Universidade do Minho
Escola de Engenharia

Lídia Sofia Sequeira

Study of new methods for the characterisation and the preservation of diatom cultures

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Trabalho realizado sob a orientação da
Doutora Ana Nicolau
e do
Doutor Cledir Santos

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Declaração

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Abstract

Diatoms are microscopic, unicellular photosynthetic microrganisms which possess rigid cell walls (frustules) composed by amorphous silica. As photoautotrophic, their cultures are affected by light. Other limiting factors are nutrients content (phosphate, nitrogen and silicon), pH and temperature. Diatoms can be explored in Biotechnology (pharmaceutical industry, cosmetics and biofuel) and the frustule itself is of vast interest to Nanotechnology. However, the success of industrial applications using diatoms depends on the choice of the species with the most relevant properties for the required application. As a result, the methods of taxonomic characterisation must be improved.

The work presented in this thesis aims at investigating new methods for the characterisation and preservation of diatoms (*Seminavis robusta*, *Cosciscodiscus*, *Thalassiosira*, *Cyclotella meneghiniana*) and, more specifically, the possibility of using the MALDI-TOF ICMS technique for their characterisation. The immobilisation of the marine centric diatom *Coscinodiscus*, in gelatine and pectin beads as a method of long-term preservation was studied as a way of preserving the genomic, morphological and physiological characteristics. Finally, surfactants were used in an attempt to detach the *pennate* diatom *Seminavis robusta* through chemical action.

From these studies, it was concluded that: (1) diatoms generate MALDI-TOF ICMS fingerprints that can be used in their identification and characterisation; (2) diatoms immobilisation in pectin and gelatine beads did not have any advantages over the existing methods; (3) the chemical action proved to be an alternative method to detach diatoms. However, more studies about the characterisation and detachment are required.

Resumo

As diatomáceas são seres microscópicos, unicelulares, eucariontes e fotossintéticos que possuem paredes celulares rígidas (frústulas) compostas por sílica amorfa. Sendo fotoautotróficas, as diatomáceas são especialmente sensíveis ao efeito da luz. Outros factores limitantes são os nutrientes (fosfato, nitrogénio, silício), o pH e a temperatura. As diatomáceas podem ser exploradas na Biotecnologia (indústria farmacêutica, cosmética e de biocombustíveis) e a frústula tem despertado um grande interesse para aplicações nanotecnológicas. No entanto, o sucesso das aplicações industriais usando diatomáceas depende da escolha da espécie com as propriedades mais adequadas para a aplicação desejada. Deste modo, os métodos de caracterização taxonómica são fundamentais e devem ser optimizados.

O trabalho apresentado nesta tese teve como objectivo investigar novos métodos de preservação e caracterização de diatomáceas (*Seminavis robusta*, *Cosciscodiscus* sp., *Thalassiosira* sp., *Cyclotella meneghiniana*) e, mais especificamente, a possibilidade de utilizar a técnica de MALDI-TOF ICMS para a caracterização destes microrganismos. A imobilização das diatomáceas cêntricas e marinhas, no caso *Coscinodiscus*, em esferas de gelatina e pectina como um método de preservação a longo prazo, foi estudada como forma de preservar as características genómicas, morfológicas e fisiológicas. Finalmente, os surfactantes foram usados numa tentativa de destacar as diatomáceas *pennate* da espécie *Seminavis robusta* por acção química.

Após o presente estudo, pôde concluiu-se que: (1) as diatomáceas são capazes de gerar *fingerprints* obtidos em MALDITOF ICMS que poderão ser usados na sua identificação e caracterização; (2) a imobilização de diatomáceas em esferas de pectina e gelatina não apresenta vantagens em relação aos métodos já existentes; (3) a acção química provou ser um método alternativo para destacar diatomáceas. No entanto, mais estudos sobre a caracterização e destacamento são necessários para se cumprirem totalmente os objectivos iniciais.

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List of abbreviations

AS	Anionic surfactant
$CaCl_2$	Calcium chloride
$CaCl_2.2H_2O$	Calcium chloride dihydrate
$CaCl_2.6H_2O$	Calcium chloride hexahydrate
$CuSO_4.5H_2O$	Copper sulfate pentahydrate
CMC	Critical micelle concentration
DHB	2,5 % dihydroxybenzoic acid
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
EPS	Extracellular polymeric substance
FDA	Fluorescein diacetate
Fe	Iron
$FeCl_2.6H_2O$	Iron chloride hexahydrate
G1/2 phase	Gap 1/2 phase of cell cycle
GTA	Glutaraldehyde
HCl	Hydrochloric acid
H_3BO_3	Boric acid
K_2HPO_4	Dipotassium phosphate
LAS	Linear alkylbenzene sulfonates
LB	Luria-Bertani
M phase	Mitosis phase of cell cycle
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation – Time Of Flight
MALDI-TOF ICMS	Matrix Assisted Laser Desorption Ionisation –Time Of Flight Intact Cell Mass

	Spectrometry
MALDI-TOF MS	Matrix Assisted Laser Desorption Ionisation – Time Of Flight Mass Spectrometry
$MgSO_4.2H_2O$	Magnesium sulfate dihydrate
$MnCl_2.4H_2O$	Manganese chloride tetrahydrate
mPBS	Marine phosphate buffered saline
MUM	Micoteca da Universidade do Minho
m/z	Mass to Charge Ratio
N_2	Nitrogen
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
Na_2HPO_4	Sodium phosphate dibasic
$NaH_2PO_4.2H_2O$	Sodium dihydrogen phosphate dyhydrate
Na_2EDTA	Ethylenediaminetetraacetic acid disodium salt
$Na_2MoO_4.2H_2O$	Sodium molybdate dihydrate
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
$NaSiO_2.9H_20$	Sodium metasilicate nanohydrate
Nd:YAG	neodymium-doped yttrium aluminium garnet
PAE	Protistology and Aquatic Ecology
PUFAs	Polyunsaturared fatty acids
S phase	Synthesis (DNA replication) phase of cell cycle
SARAMIS™	Spectral Archiving and Microbial Identification System
SEM	Scanning Electron Microscopy
SDS	Sodium dodecyl sulphate
SDV	Silica deposition vesicle
TFA	Trifluoroacetic acid
UK	United Kingdom

WC	Wright´s Chu
$ZnSO_4.7H_2O$	Zinc sulfate heptahydrate

“Most diatoms are beneficial to the oceans’ ecosystems and ultimately to human health”

(Trainer *et al*. 2008)

1 General introduction and thesis outline

Diatoms (class *Bacillariophyceae*) are unicellular photosynthetic eukaryotes (Huysman et al. 2010), which have a particular cell wall, composed of silica. These microorganisms are divided in two major architectural types: “centric” diatoms, a paraphyletic group with radially patterned valves; and “pennate” diatoms, a monophyletic group characterised by a feather-like valve structure (Scala and Bowler, 2001; Gillard et al. 2008). Diatoms are one of the predominant contributors to global carbon fixation (Parkinson *et al.* 1999; Scala and Bowler, 2001; Sarthou *et al*. 2005).

Recently, the interest in diatoms has increased since they have many possible applications (e.g. aquaculture, nanotechnology, among others). Consequently, proper diatom identification processes are necessary in order to select the best species for each given purpose. Diatom taxonomic classifications were originally based on optical microscopic analysis; this identification is currently made by Scanning Electron Microscopy (SEM) because the frustule structures are the basis for species identification. However, this technique is subject to misidentification (Scala and Bowler, 2001).

The aim of this work was the development of new methods for the characterisation and cultivation of diatoms. One of the studies performed in this work was based on the use of immobilisation in gelatine beads, for long-term preservation of diatoms as an alternative to the existing maintenance strategies, that is, obscurity in cold room or cryopreservation. Additionally, the possibility of detaching diatoms through chemical action was studied. And finally, the possibility of using the MALDI-TOF ICMS technique as a fast, low cost and reliable method for characterisation of diatom strains was evaluated.

This thesis consists of 6 chapters. In the first Chapter, the subject and the theoretical framework of the work are presented. In Chapter 2, the biology and morphology of diatoms, as well as their specified characterisation, is presented. In Chapter 3, the possibility of using the MALDI-TOF ICMS technique to characterise the diatom strains is discussed. In Chapter 4, the long-term preservation of the centric diatom *Coscinodiscus* sp. in gelatine and pectin beads is studied. In Chapter 5, a method to detach benthic diatoms, *Seminavis robusta* 84A in this case, through the action of surfactants is presented. Finally, in Chapter 6, general conclusions of the thesis are summarized and perspectives for future research are provided.

2 Diatoms Classic Characterisation

ABSTRACT. Before working with any living being it is necessary to understand its way of living. In this chapter, the unique characteristics of diatoms and their growth are described.

2.1 Introduction

2.1.1 General Overview on Diatoms

Diatoms (division *Heterokotophyta*, class *Bacillariophyceae*) are microscopic organisms ranging in general from 20 to 200 μm (Figure 2.1) (Scala and Bowler. 2001; Gordon *et al*. 2008) and live in aquatic ecosystems, freshwater or marine (Trainer *et al*. 2008; Groger *et al*. 2008). These microorganisms are divided in two types: benthic - attached to submerged surfaces - and planktonic - free swimming in open water. Benthic diatoms can grow in sediments or on the surface of plants (epiphytic), animals (epizootic), or rocks (epilithic) (Winter and Duthie, 2000; Trainer *et al*. 2008). Both benthic and planktonic diatoms can be found as single cells, while others form long chains of adjacent cells, either by end to end junction or by joining their protruding spines or setae (Trainer et al. 2008).

Figure 2.1. Diversity of diatoms (Gordon *et al.* 2008).

Diatoms are the most diverse group within algae, reaching about 200 000 species (Holtermann *et al*. 2010) and, like plants, they contain chlorophyll and other pigments that capture the energy of sunlight and convert carbon dioxide and water molecules into carbohydrates *via* photosynthesis (Trainer *et al*. 2008). Thus, they are a major contributor to global carbon dioxide fixation. They are also responsible for 20 % to 25 % of the world net primary production, and roughly for 40% of annual marine biomass production supporting most of the world fisheries (Gordon *et al*. 2008; Trainer *et al*. 2008) .

The name *diatom* comes from the Greek: *dia* "through" and *temein* "to cut", meaning "cut in half" referring to their highly ornamented siliceous cell wall – or frustule – divided in two twin parts (Trainer *et al*. 2008). The frustule is composed of two overlapping halves (or valves) that fit together like a petri dish in which the upper half – the epitheca - covers the lower half – the hypotheca (Figure 2.2). Generally, the diatoms are categorized into two major groups based on how the silica (SiO_2 = glass) ribs on the valve radiate: centrics - a paraphyletic group with discoid or cylindrical cells and radially symmetrical valves - and pennates - a monophyletic group having 'feathery' patterned and more or less bilaterally symmetrical valves (Round *et al*., 1990; Graham and Wilcox, 2000; Scala and Bowler, 2001; Trainer *et al*. 2008; Gillard *et al*. 2008; Hildebrand, 2008). These two groups consist of about 285 genera (Round *et al*. 1990) and 200 000 morphologically different species. It is worth noting that, on the basis of molecular genetic analyses, there are probably over 100000 (pseudo)cryptic species. Motility is different in these two groups: centric diatoms (Figure 2.2.a) have flagellated gametes but are otherwise swept passively by currents; pennate diatoms (Figure 2.2.b), on the other hand, are capable of limited motility (Trainer *et al*. 2008).

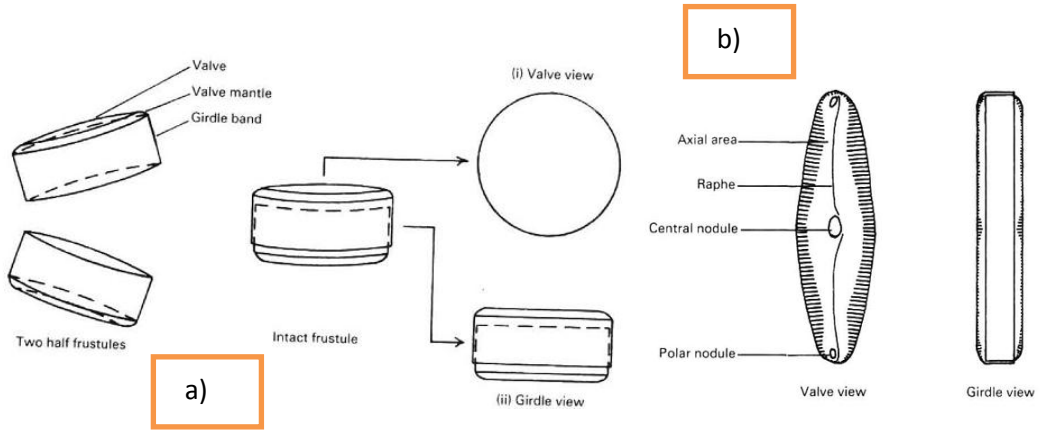


Figure 2.2. Schematic diagram of the diatom cell wall structure a) in centric diatoms; b) in *pennate* diatoms (Al-Kandari *et al*. 2009).

The frustule is composed of solid silica, being the hypotheca slightly smaller than the epitheca (Figure 2.2). This causes a cell size reduction during the vegetative cell division carried out by mitotic division. From time to time, the diatoms restore the original cell size *via* sexual reproduction. These two stages have different time laps: a prolonged vegetative stage (mitotic division of cells) lasting from months to years, a comparatively short stage of sexual reproduction (gametogenesis and fertilization) lasting several hours and then a complex developmental process (leading to the formation of the new vegetative cells) taking from hours to one week or more. The vegetative cell cycle (Figure 2.3) in diatoms consists of interphase (G1, S and G2 phases) and mitosis (M phase), the two daughter cell protoplasts still contained within the mother cell and not separated (Hildebrand, 2008). Cells in G2 and M cannot be distinguished on the basis of DNA content by flow cytometry; therefore, the term "G2+M" is used to describe this situation. During the G2+M step, the valves in of daughter cells are synthesized in the silica deposition vesicle (SDV) and once completed, are exocytosed, becoming extracellular. Cell separation occurs after synthesis of new valves in each of the daughter cells. As said above, an additional special feature of the diatom life cycle is the size-dependent control of sexuality in which only cells of a particular size range (viz. comparatively small cells) are able to become sexualized (Trainer *et al*. 2008; Hildebrand, 2008).

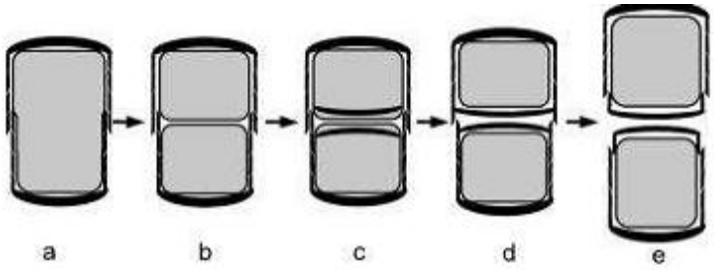


Figure 2.3. "Schematic representation of diatom cell division: a) vegetative mother cell (S phase), b) after cytokinesis, showing two daughter cell protoplasts within the mother cell thecae, c) valve synthesis occurring within the daughter cell, d) daughter cell valves after exocytosis, and e) daughter cell separation" from Hildbrand (2008).

2.1.2 Basis taxonomy of diatoms

Diatom taxonomy was primarily based on light microscopy observation of the morphologies of their frustule. The use of SEM resulted is a significant advance for taxonomy, since it revealed additional characteristics not observable by light microscopy. Based on their morphology, Round et al. (1990) proposed the classification of diatoms in three classes including the centric diatoms (*Coscinodiscophyceae*), the *pennate* diatoms without a raphe (*Fragilariophyceae*) and the *pennate* diatoms with a raphe (*Bacillariophyceae*) (Al-Kandari *et al*. 2009).

2.1.3 Industrial applications

Due to their high productivity, intra-and extracellular composition and single cell wall composition and morphology, diatoms have sparked a growing interest for several industrial applications (Lopez *et al*. 2005).

One example comes from aquaculture since algae are used in biomass production for food and feed. Also, diatoms are a natural source of relevant biotechnology products, such as amino-acids and polyunsaturated fatty acids (PUFAs) for, respectively, cosmetic and pharmaceutical applications (Lebeau and Robert 2003; Lopez *et al*. 2005). Due to the algal biomass main components, it is possible to use diatoms in the production of biofuel: carbohydrates for ethanol production *via* fermentation, proteins for methane production *via* anaerobic gasification and natural oils for biodiesel production (Bozarth *et al*. 2009). Diatoms have the ability to accumulate up to 60 % of their weight in cytoplasmic oil drops (Lebeau and Robert 2003). The resulted products of their metabolism are often released into the medium, such as the blue-green pigment called "marennine". Also, diatom metabolites (e.g. domoic acid) can be used in pharmaceutical applications. Finally, diatoms can also be applied in the nanotechnology industry, as a result of their ability of surpassing some of the modern engineering competences (Bozarth *et al*. 2009) as their formation does not require high temperatures, high pressure or the use of caustic chemicals, being accomplished under mild physiological conditions (Lopez *et al*. 2005). In this way, the frustules can be explored for various applications, such as abrasive products, filter agents for water purification, gel filtration for protein purification, biosensors, immunoisolation, photonics, drug delivery (Parkinson *et al*. 1999).

2.2 Materials and methods

2.2.1 Cell cultures

Stock cultures of the diatoms species and strains were obtained from the Culture Collection of the Laboratory of Protistology and Aquatic Ecology (PAE) in Ghent University (http://www.pae.ugent.be/collection.html).

