(A) 75 g sodium nitrate (Panreac);

(B) 5.65 g sodium di-hydrogen phosphate dihydrate (Panreac);

(C) 3.15 g iron chloride hexahydrate (Fluka), 10 mg copper (II) sulphate pentahydrate (Merck), 4.16 g ethylenediaminetetraacetic acid disodium salt (Panreac), 22 mg zinc sulphate heptahydrate (Fluka), 180 mg manganese (II) chloride tetrahydrate (Fluka), 10 mg cobalt (II) chloride hexahydrate (Fluka), 6 mg sodium molybdate dihydrate (Merck);

(D) 30 g sodium metasilicate monahydrate (Sigma);

(E) 0.5 mg cyanocobalamin (Sigma-Aldrich), 100 mg thiamine hydrochloride (AppliChem), 0.5 mg biotin pure (AppliChem).

Add 1 ml of stock solutions A, B, C, and D to the previously filtered seawater (while stirring). Make up to 1 L with filtered seawater.

Adjust pH to 8.0 with NaOH (1 M) or HCL (1 M).

Sterilize in autoclave (120 ºC, 15 minutes).

When culture medium is at room temperature (20 ºC), add 1 mL of stock solution E.

Keep all stock cultures and culture medium in the dark at 4 ºC.

(Guillard, Rhyther, 1975)

ANNEX II –LATEX COATINGS PROTOCOL

Mixture composition:

- Glycerol + sucrose -> cell protection
- Centrifuged cells (with less water content possible)
- Latex – biocide free

Adding order:

1. 1.2 g of centrifuged cells
2. 350 µL sucrose (0.58 g mL-1)
3. 150 µL glycerol [50 % (v/v) (used by researchers) or 100 % (v/v) (used by the Principal Investigator)]
4. 1 mL biocide free Latex (for example latex SF012).

Blend the mixture gently in order to avoid the formation of air bubbles
Substrate (or mask, or surface that defines the shape of the coating) composition: polyacetate.

Surface cleaning:

1. Ethanol 70 %
2. HCl 1 M
3. H_2O sterile

(use just a few drops embedded on a clean tissue)

Use a meyer rod size 26 if you wish to obtain a coating 20 µm thick

Attention! Slide the meyer rod – do not roll it– Clean the rod immediately after use with hot water and ethanol 70 % (latex damages the meyer rod)

Let the coatings rest for 1 hour at approx. 30 ºC with an air humidity of approx. 50 %. The coatings are now ready to be used.