2.2.2 Culture conditions

2.2.2.1 Culture media

For the marine species *Seminavis robusta*, *Coscinodiscus* sp. and *Thalassiosira* sp., the f/2 medium was used and for t,he freshwater species *Cyclotella meneghiniana, the* WC medium was used. The f/2 medium (Guillard, 1975) is a common and widely used general enriched seawater medium designed for growing coastal marine algae, especially diatoms. The concentration of the original formulation of the "f Medium" (Guillard and Ryther 1962) was reduced by half. The WC medium is derived from the Chu #10 Medium (Chu 1942). The medium was originally formulated to aid Richard Wright in his efforts to cultivate cryptophytes (WC = Wright's Chu #10), and first published by Guillard and Lorenzen (1972). Subsequently, Guillard has modified the medium being referred to, in the present work, as WC medium. Stock of all ingredients and culture media were stored in Schott® glass bottles (Duran) and kept in the refrigerator at 5°C.

2.2.2.1.1 f/2 medium (Guillard, 1975) with natural seawater

Seawater, collected from the northwestern Portuguese coast (Vila de Conde) was filtered using a filtration apparatus, consisting of an Erlenmeyer flask, a funnel, a 55 mm Ø filter paper circle (VWR® European Cat. No. 516-0870) and of a rubber tube that connected the flask to the air pump. A volume of 1 L seawater was filtered over each filter paper. The filtered seawater was transferred to Schott® (Duran) glass bottles of 1 to 2 L volume.

The f/2 medium was prepared by adding to 950 mL of filtered seawater, 1mL of major nutrients stock solutions and also 1 mL of trace metals solution (table 2.1). The f/2 medium was sterilized using an autoclave (uniclave 88) at 121 ˚C for 15 minutes. Before autoclaving, the pH was adjusted to 8 with 1 N HCL or 1 N NaOH. Autoclaved bottles were cooled and the vitamins stock solution was added (table 2.2)

Table 2.1. Marine enrichment f/2 medium (Guillard, 1975)

Component	Stock Solution
Major nutrients (mg/mL in distilled water)	
$NaNO_3$	75
$NaH_2PO_4.H_2O$	5
$Na_2SiO_3.9H_2O$	30
Trace metals (mg/mL in distilled water)	
Na_2EDTA	4.36
$FeCl_3.6H_2O$	3.15
$CuSO_4.5H_2O$	0.01
$ZnSO_4.7H_2O$	0.022
$CoCl_2.H_2O$	0.01
$MnCl_2.H_2O$	0.18
$Na_2MoO_4.2H_2O$	0.006
Vitamins (µg/mL in distilled water)	
Thiamine.HCl	100
Biotin	0.5
Cyanocobalamin	0.5

2.2.2.1.1.1 WC medium (Guillard and Lorenzen, 1972)

Stock solutions of the components for freshwater WC medium are shown in table 2.2. Similar components to the f/2 medium were prepared only once. Other major nutrients were individually prepared. Components were added to distilled water in Schott bottles (Duran) using 1 mL stock solution/L (except for the vitamins stock solution). The mixed solutions were autoclaved at 121°C for 15 minutes.

Table 2.2. Freshwater enrichment WC medium (Guillard and Lorenzen, 1972)

Component	Stock Solution
Major nutrients (mg/mL in distilled water)	
$NaNO_3$	85.01
$NaHCO_3$	12.60
$Na_2SiO_3.9H_2O$	28.42
$CaCl_2.2H_2O$	36.76
$MgSO_4.7H_2O$	36.97
K_2HPO_4	8.71
Trace metals (mg/mL in distilled water)	
Na_2EDTA	4.36
$FeCl_3.6H_2O$	3.15
$CuSO_4.5H_2O$	0.01
$ZnSO_4.7H_2O$	0.022
$CoCl_2.H_2O$	0.01
$MnCl_2.H_2O$	0.18
$Na_2MoO_4.2H_2O$	0.006
H_3BO_3	1
Vitamins (µg/mL in distilled water)	
Thiamine.HCl	100
Biotin	0.5
Cyanocobalamin	0.5

2.2.3 Culture growth

The *Seminavis robusta* cultures were re-inoculated by adding 2 mL of the old culture (gently scraping with a cell scraper) in f/2 medium, in a total volume of 50 mL. Cells were cultivated in 75 cm^2 tissue culture flasks with filter cap (Orange Scientific®). Incubation was at room temperature by a 12:12 h light:dark cycle (85 µmol photons/m^2/s from cool-white fluorescent lamps). Cultures were observed weekly under the inverted microscope to assess growth and possible contaminations. On the other hand, planktonic species (*Thalassiosira* sp, *Coscinodiscus* sp, *Cyclotella meneghiniana*) were harvested and re-inoculated by adding 12 mL to the f/2 medium, to complete a total volume of 250

mL. Harvesting and inoculation of cells were done in the close vicinity of a flame. The cultures used were uni-algal but not axenic, although bacterial numbers were minimal at all stages.

2.2.4 Assessment of the viability

2.2.4.1 Observation under the inverted microscope

A sample of 1 mL of recovered cells suspension was transferred to 3 wells of a 6 well-plate. Settled cells were observed under the inverted microscope (Nikon).

2.2.4.2 Observation under the light microscopy

A sample of 30 µL of recovered cells suspension was transferred to a glass slide and covered with a coverslide. Cells were observed under the light microscopy (LEITZ).

2.2.4.3 Epifluorescence microscope (diatom viability using FDA)

The viability of the diatoms was assessed using the fluorescein diacetate (FDA) staining method. One milliliter of the benthic diatoms culture and the planktonic species were removed, the first gently scraped with a cell scraper. These samples were incubated with 10 µL FDA stock solutions (Michels et a. 2010) for 20 min in the dark at room temperature. The samples were observed under an epifluorescence microscope (OLYMPUS BX51) using a fluorescence emission of fluorescein detected at 525 nm (Holm et al. 2008, Michels et a. 2010).

2.2.5 Growth curve

2.2.5.1 Inverted microscope

All *Seminavis robusta* cultures were directly observed every eight hours under the microscope. The planktonic species, including *Thalassiosira* sp, *Coscinodiscus* sp, *Cyclotella meneghiniana*, were assessed in a different way: 1 mL of culture was removed every eight hours and inspected under the inverted microscope (Nikon).

2.2.5.2 Use of Coulter Counter

Seminavis robusta cultures were gently scraped with a cell scraper and centrifuged (10 min at 3000xg, in centrifuge 2 HERAEUS MEGAFUGE 1.0R). The pellet was collected and resuspended in 20 mL of diluent. After this, cells were counted in a Bechman Coulter (Z_2 Couter® Particle count and size analyser) every eight hours.

2.2.5.3 Fluorimetry

The benthic *Seminavis robusta* was gently scraped with a cell scraper and centrifuged (10 min at 3000 g), being the *pellet* collected and resuspended in 2 mL of mPBS (Cerda-Cuellar et al. 1997). The centric genera were removed by a volume of 2 mL of culture. The cell suspensions were analysed by flourimetry (Fluorescence Spectrometer Jasco FP6200). For that, 2 ml of cell suspension were diluted in mPBS (to fit the calibration curve) and placed in a fluorimeter cell (cuvette with at least three polished windows and no background fluorescence). The emission at 685 nm was read after excitation at 440 nm (Ikeda *et al*. 2008). All readings were done in triplicate every eight hours.

2.3 Results and discussion

2.3.1 Characterisation

In order to fully characterise the diatoms, these were observed under three types of microscopes (light microscopy, inverted microscope and epifluorescence microscope).

2.3.1.1 Pennate diatoms

Seminavis robusta (Figure 2.4) is a small *pennate* of the marine epipelon and epiphyton (Garcia, 2007). The most important characteristics for their identification are: the outline of the ventral margins, the shape of the apices, the length of the *striae* on the dorsal valve face, the presence or the absence of striation interruption on the ventral valve face and the shape and the position of the raphe in relation to the ventral margin (Garcia, 2007).

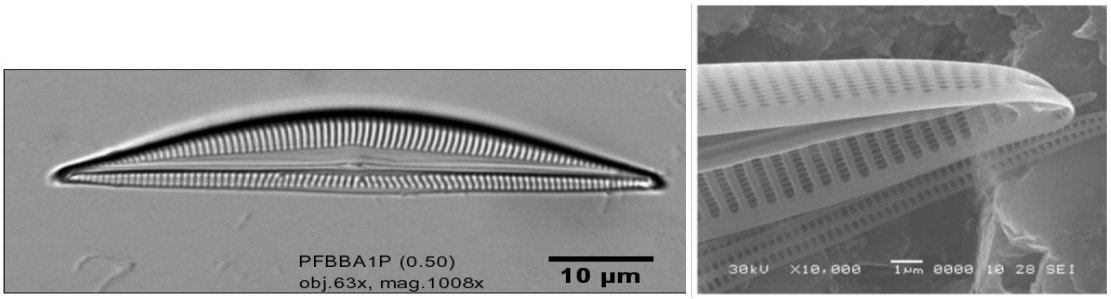


Figure 2.4. *Seminavis robusta*.

All the *Seminavis* strains used in this work (Figure 2.5) have the same shape but different sizes and were of different mating types. *Semivavis robusta* 84A (Figure 2.5 a) and 85A (figure 2.5 d) have a size of about 45 µm and *Semivavis robusta* 85As (Figure 2.5 g) and 85Bs (Figure 2.5 j) have a size of about 25 µm.

Under the light microscopy, it was possible to verify the elongated frustule and two chloroplasts. As described in the literature, the frustule is generally elongated with a longitudinal axis of symmetry (rib) in each valve (bilateral symmetry) (Kröger and Poulsen, 2008).

With the inverted microscope, the spatial organisation in culture was studied. As in light microscopy, it was possible to check again the elongated frustule, but not the presence of the chloroplasts.

Under the epifluorescence microscope, the FDA was used to determine if the cells were intact. With FDA, it was possible to see the chloroplasts in red and the membrane in green.

Light microscopy | Inverted microscope | Epifluorescence microscope (FDA)

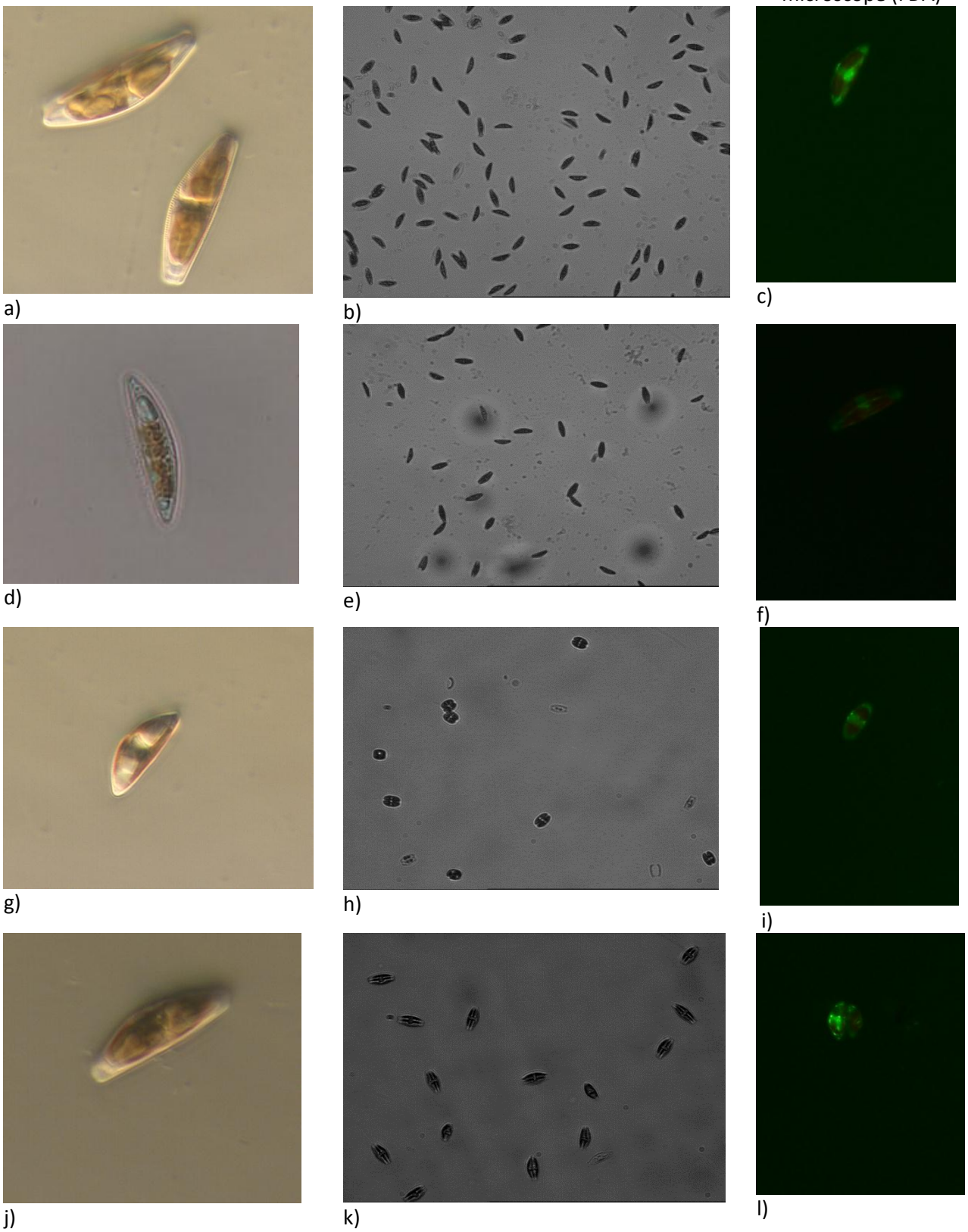


Figure 2.5. Living cells of *Seminavis robusta*. a, b, c) strain 84 A; d, e, f) strain 85 A; g, h, i) strain 85 As; j, k, l) strain 85 Bs. a, b, c, d, e, f, g, h, l, j and l) using the objective of 40x magnification; k) using the objective of 60x magnification.

2.3.1.2 Centric diatoms

Three centric diatoms (*Coscinodiscus* sp., *Thalassiosira* sp. and *Cyclotella meneghiniana*) were studied.

Coscinodiscus is a big centric diatom with a diameter of 30 to 500 µm. These marine diatoms have numerous chloroplasts. The cell is described as being disc-shaped, cylindrical or wedge-shaped, and solitary.

Thalassiosira is an important centric diatom, due to its presence in marine environments with more than 100 already identified species. The first genome sequenced among diatoms was the *Thalassiosira pseudonana*, which was important to enable the unravelling of the genetic basis of the unique properties underlying the ecological and evolutionary success of diatoms. The morphological identification of the genus *Thalassiosira,* with diameters ranging from 3 to 186 µm, is based on ultra-structure details as the number and the location of the *rimoportulae* and *fultoportulae* processes on the valve (Garcia and Odebrecht, 2009).

The last centric diatom used in this work is the *Cyclotella meneghiniana*, being one of the most extensively studied freshwater diatoms species (Beszteri *et al*. 2005, Beszteri *et al*. 2007). Their cells are described as barrel-shaped, with more-or-less tangentially undulate valves, strongly striated from the marginal area to halfway towards the middle, widening towards the margins and narrowing towards the centre (8-9 in 10 µm); the central area is plain, or with faint radiate, punctate striae, sometimes with a few solitary large punctae; their diameter is 10 to 30 µm.

In centric diatoms (Figure 2.6), as in *pennate* diatoms, light, inverted and epifluorescence microscopes were used to characterise the cells. Centric diatoms have the same shape (circular), however there is a big difference among them when size is considered, the *Coscinodiscus* sp. strain used in the present study (Figure 2.6 a) being the largest with about 170 µm, followed by the *Thalassiosira* sp. Strain (figure 2.6 d) with about 70 µm and finally *Cyclotella meneghiniana* (Figure 2.6 g) with about 10 µm.

With the light microscope, it was possible to see the circular frustule in all centric diatoms and the chloroplasts distribution. *Coscinodiscus* sp. has many small chloroplasts; *Thalassiora* sp. has numerous big chloroplasts.

Diatoms Classic Characterisation

Using the inverted microscope it was possible to observe the spatial distribution of the diatoms in culture.

Under the epifluorescence microscope and using FDA, the membrane and porous were seen in green. *Thalassiosira* has more pores than the other centric diatoms.

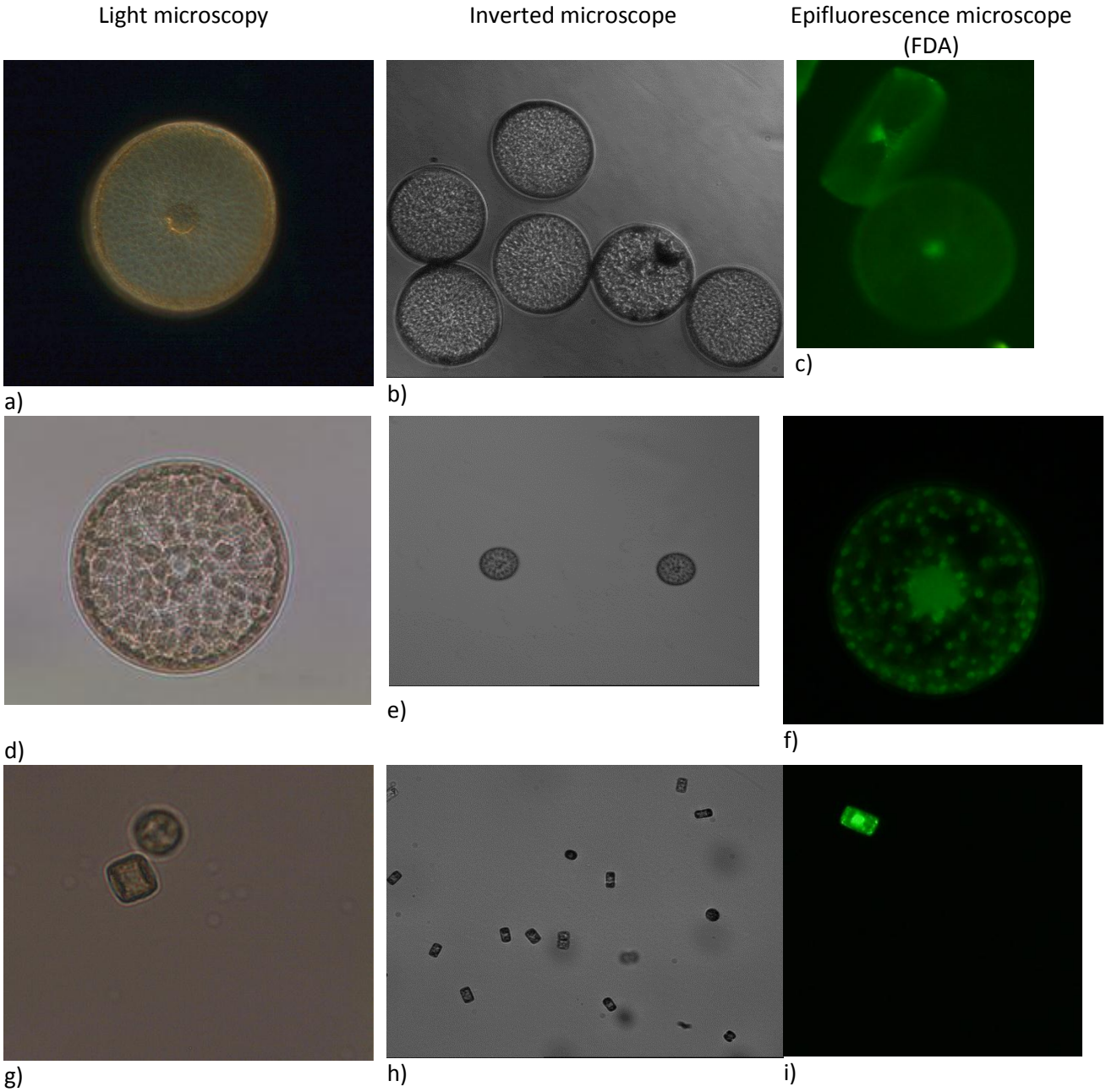


Figure 2.6. Living cells of centric diatoms. a, b, c) strain *Coscinodiscus* sp.; d, e, f) strain *Thalassiosira* sp.; g, h, i) strain Cyclotella meneghiniana. a, b, c, d, e and f) using the objective of 10x magnification; g, h and i) using the objective of 60 x magnification.

2.3.2 Growth curve

2.3.2.1 Benthic diatom Seminavis robusta

To study the *Seminavis robusta* growth, the Coulter Counter and the inverted microscope were used. Figure 2.7 describes the growth curve of all *Seminavis* strains using the Coulter. The growth curve is very similar for all diatoms, being the only significant difference the concentration. The same can be observed in the *Seminavis* growth curve using the inverted microscope (Figure 2.8). The exponential phase is very short (90 hours) and there is not a lag phase.

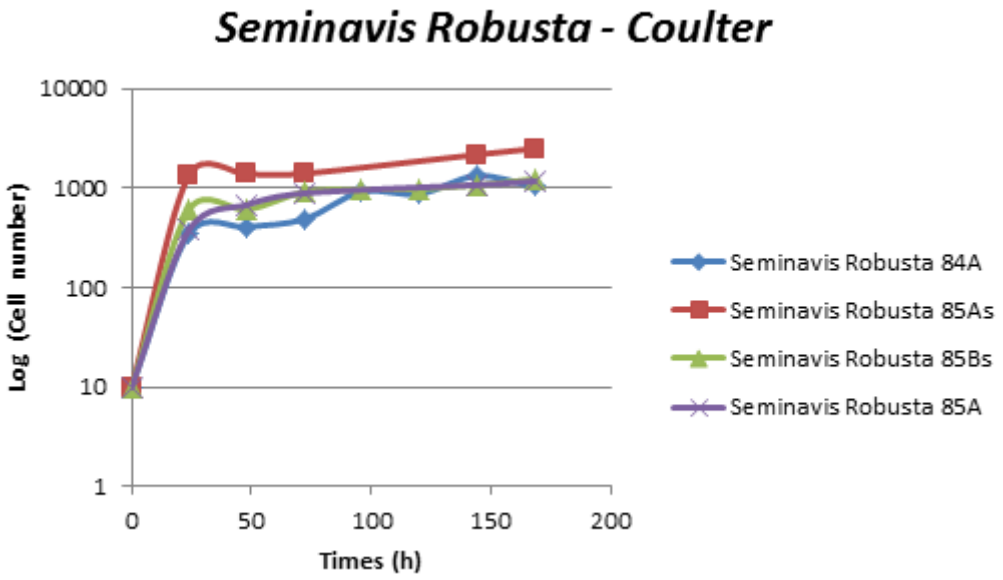


Figure 2.7. All *Seminavis robusta* growth curve using Coulter.

Seminavis robusta showed one particular feature during its growth: during the stationary phase, by approximately 150 hours, the diatoms spontaneously detach from the wall of the culture flasks. That explains the decrease of the cell number assessed by inverted microscopy, as the cells in different plans were difficult to count.

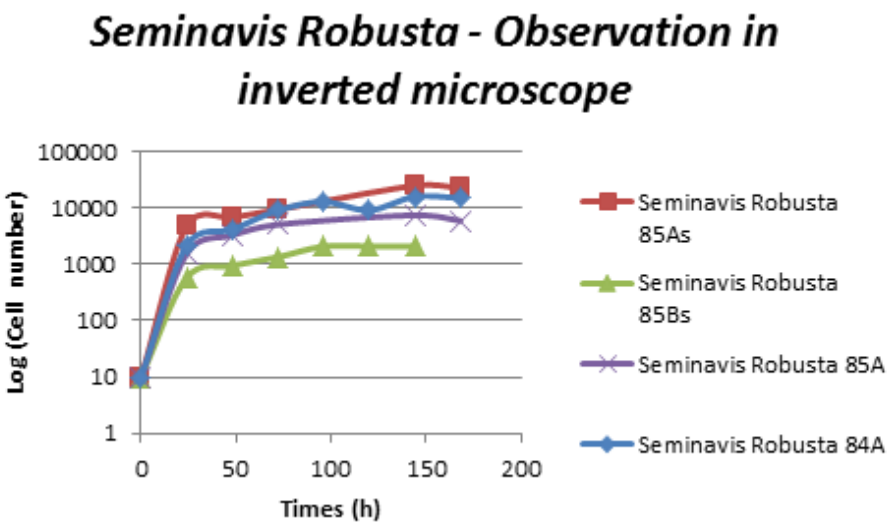


Figure 2.8. All *Seminavis robusta* growth curve using inverted microscope.

Figure 2.9 shows the differences in *Seminavis robusta* 84 A growth using the three different methods used (Coulter Counter, inverted microscope and fluorimetry). The observation under the inverted microscope was the method the most affected by errors, being the curves obtained by the fluorimetry and by the Coulter Counter equivalent. Nevertheless, the pattern of the growth curve, comprising the two phases – exponential phase and stationary phase – are consistently observed in the curves generated by the three methods. *Seminavis robusta* does not have a lag phase: the exponential phase ends *circa* the 90 hours but the culture only is in a true stationary phase at 140 hours. The coincidence in the curves that resulted by the fluorimetry and the Coulter Counter stands for the significance of the results obtained by both methods.

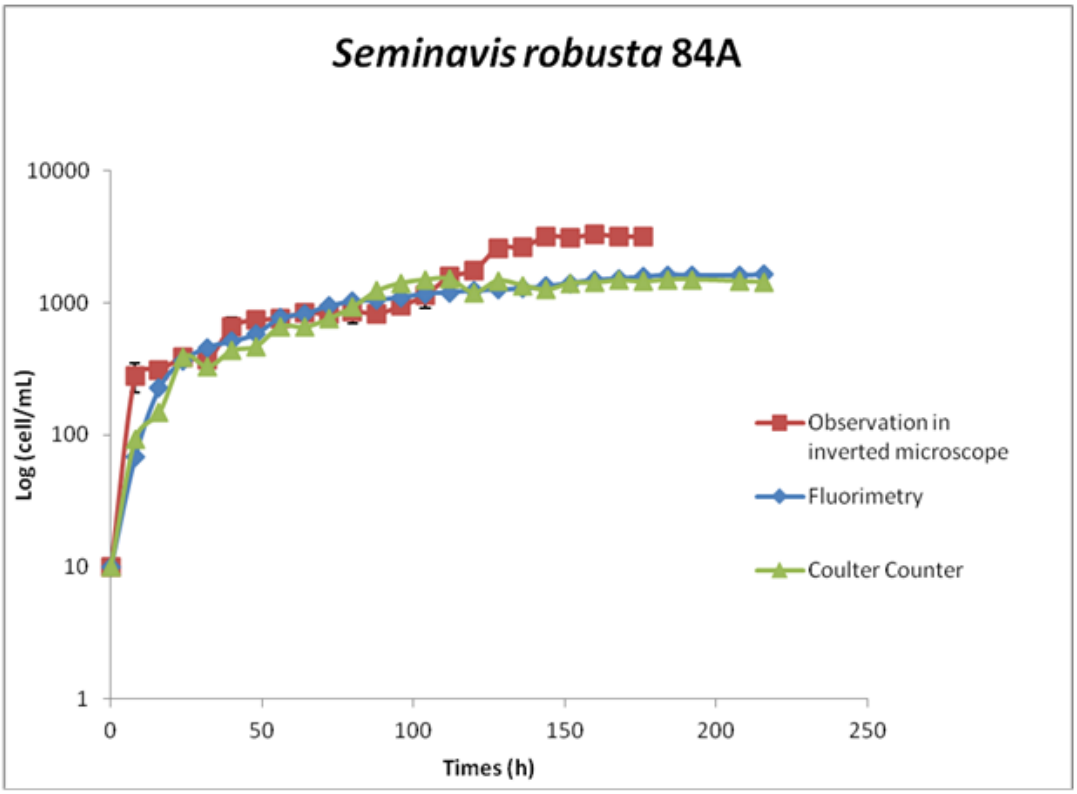


Figure 2.9. *Seminavis robusta* 84A growth curve using three different methods (Coulter, inverted microscopy and fluorimetry).

2.3.2.2 Centric diatoms

The same methods (Coulter, inverted microscope, fluorimetry) were used for the study of the growth of the centric diatoms, but the cell size and the concentration of the cultures prevented the simultaneous use of the three methods in all cultures.

In the case of *Coscinodiscus*, the Coulter Counter was not used because the cell size is bigger than the probe detection limit. All the same, the results of fluorimetry and inverted microscopy were similar (Figure 2.10). Unlike *Seminavis robusta*, *Coscinodiscus* has a prolonged lag phase (lasting 90 hours), the cell needing more time to reach the stationary phase and being the curve during the exponential phase not very sharp.

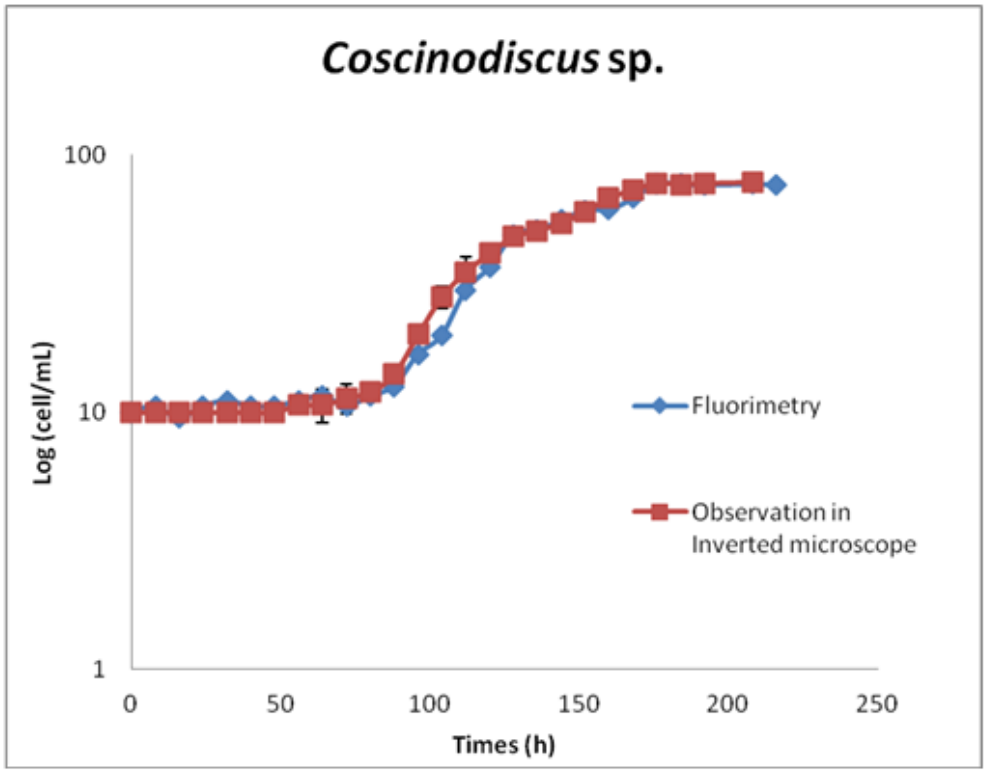


Figure 2.10. *Coscinodiscus* sp. growth curve using two different methods (inverted microscopy and fluorimetry).

In the case of *Thalassiosira,* only the inverted microscope was used, since the culture was not sufficiently concentrated and neither the Coulter Counter nor the fluorimeter could be used. The use of centrifugation was considered to concentrate the samples and consequently to enable fluorimetry, but this approach had to be abandoned because huge volumes of culture would be needed. The growth curve (Figure 2.11) is similar to the one of *Coscinodiscus*. That is, the *Thalassiosira* growth curve has an extended lag phase (with 90 hours) and a smooth exponential phase. The *Thalassiosira* concentration was very low not reaching 10 cells per milliliter.

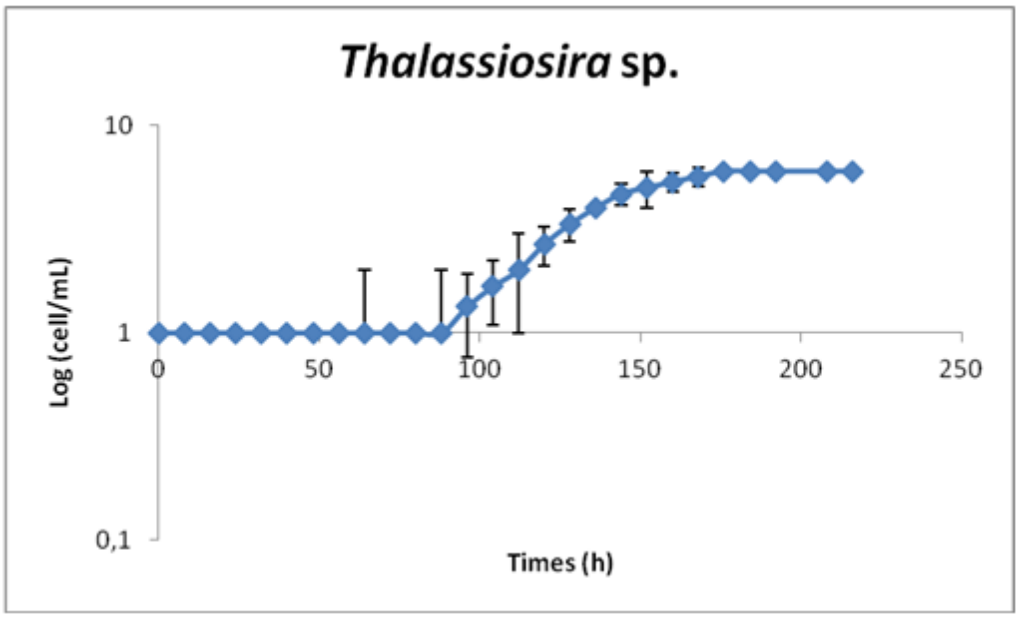


Figure 2.11. *Thallassiosira* growth curve using inverted microscopy.

Cyclotella was the only freshwater diatom studied in this work. Here, as in *Coscinodiscus*, the Coulter Counter could not be used as the size of *Cyclotella* is beneath the limit of detection. The curve (Figure 2.12) shows three well-defined phases (lag, exponential and stationary). Here, as it happened with *Seminavis robust,* the inverted microscope was error affected, because *Cyclotella* is very little and observation and counting in different plans of the microscope was limited.

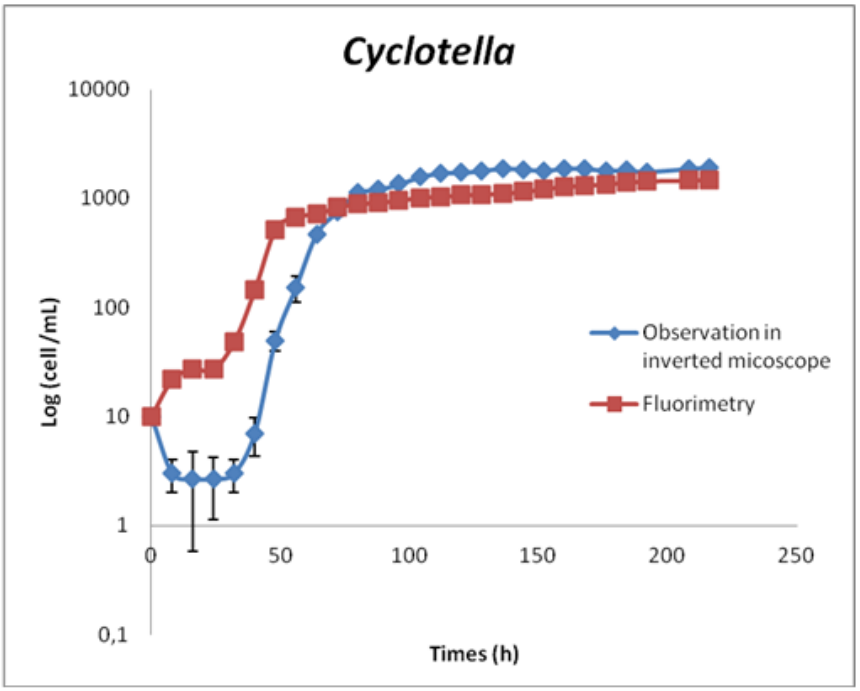


Figure 2.12. *Cyclotella* growth curve using two different methods (inverted microscopy and fluorimetry).

2.4 Conclusions

The growth curves of all *Semivavis robusta* strains have the same shape, which was expected since they all belong to the same species. No morphological differences were observed among the strains with the exception of the cell size.

The three centric diatoms, *Talassiosira*, *Coscinodiscus* and the marine *Cyclotella,* were different in size and in chloroplasts distribution and their concentrations during the experiments were very low, which can be due to the high temperatures in the lab, around 30 °C.

All diatoms showed different growth curves patterns. All *Seminavis* strains belong to the same genus and they show the same behaviour. *Talassiosira* and *Coscinodiscus* were grown in the same conditions and have similar growth curves, even though they attend very different concentrations.

By the results, we can conclude that the three methods, the Coulter Counter, the inverted microscope and the fluorimetry were applicable methods for the study of diatom growth. However, these methods sometimes cannot be used due the cell size or population density. Furthermore, the inspection under the inverted microscope can result in significant errors, as the focusing of several focal plans cannot be efficiently accomplished rendering the results affected by errors.

3 Diatom characterisation by MALDI-TOF ICMS

ABSTRACT. Diatom identification is sometimes intricate because it is based on the frustule morphology. To overcome the current difficulties, MALDI-TOF ICMS technique is proposed as a fast, inexpensive and reliable method. The use of MADI-TOF ICMS for diatoms identification is evaluated.

3.1 Introduction

The analysis and characterisation of macromolecules and their complexes which are the core of life, as well as the spectral typing of microbial cells for their taxonomic classification/identification and authentication using spectral analysis by Matrix Assisted Laser Desorption Ionisation – Time Of Flight Mass Spectrometry (MALDI-TOF MS) are both modern approaches for the life sciences and biotechnology studies (Shaah and Gharbia, 2010). MALDI-TOF MS emerged in the late 1980s as a sound technique to investigate the mass spectrometry of molecular high-mass of organic compounds through a soft ionisation of the molecules resulting in minimum fragmentation (Tanaka et al. 1998).

The MALDI-TOF MS technique has contributed to increasing scientific knowledge about the microorganisms and is now used as a reliable tool for rapid tests in hospitals and health centres. In this case the interest of the art in question is the analysis of the intact cell. The spectrum generated is analysed as fingerprint and the technique is called MALDI-TOF IC (Intact Cell) MS. In the MALDI-TOF ICMS technique the microbiological sample is covered with a UV-absorbing organic compound called matrix leading to a crystallised mixture. Then the crystallised sample is placed in a vacuum system that is targeted and irradiated with a pulsed light from a laser (e.g. N_2, Nd:YAG or other) . The charged matrix molecules and/or clusters transfer protons onto the sample molecules (e.g. peptide or proteins) in the expanding plume. The generated ions are accelerated into the TOF analyser, in which ions are separated according to their "time-of-flight" which is a function of molecular mass to charge. The TOF analyser determines the molecular mass to charge (m/z) ratio of ions by measuring velocities from accelerating ions to defined kinetic energies after calibration of the instrument with molecules of known molecular mass (Figure 3.1). In MALDI-TOF ICMS technique the linear mode set covers a huge mass range capable to get the appropriate fingerprint for each kind of microorganism (e.g. 2 - 20 kDa) (Santos *et al*. 2010).

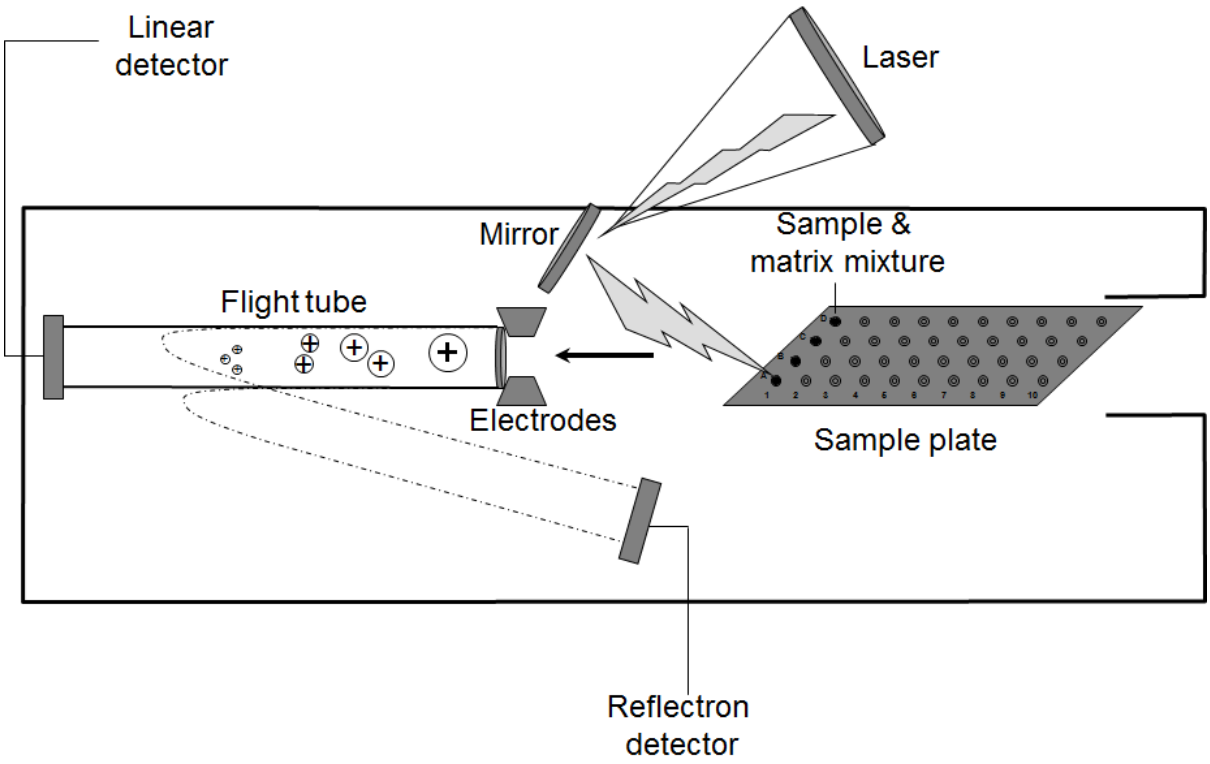


Figure 3.1. Schematic representation of MALDI-TOF MS operation: After molecule ionisation the separation of ions occurs into the flight tube based on their molecular masses (Santos et al. 2010).

The success of diatoms in biotechnological and nanotechnological application primarily depends on the correct diatom identification and characterisation. Novel identification methods need to be fast, inexpensive and reliable (Santos *et al*. 2010; Shah, 2005). MALDI-TOF ICMS is a powerful technique with all of these adjectives. This technique has been proposed as an identification method for bacteria (Shaah and Gharbia, 2010), filamentous fungi (Santos *et al*. 2010), yeasts (Santos *et al*. 2011) and virus (Franco *et al*. 2010; McAlpin *et al*. 2010).

MALDI-TOF MS technique has been used for diatoms characterisation through the analyses of lipids (Vieler *et al*. 2007), chlorophylls (Suzuki *et al*. 2009), silaffins (Sumeper *et al*. 2007) and polyamines associated with silica (Sumper *et al*. 2006; Knott *et al*. 2007). In the works presented above, authors used a mass range between 100 and 3 000 Da. However, the remarkable reproducibility of the MALDI-TOF ICMS technique for the most common microorganisms (e.g. bacteria, filamentous fungi and yeasts) is based on the measurement of constantly expressed and highly abundant proteins. The usually observable molecular mass range is between 2 000 and 20 000 Da, where important proteins and other macromolecules appear, which is an advantage because these can be easily used as biomarkers (Santos *et al*. 2010).

In this work, seven isolates of diatoms belonging to four different genera namely, *Seminavis*; *Coscinodiscus*; *Thalassiosira* and *Cyclotella*, were analysed by MALDI-TOF ICMS. All isolates were obtained from the culture collection of the Laboratory of Protistology and Aquatic Ecology of University of Ghent (Ghent, Belgium). The molecular mass range between 2 000 and 20 000 Da was used as an attempt to generate a specific fingerprint for each diatom isolate.

3.2 Materials and methods

3.2.1 Growth conditions

All 7 diatoms isolates analysed in this study were obtained from the Laboratory of Protistology and Aquatic Ecology culture collection (PAE, http://www.pae.ugent.be/collection.html). *Escherichia coli* strain DH5α was obtained from the Micoteca da Universidade do Minho (MUM, www.micoteca.deb.uminho.pt). All cultures were maintained in falcon tubes and preserved in a dark room at 4.0 ±1.0 °C. Tubes were opened and strains sub-cultured according to the instructions issued by PAE. Homogenous inocula of diatoms cells were grown and maintained on f/2 medium. *Escherichia coli* cells were grown and maintained on Luria-Bertani agar medium (LB: 10 g·l 1 Bacto-tryptone, 5 g·l-1 Bacto-yeast extract, 10 g·l-1 NaCl).

3.2.2 MALDI-TOF Analysis

Benthic diatoms were initially detached from the culture flask through mechanical action, with a cell scraper (Cat # 5560500 - 23 cm - Orange Scientific). Centric diatoms were cultivated in 250 mL *Elermeyers* flasks. All the cultures were centrifuged at 3000 g for 10 minutes using a centrifuge 2 HERAEUS MEGAFUGE 1.0R. The pellet was collected, placed in an *Eppendorf* and centrifuged once again for 4 minutes using a micro-centrifuge Sigma 112 – B. Braun Botech international. The pellet obtained was transferred into an *Eppendorf* containing 30 µL acetonitrile aqueous solution (60 % acetonitrile in 40 % ultra-pure water), and vortexed for 1 minute. Half a microliter of the green suspension obtained above was placed on the MALDI sample plate. When the liquid phase was almost evaporated, 0.5 µL DHB matrix solution (75 mg/mL 2,5-dihydroxybenzoic acid [DHB] in ethanol/water/acetonitrile [1:1:1; v/v/v] with 0.03% trifluoroacetic acid [TFA]) was added and mixed gently. Finally, all samples were air dried at room temperature and analysed in quadruplicate.

The MALDI-TOF ICMS analyses were performed in the Platform of Structural Analysis of the Centre of Biological Engineering of University of Minho on an MALDI-TOF Axima LNR system (KratosAnalytical, Shimadzu, Manchester, UK).The instrument was equipped with a nitrogen laser (337 nm), where the laser intensity was set above the threshold for ion production. *Escherichia coli* DH5α strain with known mass values of ribosomal proteins was used as an external calibrant. The mass range from 2 000 to 20 000 Da was recorded using the linear mode with a delay of 104 ns and an acceleration voltage of + 20 kV. Final spectra were generated by summing 20 laser shots accumulated per profile and 50 profiles produced per sample, leading to 1 000 laser shots per summed spectrum. The resulting peak lists were exported to the SARAMIS™ software package (Spectral Archiving and Microbial Identification System, AnagnosTec, Germany, www.anagnostec.eu) where the final identifications were achieved. This software uses a point system based on peak list with mass signals weighted according to their specificity. The similarity between individual spectra is expressed as the relative or absolute number of matching mass signals after subjecting the data to a single link agglomerative clustering algorithm. Microbial identifications by the SARAMIS™ package are based on the presence or absence of each peak in the spectra.

3.3 Results and discussion

The genetic and proteomic information are not available on the literature for the diatoms species studied in this work. The mass range from 2 000 to 20 000 Da was chosen taking into consideration the proteomic information available for bacteria (Shaah and Gharbia, 2010), filamentous fungi (Santos *et al*. 2010) and yeasts (Santos et al. 2011). However, through the results obtained preliminary in this work for all diatoms isolates it was possible to observe that the chemical compounds present on each diatom spectral fingerprint presented variations as a function of the isolate age. Moreover, for the same culture duplicate or triplicate MALDI-TOF ICMS samples presented different mass spectra. Figure 3.2 shows the duplicate spectra obtained from the same culture for *Seminavis robusta* strain 85A at 5 days old. The observation of these spectra leads to the conclusion that the majority of the compounds evaluated within the mass range from 2 000 to 20 000 Da are not ribosomal proteins (Appendices B).

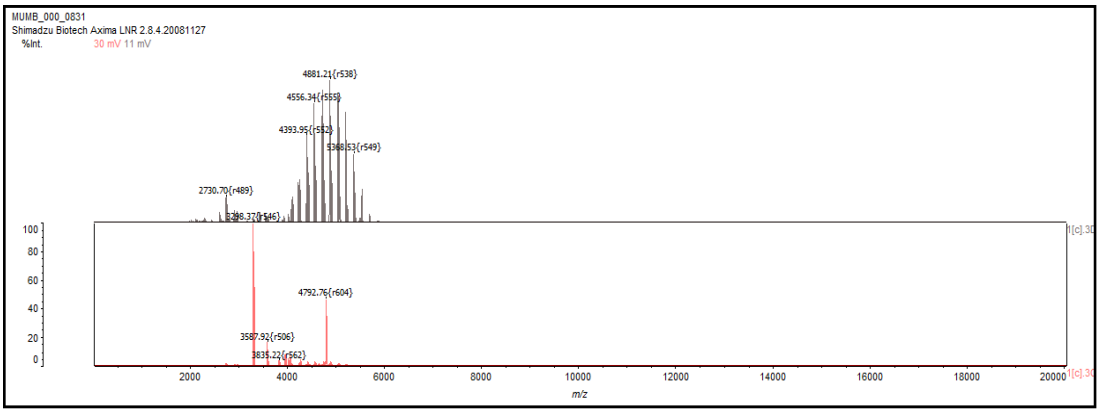


Figure 3.2. Duplicates MALDI-TOF ICMS mass spectra for *Seminavis robusta* strain 85A at 5 days old.

In order to understand the change in molecular masses, the mass analyses of mature diatoms was performed at the stationary phase. It was developed according to the growth curve (Chapter 2) for each diatom species (*Seminavis robusta* strains 84A, 85A, 85As, and 85Bs, *Cyclotella meneghiniana*, *Coscinodiscus* sp. and *Thalassiosia* sp.). All of the mass spectra obtained in these analyses show similar behaviour as observed for *Seminavis robusta* strain 85A mass spectra at 5 days old described above (Figure 3.2). Considering both the growth curve obtained by classical techniques (Chapter 2) and the mass fingerprint by MALDI-TOF ICSM discussed above incongruent results are observed. The growth curves by classical techniques indicate that the stationary phase was 6 days old for all *Seminavis robusta* and 7 days old for *Cyclotella meneghiniana*, *Thalassiosia* sp. and *Coscinodiscus* sp.. On the other hand, MALDI-TOF ICMS spectra performed at and/or after these diatoms ages indicate to a potential different growth phase inside the same diatoms cultures.

Since the mass spectra change with the diatoms age the spectral change *versus* the diatom age was assessed. For each diatom strains a specific time course of the spectrum *versus* time was assessed based on the follow schedule: 7, 9, 13, 14, 18 and 30 days old. However, results indicate that because of the *in situ* bio-compounds extraction quality for some of the diatoms isolates the mass spectra where not generated for this entire schedule. Moreover, all the diatoms evaluated in this study presented a specific age where the mass spectrum becomes reproducible. For all *Seminavis robusta* and *Cyclotella meneghiniana* this time was 9 days old (Figure 3.3) and for all *Thalassiosia* sp. and *Coscinodiscus* sp. it was 13 days old (Figure 3.4).

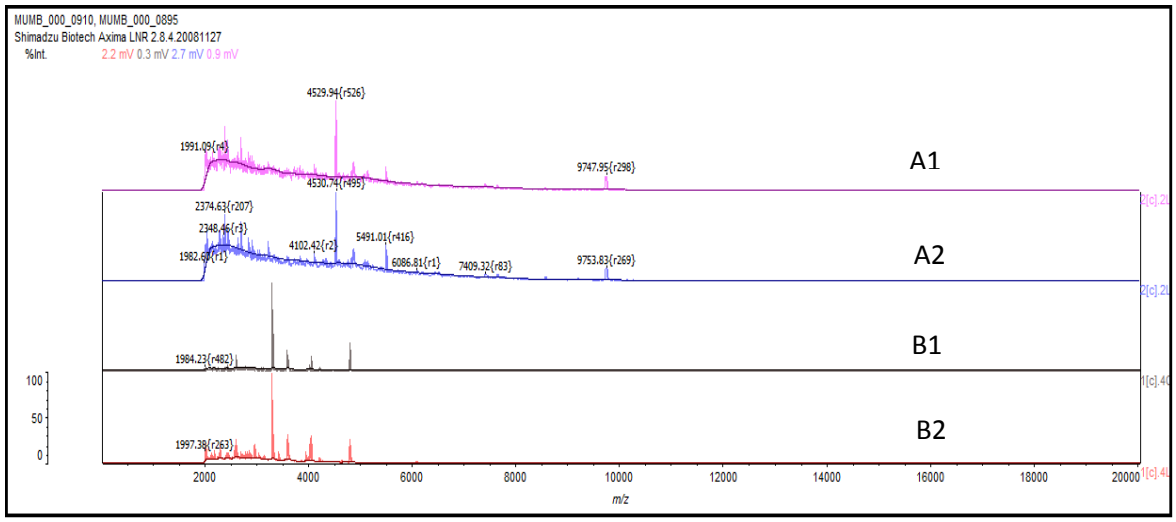


Figure 3.3. Reproducible MALDI-TOF ICMS mass spectra for (A1-2) *Cyclotella meneghiniana* and (B1-2) *Seminavis robusta* strains 84A at 9 days old.

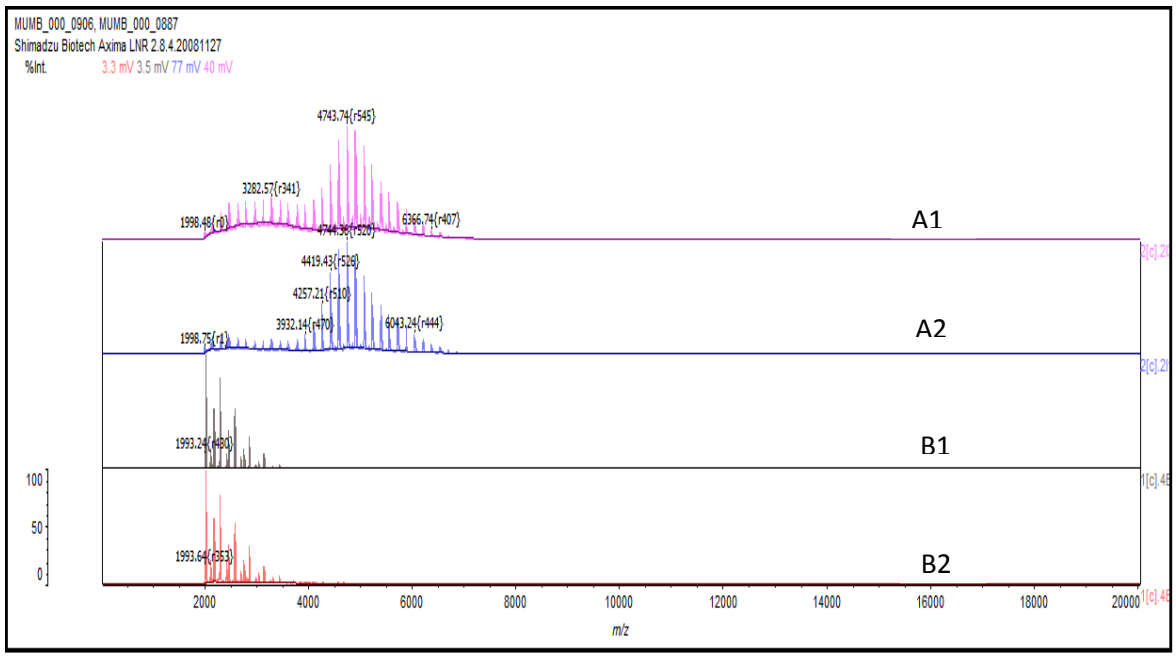


Figure 3.4. Reproducible MALDI-TOF ICMS mass spectra for (A1-2) T*halassiosia sp.* and (B1-2) *Coscinodiscus sp.* at 13 days old.

Furthermore, there was no compounds stability over time. Spectral data obtained for *Coscinodiscus* sp. at 13, 18 and 30 days old culture (Figure 3.5) is an example of instability over time for this species.

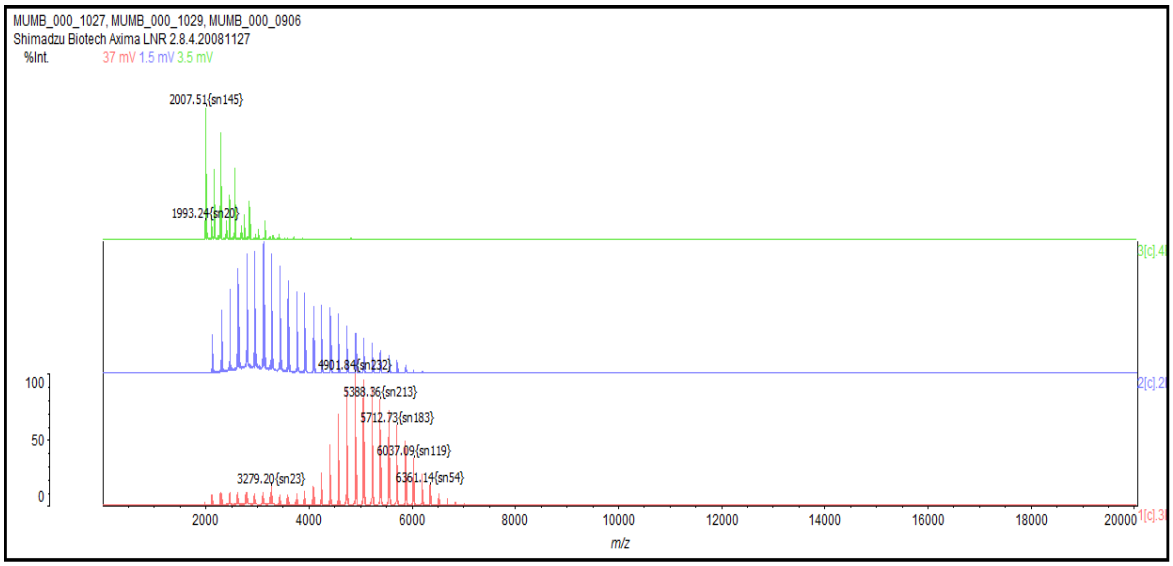


Figure 3.5. MALDI-TOF ICMS mass spectra for *Coscinodiscus* sp. at 13, 18 and 30 days old from top to bottom.

Using the first reproducible spectral data (Appendices B) including all peaks obtained from 2 000 to 20 000 Da a cluster analysis of the MALDI-TOF ICMS mass spectral data was assembled. This statistic analysis based on the mass spectral signatures allowed the grouping of all isolates into clusters according to their species designation (Figure 3.6). The dendrogram presents two main clusters with the two different pairs *Thalassiosia* sp./*Cyclotella meneghiniana* and *Seminavis robusta*/*Coscinodiscus* sp. grouped at a threshold of about 30 % similarity. The pairs *Thalassiosira* sp./*Cyclotella meneghiniana* was distinct at a threshold of about 44 %. Additionally, the pair *Seminavis robusta*/*Coscinodiscus* sp. can be identified at a threshold of 38 %. Overall, the clustering shows the isolates grouped altogether according to their species. Furthermore, *Seminavis robusta* strains 84A, 85A, 85As and 85Bs grouped altogether without a clear spectral differentiation for each strain.

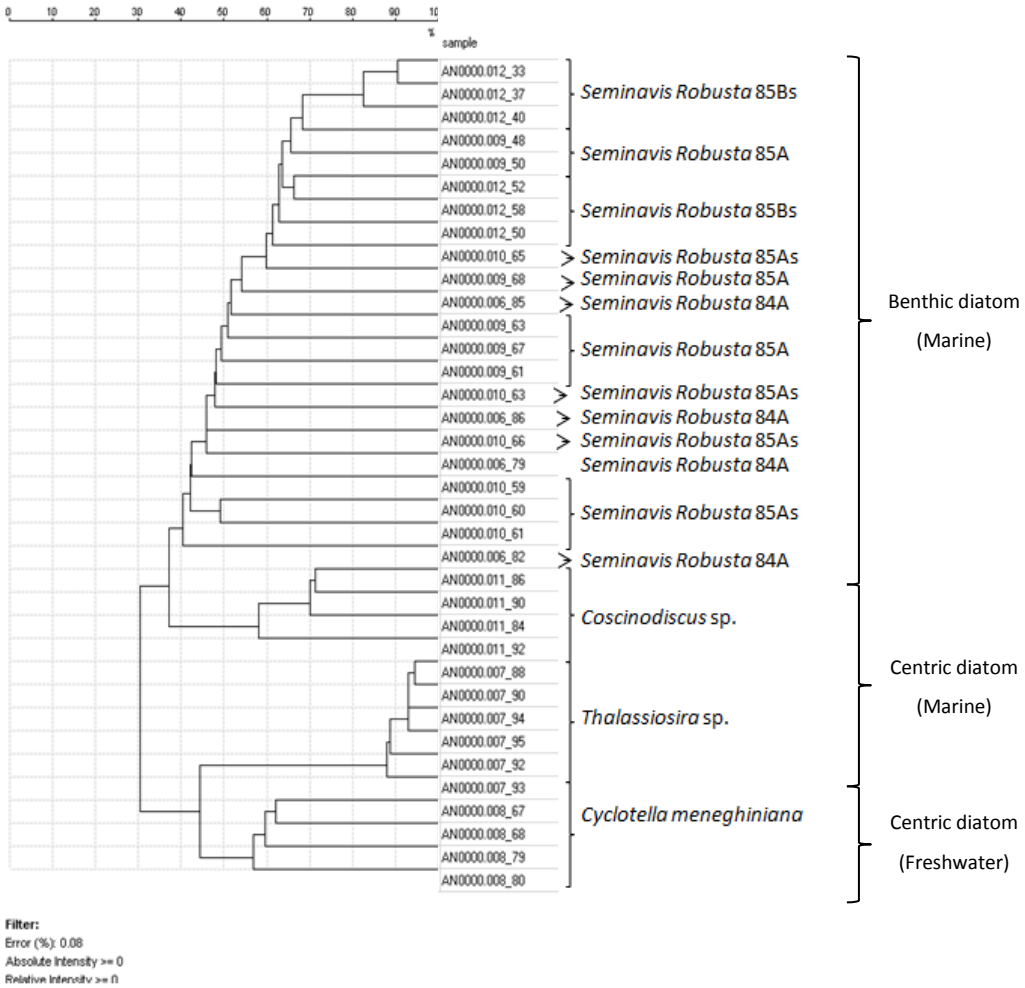


Figure 3.6. MALDI-TOF ICMS spectra-based dendrogram of the diatoms isolates studied in this work.

3.4 Conclusions

MALDI-TOF ICMS, when the microbial age is known, appears to be a powerful highthroughput mass spectra method to discriminate diatoms genera. This technique may be alternative to classical and molecular biology methods that are time consuming and expensive approaches. The MALDI-TOF MS equipment is straightforward to operate and cost-effective on an individual sample basis. The best growth time for the diatom identification by MALDI-TOF ICMS was 9 days old for *Seminavis robusta* and *Cyclotella meneghiniana* and 13 days old for *Thalassiosia* sp. and *Coscinodiscus* sp.. All of the diatoms evaluated in this study present a specific age where the mass spectrum becomes reproducible. However, the reproducibility is not accompanied by the compounds

stability. Based on the chemical variability of the molecular mass of some of the compounds evaluated within the mass range from 2 000 to 20 000 Da it is possible to conclude that the majority of the compounds presented on these fingerprints are not ribosomal proteins. The isolation and analysis of the diatom ribosomal proteins by MALDI-TOF MS could lead to a better understanding on this subject, inclusively the percentage of these proteins on the mass range evaluated in this work.

So, it is possible conclude that the MALDI-TOF ICMS have powerful to physiological characterisation of diatom cultures.

4 Immobilisation of diatoms for long-term preservation

ABSTRACT. In every collection there is a need to preserve the genomic, the morphological and the physiological features of the specimens. In the case of long-term preservation of the marine centric diatom *Coscinodiscus* sp., the strategy of conserving its cultures in obscurity is the most commonly used, but this method can only be used for, at most, six months. In the present work, new methods of preservation were studied - immobilisation in gelatine and pectin beads - to try to improve the preservation time. Immobilized cells were stored for 4 weeks in absolute darkness at 4 °C, in gelatine beads. The immobilised cells were found dead when their viability was assessed.

4.1 Introduction

Cryopreservation consists in freezing the tissues (microorganisms, among others) at the temperature of liquid nitrogen (- 196 °C). This method is widely used for suspending the metabolic activity and keeping the organisms characteristics, that is, the genomic, the morphological and the physiological stability of preserved cells. The most commonly employed cryopotectants for diatoms are dimethyl sulphoxide (DMSO) and methanol. However this method is not suitable for the preservation of the diatom frustules. Indeed, as frustules are made of silica, they become very fragile at very low temperatures and tend to break up (Tzovenis *et al*. 2004; Gwo *et al*. 2005; Mitbavkar and Anil, 2006).

Obscurity is another method used to preserve diatoms. It consists in keeping the diatoms harvested during the exponential phase at 4 °C in the dark, for a period between 4 and 5 months. Diatoms were preserved between 4 and 5 months since the cells grow slowly (as diatoms are photosynthetic autotrophic microorganisms, they are growing in unfavourable conditions) (Montainia, 1995; Gillard *et al*. 2008).

There is another method of long-term preservation: the immobilisation. Cell Immobilisation is defined as a "natural or artificial method, to prevent cells from moving independently from its original location to all parts of an aqueous phase in a system" (de-Bashen and Bashan, 2010) with preservation of a required catalytic activity. In this work, the immobilisation of diatoms was done through different polymers described below.

Gelatine is an interesting candidate to immobilise diatoms for long-term preservation because it is a non-toxic, inexpensive and non-immunogenic material; nevertheless, it is biodegradable (Vandelli *et al*. 2004; Ratanavaporn *et al*. 2006; Huang *et al*. 2008).

Gelatine is a protein that it is derived from collagen by hydrolysis and denaturation (results of breaking of the collagen triple helix). These will cause an alteration in the collagen molecule, but not in their chemical composition.

To get the gelatine from denatured collagen, two basic processes are used: thermal treatment and hydrolytic degradation of covalent bonds. Thermal treatment (40 °C) occurs in the

presence of water (destroys both hydrogen and electrostatic interactions). Hydrolytic degradation of collagen can occur under acidic or basic conditions, leading to the formation of gelatine type A (acid pig skin) or type B (limed ossein), type C (limed bovine and cattle hide) and type AB (acid ossein) (Bosch and Gielens, 2003; Habraken *et al*. 2008). After hydrolysis and denaturation, gelatine chains undergo a gradual conformational change, known as the coil-to-helix transition. During this process, there is an increase in viscosity. Gelatine can be made into roll film and drug capsules which can also be used as a biomaterial in biomedical applications, such as in drug delivery systems (Zhang *et al*. 2006). Crosslink of gelatine is possible if aldehydes such as formaldehyde and glutaraldehyde are used. The crosslinked gelatine can form toxic products between the gelatine and the crosslinker (Vandelli *et al*. 2004).

Another candidate for the immobilisation is pectin. Pectin is a ubiquitous component (anionic polysaccharides) of the plant cell walls (Sila *et al*. 2009, Sørensen et al. 2009). Chemically, pectin is predominantly a linear polymer of mainly α-(1-4)-linked D-galacturonic acid (Sriamornsak *et al*. 2008, Sørensen *et al*. 2009, Souza *et al*. 2009). Pectin like gelatine can be used in drug delivery systems. This is because pectin can form gels by cross-linking with calcium ions. Intermolecular cross-links are formed between the divalent calcium ions and the negatively charged carboxyl groups of the pectin molecules, called an 'egg-box' conformations with interstices in which the calcium ions may pack and be coordinated (Sriamornsak *et al*. 2008).

4.2 Materials and methods

4.2.1 Cell cultures and culture conditions

The cell culture and culture condition are the same as described in Chapter 2.

4.2.2 Immobilisation

4.2.2.1 Gelatine

The gelatine solution (type A) is made at a concentration of 20 % (w/v) (5 g of gelatine was dissolved in 25 mL f/2 medium) in f/2 medium (Habraken *et al*. 2008). After this, the autoclave is used (15 min, 121 °C) as the gelatine solution needs to stay sterile. The gelatine solution is cooled, until its temperature reaches 30 °C after which it is placed in a hotplate. This is when the diatoms (25

mL of the culture) are mixed in the gelatine solution (800 rpm). The resulting solution is cooled for 30 min at room temperature. The solution (gelatine + diatom) was added drop-wise into paraffin oil (ice bath), while the mixture was mechanically stirred at 10 rpm to form an emulsion (Vandelli *et al*. 2004; Huang *et al*. 2008). The formed gelatine particles were filtered washed with f/2 medium and placed in petri dishes (Huang *et al*. 2008). These gelatine particles were kept at 4 °C in cold room.

The same process was repeated but with mPBS.

4.2.2.2 Cross-linking of Gelatine

The procedure of cross-linking gelatine beads is the same procedure of gelatine beads preparation, with the exception that the 8 % glutaraldehyde is added directly to the paraffin.

4.2.2.3 Pectin

Pectin (6 % w/v) was dispersed in solution with 50 % f/2medium and 50 % distilled water with agitation. The solution needs to cool down (fifteen minutes). After cooling, the pectin beads were obtained by dripping in an aqueous solution of 10 % (w/v) $CaCl_2$ in low agitation. The formed beads stayed in the solution for 30 minutes, then were separated and washed with f/2 medium, screen-filtered and dried at room temperature for 24 hours (Sriamornsak *et al*. 2008).

4.2.3 Viability

For viability determination the FDA was used (as described in Chapter 2).

4.3 Results and discussion

The stock culture was preserved by the obscurity method because it is very simple, easy and quick to carry on: a concentrated culture was placed at 4 ± 1 °C in the dark. With this method, it is not necessary to add anything to the culture, being this viable for 4 or 5 months and no contamination occurred.

Commonly referred in literature, the cryopreservation was not used because, as mentioned above, the low temperature involved can break diatoms frustule (causing their death) and the

cryopotectants are toxic for the cells. With this method it is necessary to be more careful and spend more time with sample preparation; however the cells may keep on viable for one year.

In the beads preparation, distilled water was normally used as a solvent, however in this work has been used f/2 medium and mPBS, since the diatoms (marine creatures) need salts to prevent the osmotic shock.

4.3.1 Gelatine

During this work, it was observed that it is very difficult to get diatoms concentrations higher than 20 % (w/v) (close to the solution saturation). Using higher gelatine concentration, the resulting beads were more perfect and consistent, as was expected. Figure 4.1 shows the difference in beads structure with different concentrations of gelatine. This experience was done with and without diatoms, to ensure that the problems did not arise from diatom enzymes, for instance.

a) b)

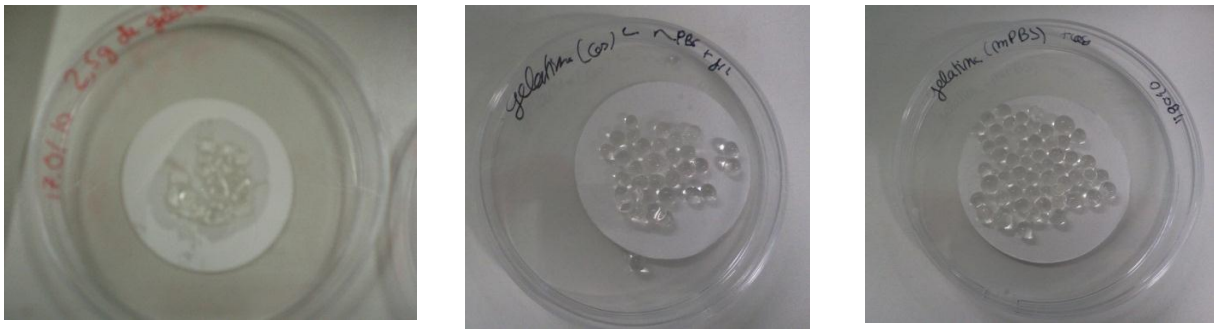
Figure 4.1. a) Gelatine beads in a concentration of 20 % (w/v); b) Gelatine beads in a concentration of 10 % (w/v).

In Figure 4.2, it is possible to observe that deterioration of the beads structure occurs over time. After 31 days, beads were completely solubilised. It seems that gelatine is not stable in the long-term (Wei *et al*. 2007), being this method difficult to use for long periods of time. The gelatine beads had, as solvent, the f/2 medium. The salt blocks some connection points and the gelatine structure is not compact enough to ensure long-term stability (Sarabia *et al*. 2000).

a) b) c)

Figure 4.2. a) Gelatine beads at time zero; b) Gelatine beads in 15 days after its production; c) Gelatine beads 31 days after its production.

A possible way to eliminate some of the salts effect is to make a medium with less salt (e.g. mPBS). In mPBS and with the same concentration of gelatine, beads had a better consistence (Figure 4.3). This is due to the fact that there is less salt available to occupy the connection points. As expected, the gelatine beads in mPBS did not dissolve over one month.



a) b) c)

Figure4.3 A – Gelatine beads in a concentration of 10 % (w/v) in f/2 medium; B - Gelatine beads in a concentration of 10 % (w/v) in $^1/_2$.of f/2 medium + $^1/_2$ of mPBS; C – Gelatine beads in a concentration of 10 % (w/v) in mPBS.

After recovering the diatoms, it was necessary to assess the diatom viability, by using FDA. Unexpectedly, all cells were dead (Figure 4.4)which may be explained by a) the initial cell concentration was low (77 cell/mL), b) the cells were destroyed when placed on the coverslide, c) the diatoms chosen (centric diatoms) were not appropriate, since all known studies about diatom immobilisation are carried on with pennate diatoms (Gaudin *et al*. 2006).

Light microscopy Epifluorescence microscopy

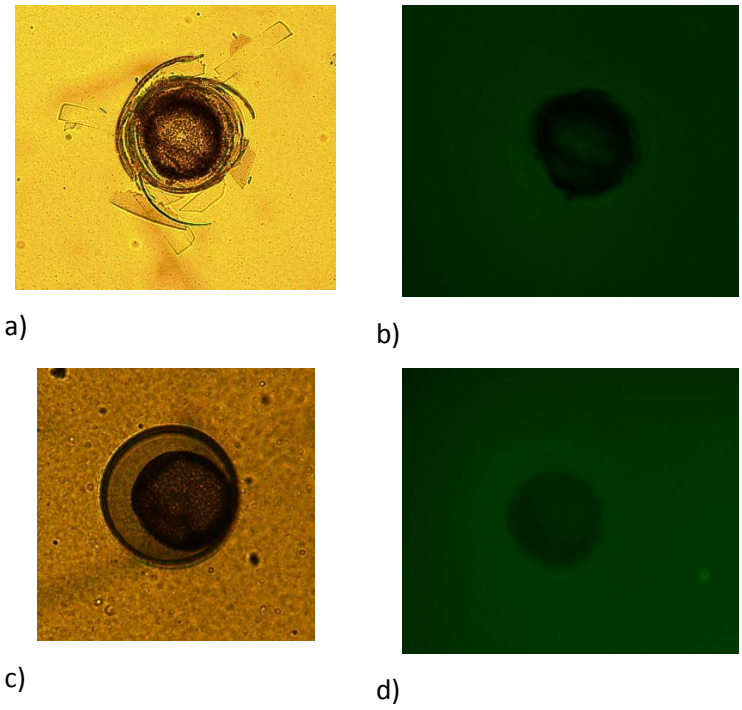


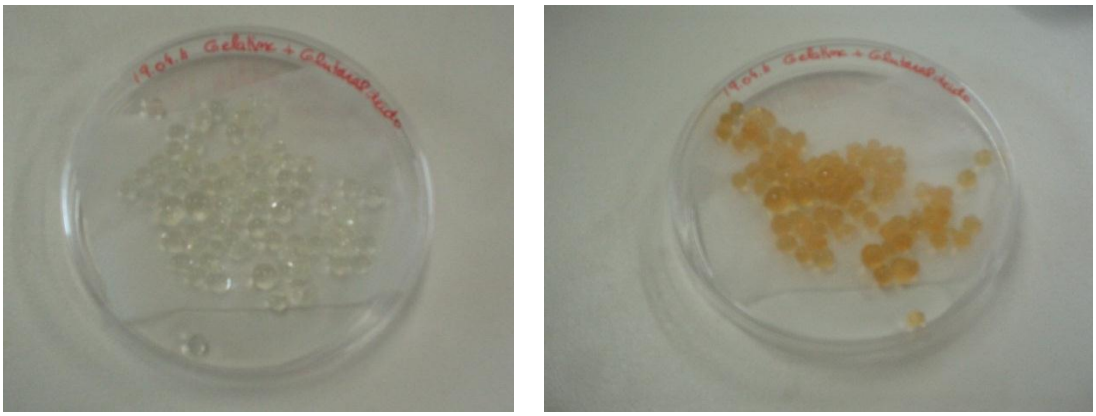
Figure 4.4. *Coscinodiscus* immobilisation in gelatine beads in mPBs – a) in light microscopy and b) in epifluorescence microscopy; c, d) *Coscinodiscus* immobilisation in gelatine beads in mPBs more f/2 medium, in light and in epifluorescence microscopy, respectively.

4.3.2 Crosslinking of Gelatine

Initially, gelatine beads were made without cross-linking because this can be toxic to the diatoms (Cortesi *et al*. 1999; Vandelli *et al*. 2004). But as the results were not satisfying, gelatine beads with cross-linking (where glutaraldehyde was proposed as connection agent) were made. Glutaraldehyde is toxic for the cell. To reduce the glutaraldehyde effect in cell, it cannot contact directly with cells. One way of doing this is to place the glutaraldehyde directly in paraffin instead of being added to the gelatine solution (Saarai *et al*. 2011).

It was difficult to dissolve the glutaraldehyde in paraffin, since the glutaraldehyde is highly cationic. To mitigate this fact, the solution was in constant agitation (200 rpm) to promote the homogeneity.

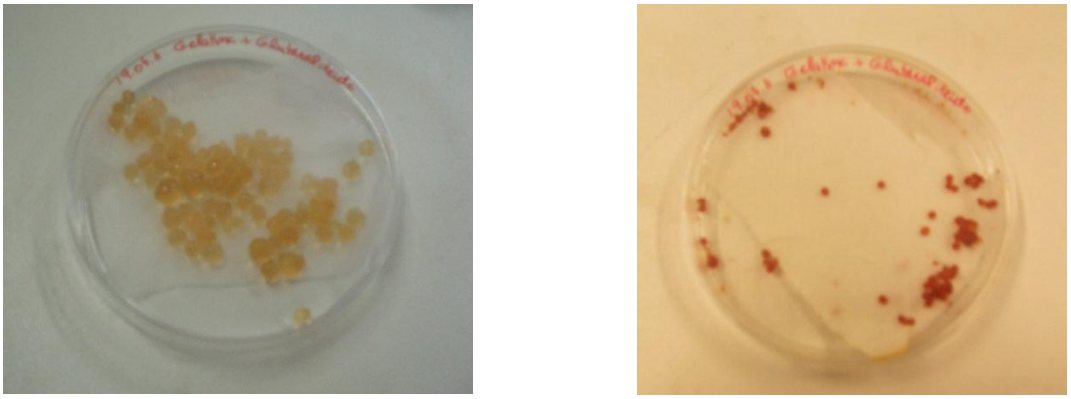
The beads formed from the cross-linked gelatine at time zero have the same texture and colour as the gelatine beads in mPBS. However, in the second day, they showed a different colour and had a smaller size (Figure 4.5).



a) b)

Figure 4.5. a) Beads at time zero; b) Beads in the second day.

As in normal gelatine beads, the gelatine beads with gluraraldyde changed their structure, by being smaller and of a different colour, over time (Figure 4.6).



1 days 30 days

Figure 4.6. a) Gelatine beads at time zero; b) Gelatine beads after 30 days.

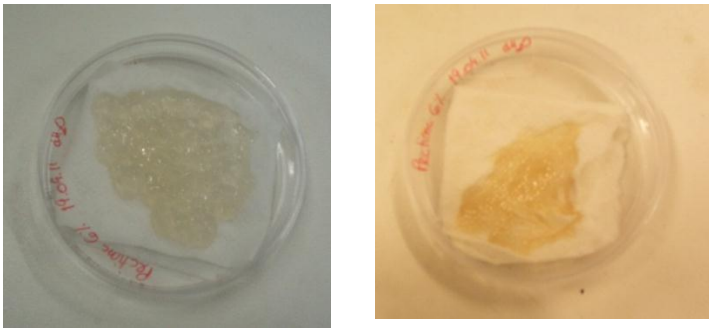
Glutaraldehyde increases the resistance of the beads to the temperature so they could stay more than one month in the cold room (4 ± 1 °C).

The disadvantage of this method is that the use of high temperatures (more than 30 °C) was essential for the cell recovery, causing subsequent cell death.

4.3.3 Pectin

Pectin proved to be a limited immobilisation method, because it was not possible to build the beads efficiently and consequently to encapsulate the cells (Figure 4.7).

This was not expected since Sriamornsak, in 2008, described this procedure in distilled water. Apparently, the salts from the f/2 medium influenced the beads structure.



0 days 1 month

Figure 4.7. a) Pectin beads at time zero; b) Pectin beads in 30 days after its production.

4.4 Conclusions

Comparing all methods it was possible to conclude that obscurity is presently the best method for the diatom conservation. It is simpler, cheaper and faster than the immobilisation method.

The immobilisation in gelatine beads was not satisfactory because the beads only lasted for one month, much lower than the six months obtained with the obscurity. On the other hand, gelatine beads in mPBS were not satisfactory as well because the cells were unviable after recovery.

When the glutaraldehyde was used, the beads lasted more than one month, but the need of using higher temperatures to solubilise the gelatine beads and to recover the cells caused the diatoms death.

The pectin proved to be a poor option for the immobilisation since it did not allow for the beads assemblage.

We may thus conclude that the methods used were not the best to immobilize this type of cells. Other ways to liquefy gelatine, alternative to heating, might prove to be more successful.

5 Diatom detachment

ABSTRACT. Benthic diatoms grow attached to a surface. This means that prior to their use, they need to be detached. Normally the detachment is performed by using scrapers (mechanic action). A less aggressive method was studied based on chemical action through the use of surfactant. The surfactants were placed in contact with the cell cultures overnight. Cells were recovered the next morning and their viability evaluated. It was concluded that chemical action can be a good method for detaching benthic diatoms.

5.1 Introduction

Most of the p*ennate* diatoms grow adhered to a surface, by secreting an adhesive mucilage composed of polysaccharides and proteoglycans (Drum and Gordo, 2003; Stanley and Callow, 2007). Prior to their use, cells need to be detached. Detaching can be achieved through mechanic action, chemical action and temperature.

When mechanical action is used, the diatom culture is gently scraped with a cell scraper. Other possible treatment is putting the diatoms at lower temperatures and in the dark (Kilroy *et al*. 2007).

Surfactants, sometimes called surface-active agents or detergents, are of widespread use around the world. They are classified in four classes (cationic, anionic, amphoteric and non-ionic) based on the ionic charge (if present) of the hydrophilic portion of the surfactant in an aqueous solution (Yüksel *et al*. 2009).

Anionic surfactants (AS) are the major class of surfactants used in detergents formulations. In this case, when the surfactant is dissolved in water the obtained electrolyte is an anion. These surfactants adsorb various types of substrates, giving them a negative charge. AS are wide-purpose detergents and exhibit a large capacity of foam production (Oliveira, 2001). An example of these surfactants is Sodium dodecyl sulphate (SDS). SDS (Figure 5.1 the SDS structure), a member of the linear alkylbenzene sulfonates (LAS) family, is used as detergent, dispersant, or anionic surfactant (Yüksel *et al*. 2009).

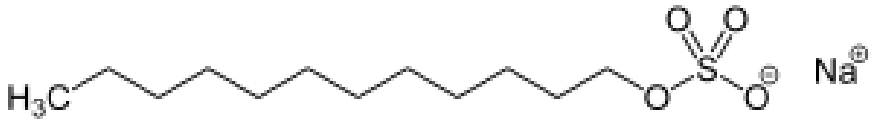


Figure 5.1. SDS structure.

Cationic surfactants like anionic surfactants are electrolytes. Cationic surfactants have a positive charge. Most of the materials, when in aqueous solution, are positively charged and the negatively charged surfactants are therefore adsorbed, directing their hydrophilic heads to the negative surface (Oliveira, 2001). One example of these surfactants is Tris base (Figure 5.2). Tris is an abbreviation of the organic compound known as tris(hydroxymethyl)aminomethane (Marcozzi *et al*. 1998; Oliveira, 2001).

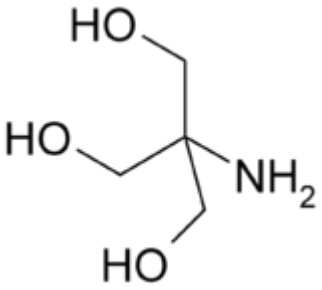


Figure 5.2. Tris base structure.

Non-ionic surfactants do not have an electrical charge, which makes them resistant to water hardness deactivation. They are excellent grease removers, being used in laundry products, household cleaners and dishwashing liquids (Oliveira, 2001).

Triton X-100 (Figure 5.3) is a commercial name for the non-ionic detergent known chemically as octylphenoxy polyethoxyethanol, with a polar group that contains polyoxyethylene residues (Robson and Dennls, 1977; Marcozzi *et al*. 1998; Liu *et al*. 2009).

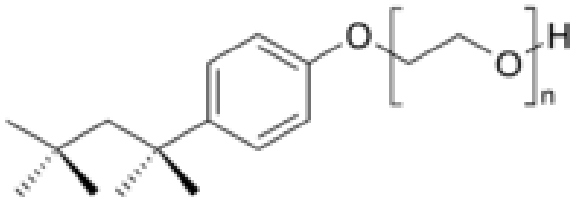


Figure 5.3. Triton x-100 structure.

5.2 Materials and methods

5.2.1 Cell cultures and culture conditions

The benthic Seminavis *robusta* 84A and culture conditions were the same described in Chapter 2.

5.2.2 Detergents

For the diatom detachment, mechanic action (by scrapers) was used as a control.

The surfactants used were sodium dodecyl sulfate (SDS), Tris Base and Triton X-100. Initially, the solutions of the SDS, tris base and Triton X-100 were prepared in mPBS in different concentrations: SDS (M= 288.38 $gmol^{-1}$ of Himedia) was used in seven different concentrations (25 g/l, 0.025 mg/mL; 0.25 mg/mL; 2.5 mg/mL; 2 % (w/v), 0.1 % (w/v) and 1 % (w/v)) (Yüksel *et al*. 2009); Tris Base (M= 11645 $gmol^{-1}$ of fisher Sciebtific) was used in three concentrations (10 mM; 17 mM; 50 mM and 50 M. And Triton X-100 (M= 646.86 $gmol^{-1}$ of Fisher Chemical) was used in six concentrations (1 % (v/v), 0.1 % (v/v), 0.001 % (v/v), 0.5 % (v/v), 0.05 % (v/v) and 0.25 % (v/v)).

To detach the cells, the culture medium was removed and 4 mL of the detergent solution was added and kept in dark overnight. Furthermore, to evaluate the effect of temperature, the culture flasks with the solutions of 2.5 mg/mL of SDS solution, 50 mM of Tris base, and 0,05 % (v/v) of Triton X-100 were made in triplicate and incubated in three different temperatures: in cold room (4 °C), at environmental temperature (25 °C) and at 30 °C.

After this, the culture was centrifuged (3000 g at 10 min), the liquid was removed and the pellet was washed with mPBS. This last step was repeated four times.

5.2.3 Viability

For viability determination was used the FDA method (described in Chapter 2).

5.3 Results and discussion

The main disadvantage of the control procedure, that is, the scraping, was the cell membrane breakage leading to the diatom death and the under-estimation of the viability assessment of the culture. For the other hand, when the scraping is used, it is not possible ensure that all diatoms are removed for the surface and the detachment sample are reproducible. Nevertheless, percentages of 80 % of viability were obtained by this method.

In this work another method for benthic diatom detach mend, *i.e.* chemical action (to use surfactants) was performed. The chemical action method aims at detaching the diatoms by destroying the adhesive mucilage that ties the walls to the flask, in a similar way as the action of trypsin in animal cell cultures.

Figure 5.4 proves that FDA was a good method to check the cell viability, since the living cells are stained with green fluorescence whereas dead cells presented no fluorescence.

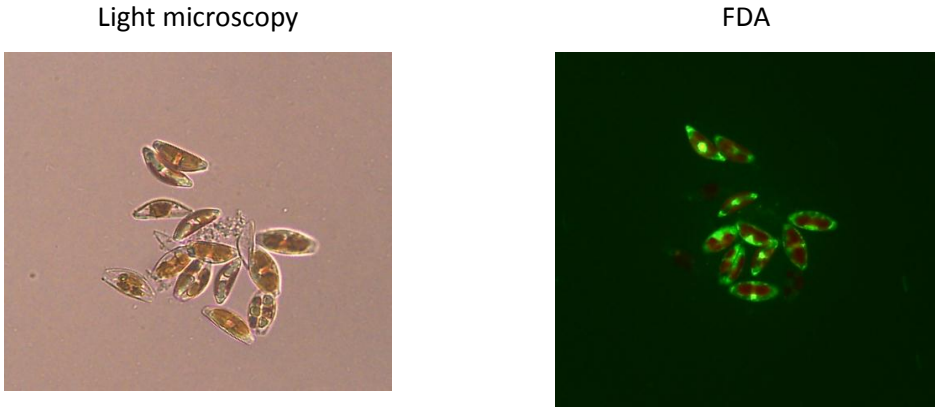


Figure 5.4 . Difference between living cell and dead cell using FDA..

In general, the surfactants concentrations were too high and besides detaching the cells, they caused cell death. Surfactants are toxic and lower concentrations should have been used to prevent toxic effects and cell death. Since these surfactants are normally used for cell lysis, higher concentrations of the surfactants were expected to result in a decrease of the cell viability. In the present work, all concentrations were above the toxic concentration and most of them caused a significant decrease in the viability of the cultures. Future work shall take this in account. The best surfactant used was the Tris base at 17 mM (cationic surfactant), the number of cells detached was similar to the number obtained with the mechanic action, using a scraper.

These problems arose because there is not much information about diatom detachment through chemical action. Furthermore, the surfactants concentration used in this work is the normally used for bacterial detachment. Control of the contact time can also be a variable to study in future works.

The surfactant effect in diatoms was affected by concentration and temperature (Table 5.1). The temperature used had a significant influence on the surfactant solubilisation: for example, the SDS was less efficient at lower temperatures (4 °C).

The detachment process was not optimized: it is necessary to find the ideal concentration and contact time for different species. It is also necessary to improve other steps such as the centrifugation time and the method for washing diatoms (mPBS washing in this case).

Table 5.1. Viability of diatom cultures after surfactant use

Technique		Viability
Scraper (mechanic action)		80 %
Tris base	0.5 M	0 %
	50 mM at 4 °C	0 %
	50 mM at 25 °C	0 %
	50 mM at 30 °C	0 %
	17 mM	70 %
Triton X-100	1 % (v/v)	0 %
	0.5 % (v/v)	0 %
	0.05% (v/v) at 30 °C	0 %
	0.05% (v/v) at 25 °C	20 %
	0.05% (v/v) at 4 °C	30 %
	0.001% (v/v)	50 %
SDS	2 % (w/v)	0 %
	1 % (w/v)	0 %
	0.5 % (w/v)	20 %
	25 mg/mL	0 %
	2.5 mg/mL at 4 °C	0 %
	2.5 mg/mL at 25 °C	0 %
	2.5 mg/mL at 30 °C	0 %
	0.25 mg/mL	30 %
	0.025 mg/mL	40 %

5.4 Conclusions

The mechanic method to detach benthic diatoms is fast, needs few work and care and maintains high viability of the cultures. However, scraping is an erratic method because the number of collected diatoms is highly variable. If we wish to achieve reproducible inoculation conditions other methods must be explored. It is necessary to optimize the process here presented using surfactants to detach the benthic cells, because the concentrations herewith presented were definitely too high for this purpose.

6 General conclusions and future prospects

Diatoms are unicellular microalgae with the ability to form complex silicate structures and with a very wide potential of applications.

The work presented in this thesis aimed at diatom characterisation, by traditional methods and by MALDI-TOF ICMS. Besides that *Coscinodiscus* sp. preservation in beads (gelatine and pectin) and the detachment of benthic diatoms were studied as they both are methods related and useful to the characterisation of diatoms: the first intends to preserve the characteristics of the cultures and the latter is crucial to get a correct sampling to further characterise the cultures.

With traditional methods, it is possible to differentiate diatoms belonging to different genus but traditional methods take a long time. These organisms present different frustule morphologies and growth rates. Diatom characterisation using MALDI-TOF ICMS was not conclusive for all diatoms, although promising. However, this study also led to the conclusion that this method is able to discriminate different diatom genus but, at least under the conditions used in this work, is not capable to differentiate isolates of the same strains.

The use of gelatine and pectin beads did not add any benefits to what already exists related to long-term preservation methods. This proves that the obscurity is a satisfactory and efficient method, and can be more practical and less laborious that immobilisation in beads. Substantial improvement can be done by changing the preparation conditions and the beads solubilisation.

Finally, promising results were obtained that pointed to the potential use of surfactants for benthic diatoms detachment. However, more studies are needed and this process needs to be optimized, namely, by using other surfactants, by identifying the optimal concentration of the surfactant and by applying different contact times.

Concerning future work, the results obtained by the present study showed that more studies are needed in order to overcome a series of unresolved questions. For example, it is necessary to study the diatom metabolism (to know the molecules that were analyzed in the MALDI-TOF) and to understand the lack of reproducibility of the MALDI-TOF spectra over time. We need to confirm that the obtained peaks are related with ribosomal proteins or other kind of macromolecules, *e.g.,* polysaccharides. If the peaks are confirmed to be ribosomal protein-related, it is needed to know the protein ribosomal mass to establish peak standards for a best MALDI-TOF analysis. Another strategywill be the development of a database with diatoms characteristic spectra. The diatoms were

analyzed and the spectra obtained for each diatom should be deposited in the database created. The spectra were obtained as the fingerprint for each diatom.

Another important feature to resolve is the low diatom concentration that usually is reached, thus new cultivation methods to increase diatom concentration are crucial.

Finally, new methods for long-term diatom preservation are required (for example, using capillaries with alginate for diatom immobilisation), since the current available preservation methods only allow diatoms to remain viable for at most one year. In preservation the diatoms with crosslink in gelatine beads used new methods for example

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Appendices

Appendices A

Calibration curves of diatoms

In order to evaluate the diatom concentration in different cell suspensions, three calibration curves correlating chlorophyll fluorescence and cell concentration for the three genera (*Seminavis robusta*, *Cosciscodiscus* and *Cyclotella meneghiniana*.) were determined. For that, different dilutions were prepared from an initial cell suspension. Cell concentration of the initial solution and of the dilutions was determined by counting cells under the microscope (Olympus). Then, chlorophyll fluorescence was evaluated by fluorimetry (Fluorescence Spectrometer Jasco FP6200; emission at 685 and excitation at 440 nm). From a linear regression, the equations in table A.1 were found:

Table A.1. Seminavis robusta, Coscinodiscus sp. and *Cyclotella meneghiniana* equation. Where, y is cell concentration (cel/ml) and x is chlorophyll fluorescence

	Equation	R^2	S_{X_0}	
Seminavis robusta	y=(1890.5±209.52)x–(133.87±123.4)	0.9908	0.028	dilutions of 1:2, 1:4, 1:8; 1:10 and 1:20
Cosciscodiscus sp.	y=(156.03±30.81)x-(49,289±19.64)	0,9886	0.0196	dilutions of 1:2, 1:4 and 1:8
Cyclotella meneghiniana	y=(1588,2±272)x-(78,87±147.87)	0,9714	0.0532	dilutions of 1:2, 1:4, 1:8; 1:10, 1:20, 1:100 and 1:150

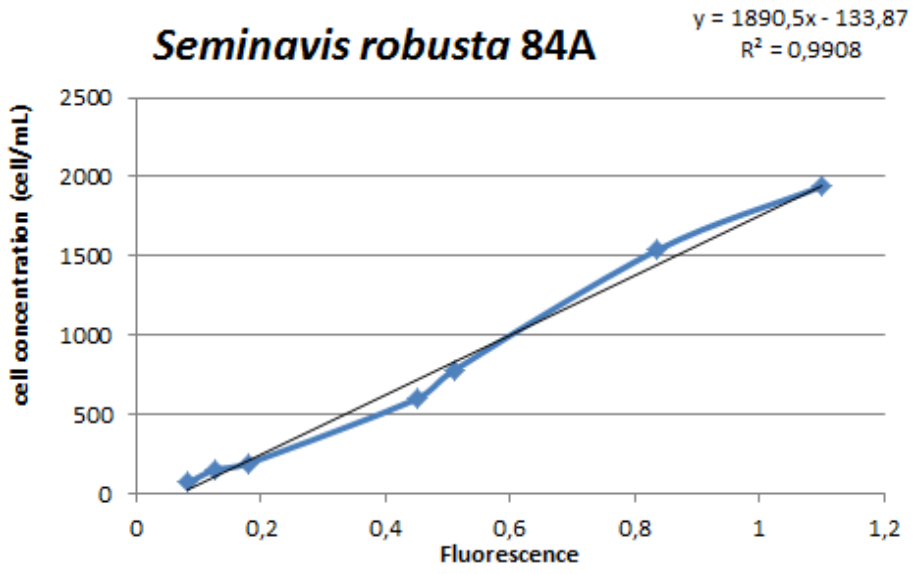

Figure A.1. Calibration curve of cell concentration for *Seminavis robusta* 84A.

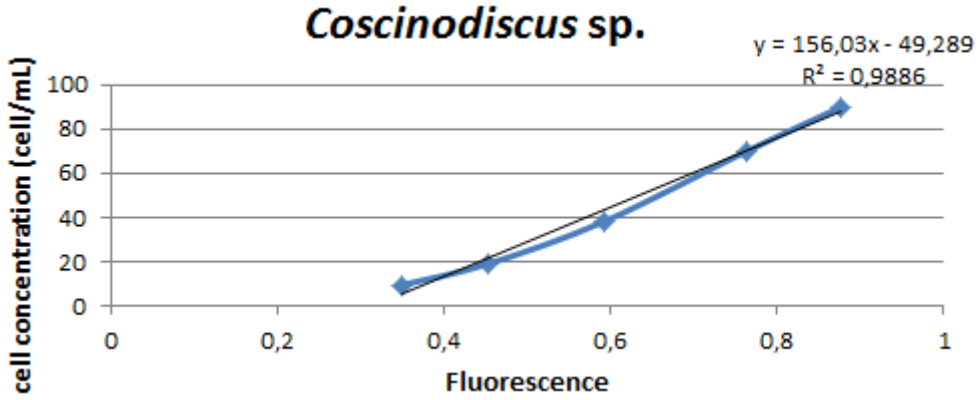

Figure A.2. Calibration curve of cell concentration for *Coscinodiscus* sp.

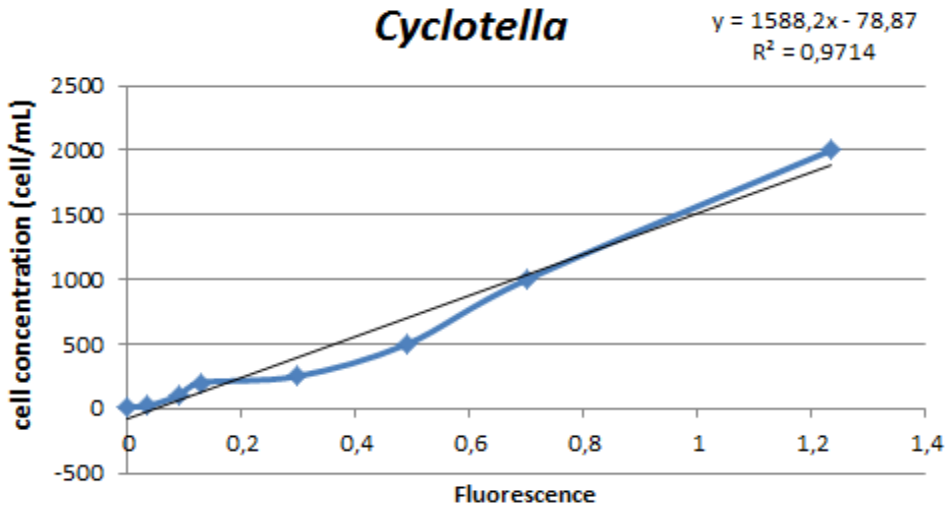

Figure A.3. Calibration curve of cell concentration for *Cyclotella meneghiniana*.

Appendices B

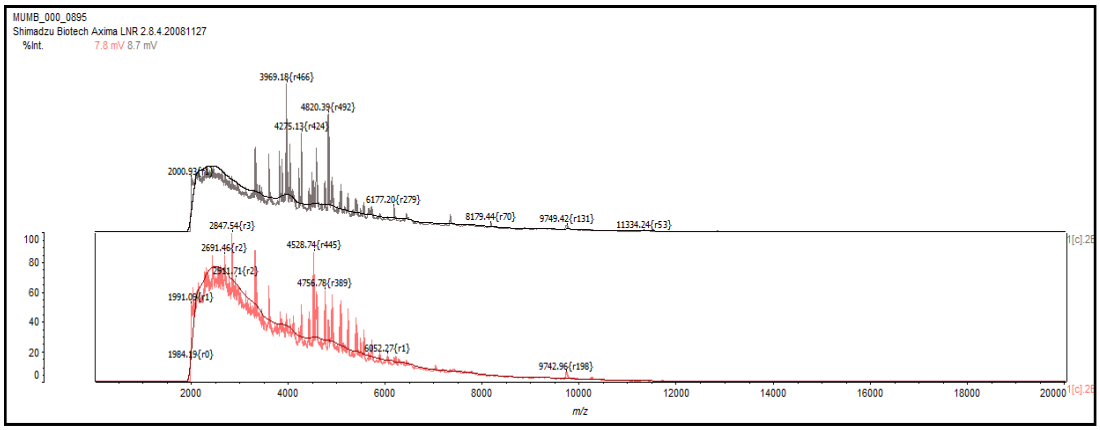

Figure B.1. Reproducible MALDI-TOF ICMS mass spectra for *Cyclotella meneghiniana* at 9 days old.

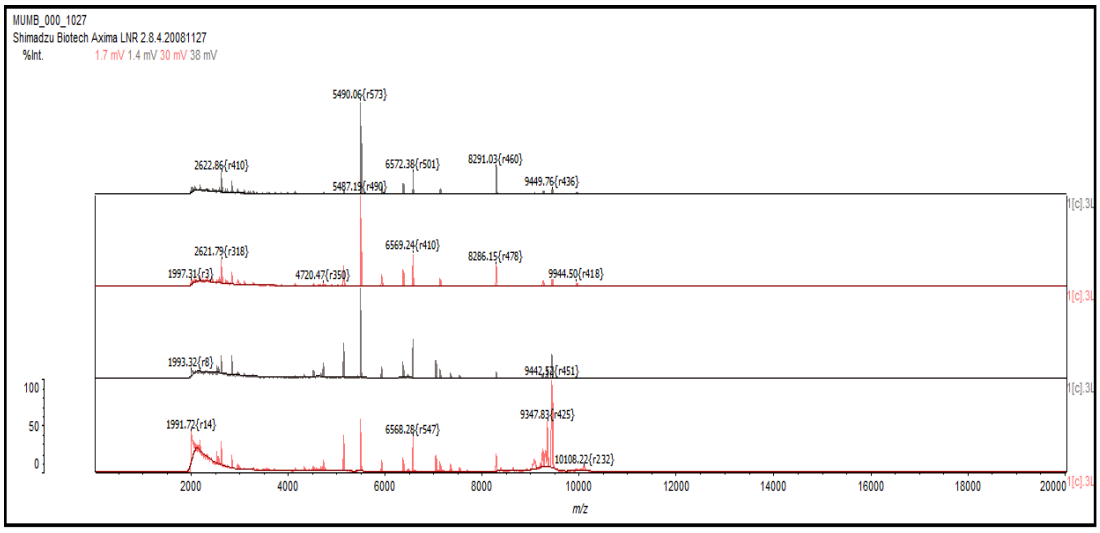

Figure B.2. Reproducible MALDI-TOF ICMS mass spectra for *Cyclotella meneghiniana* at 30 days old.

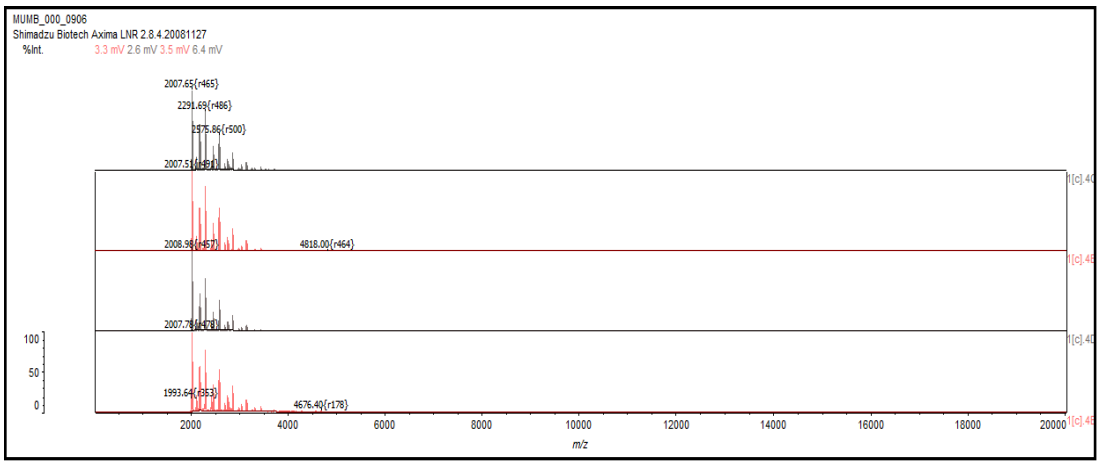

Figure B.3. Reproducible MALDI-TOF ICMS mass spectra for *Coscinodiscus sp.* at 13 days old.

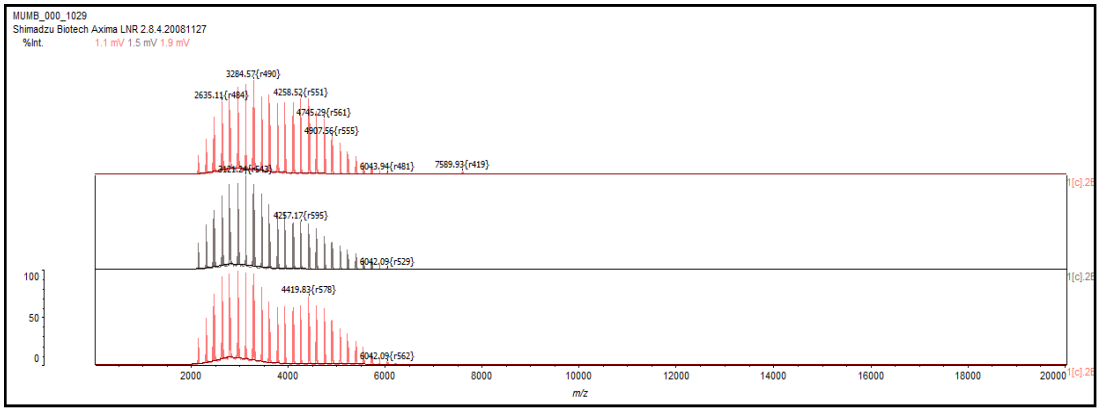

Figure B.4. Reproducible MALDI-TOF ICMS mass spectra for *Coscinodiscus sp.* at 18 days old.

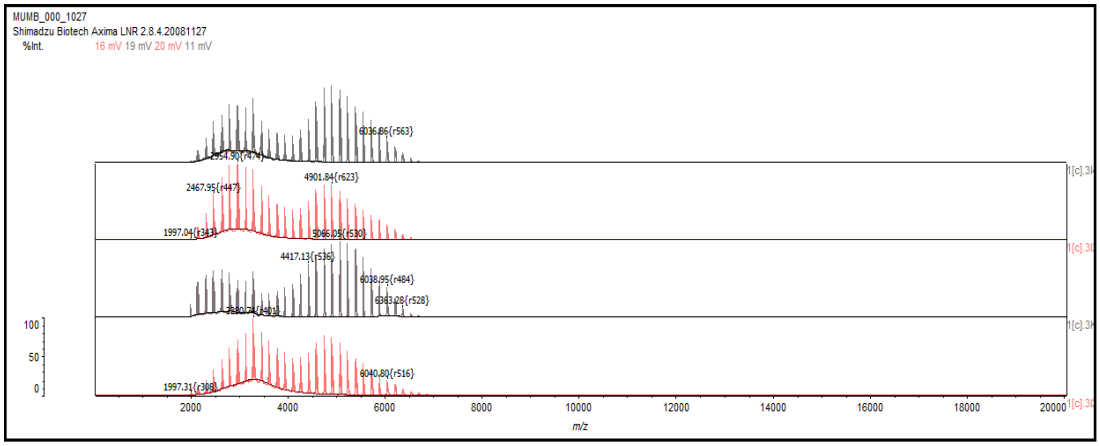

Figure B.5. Reproducible MALDI-TOF ICMS mass spectra for *Coscinodiscus sp.* at 30 days old.

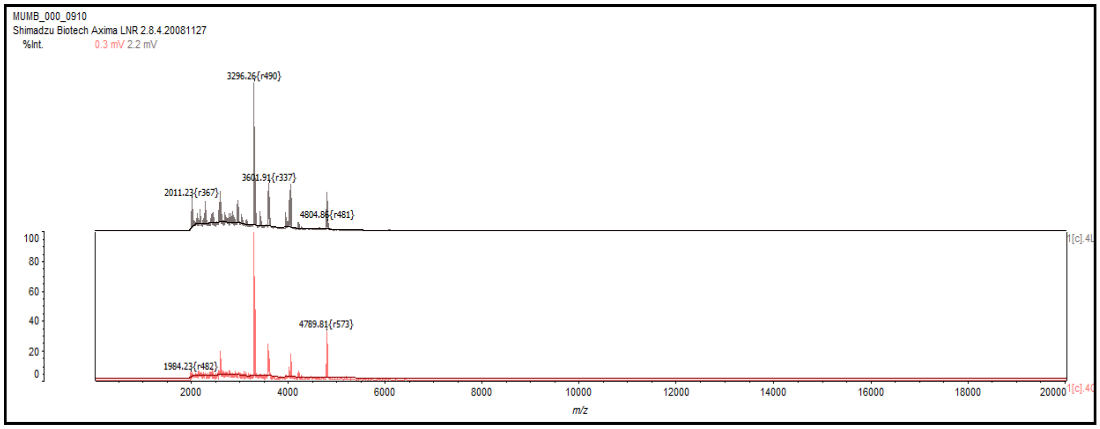

Figure B.6. Reproducible MALDI-TOF ICMS mass spectra for *Seminavis robusta* 84A at 9 days old.

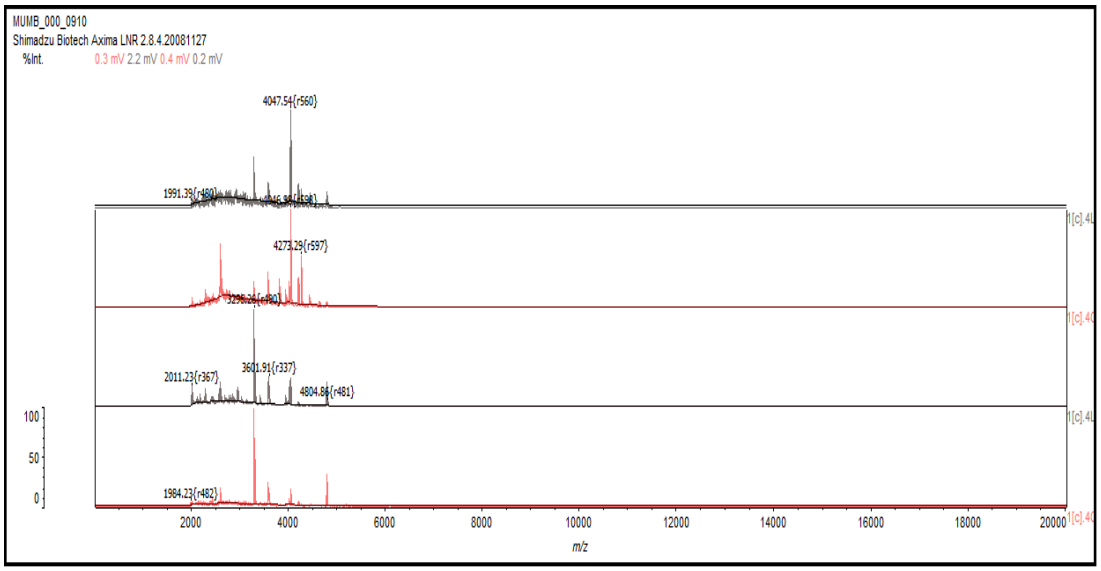

Figure B.7. Reproducible MALDI-TOF ICMS mass spectra for *Seminavis robusta* 84A at 9 days old.

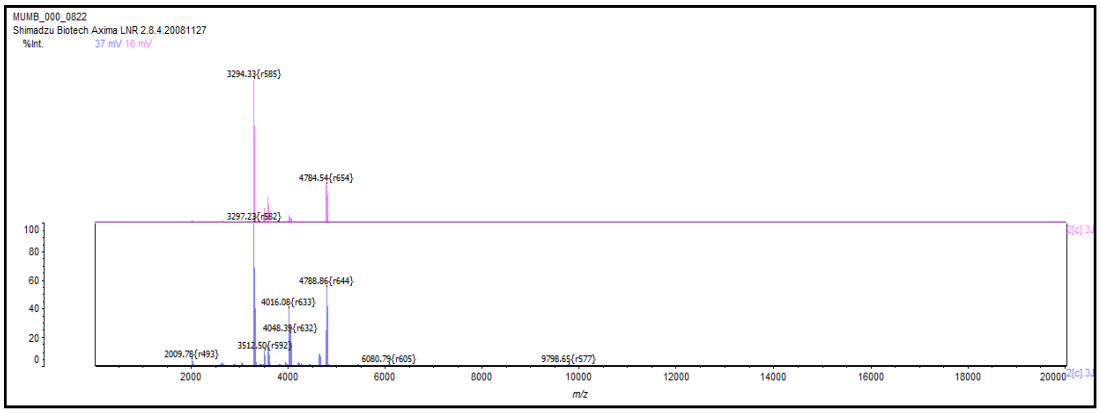

Figure B.8. Reproducible MALDI-TOF ICMS mass spectra for *Seminavis robusta* 84A at 14 days old.

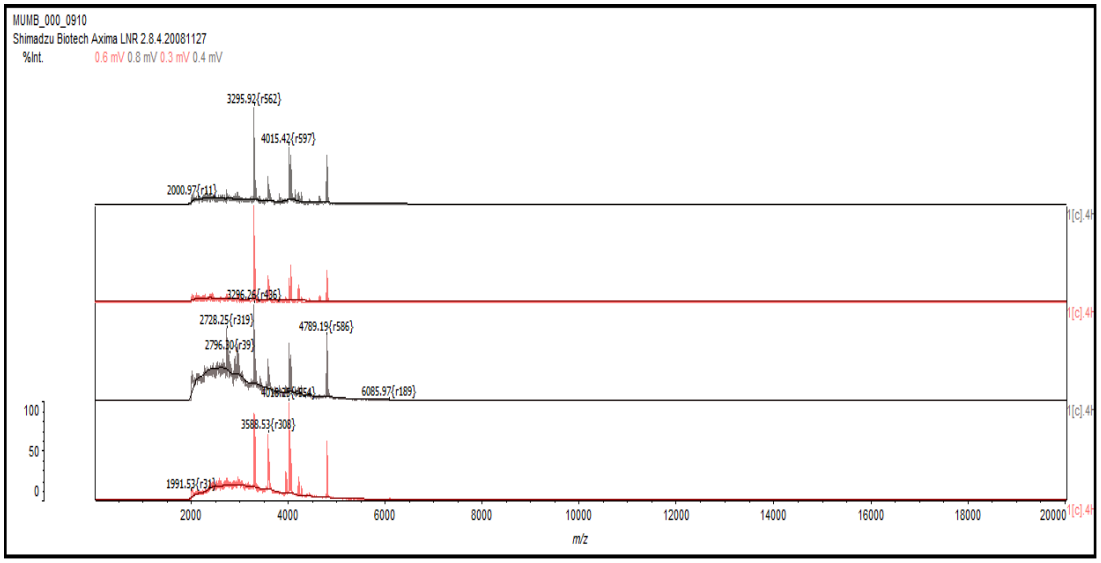

Figure B.9. Reproducible MALDI-TOF ICMS mass spectra for *Seminavis robusta* 85AS at 9 days old.

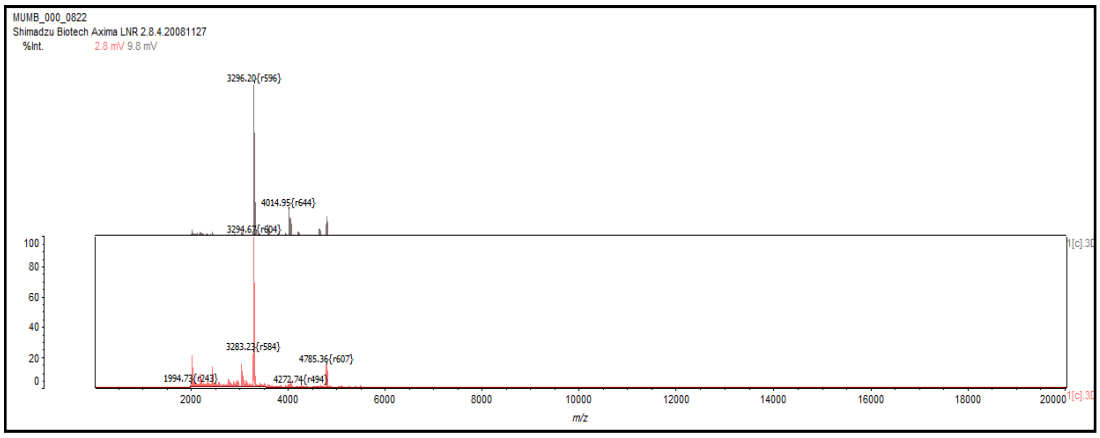

Figure B.10. Reproducible MALDI-TOF ICMS mass spectra for *Seminavis robusta* 85AS at 14 days old.

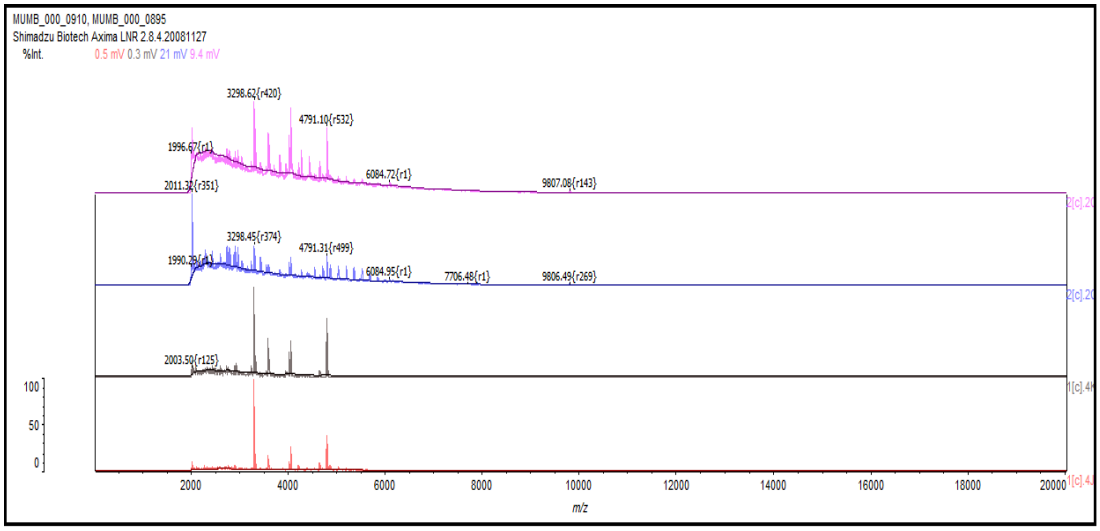

Figure B.11. Reproducible MALDI-TOF ICMS mass spectra for *Seminavis robusta* 85A at 9 days old.

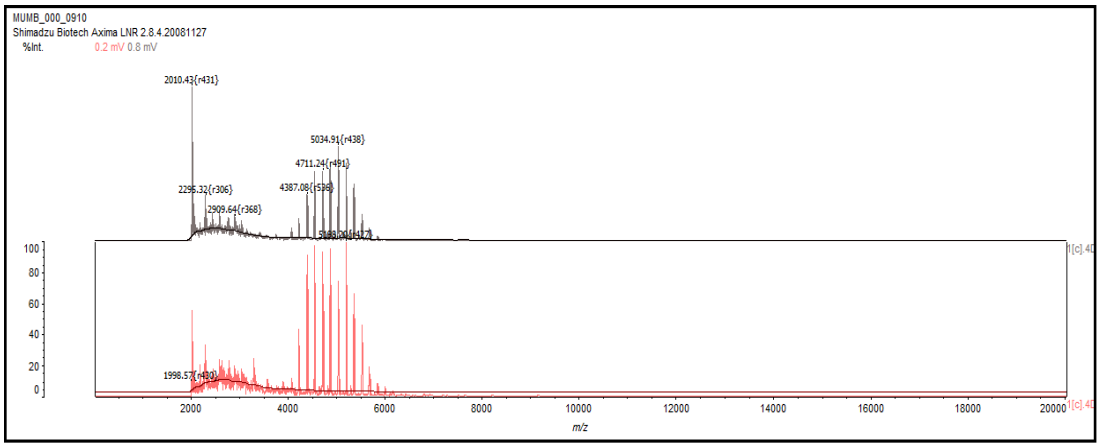

Figure B.12. Reproducible MALDI-TOF ICMS mass spectra for *Seminavis robusta* 85BS at 9 days old.