Phenotypic and genotypic characterization of Thalassiosira pseudonana (Bacillariophyceae) strains

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Phenotypic and genotypic characterization of
*Thalassiosira pseudonana* (Bacillariophyceae) strains

Cecilia Rad Menéndez
Licenciatura en Biología (University of Oviedo, Spain)
May, 2011

A thesis presented for the degree of
Master of Science by Research at the
University of Aberdeen

Partner Institute
The Scottish Association for Marine Science

Discipline
Life Sciences

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I declare that the present thesis is based on the results of investigations conducted by myself, and that it is of my own composition. This thesis has not been accepted in any previous application for a degree. All quotations have been distinguished by quotation marks and the sources of information are specifically acknowledged.

Signature:

________________________________________
Cecilia Rad Menéndez
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Abstract

Phenotypic and genotypic characterization of *Thalassiosira pseudonana* (Bacillariophyceae) strains

Cecilia Rad Menéndez

Over recent years, developments in genomics have greatly increased the quantity and quality of molecular data available to the scientific community and to date, the genome sequences of two “model” diatoms have been published: *Thalassiosira pseudonana* (Armbrust et al. 2004) and *Phaeodactylum tricornutum* (Bowler et al. 2008). These genomes have the potential to facilitate the use of genomic data to investigate diatom cell processes, increasing our understanding of these organisms and their future biotechnological applications. These developments have facilitated the genetic and phenotypic characterization of the strains of *P. tricornutum* available from culture collections (De Martino et al. 2007); however, a comparable study has not been performed for *T. pseudonana*.

In the present study a polyphasic approach has been employed to characterize ten *T. pseudonana* strains. The morphological characterization revealed that all the strains investigated were virtually identical, apart from *T. pseudonana* CS-20, which seemed to have a more weakly silicified frustule and variation in the rimoportulae position. From a biochemical perspective, based on fatty acid profiles, all the strains shared the same fatty acid composition, although variation in production was observed. Genotypic characterization based on DNA barcode genes revealed that the strains were identical; however, Amplified Fragment Length Polymorphism (AFLP) analyses indicated that the axenic strains belonged to three different clusters. Although the population variability obtained was low, it was enough to group the strains according to their biogeography. These findings will help to characterize the strains of this model organism available to researchers worldwide through culture collections. At the same time it provides valuable information about microbial populations, how they can be genetically distinct and how these differences could be related to their biogeography.

In addition, this study revealed that an optimal cryopreservation protocol has yet to be developed for this particular species, and perhaps different protocols will be needed for different strains.
List of salient points

* Scanning electron microscopy observations revealed that there is not morphological
  plasticity within the strains of *T. pseudonana* investigated, apart from strain CS-20.
  (Chapter 2).

* Chemotaxonomic markers such as fatty acid profiles were identical for all the *T.
  pseudonana* strains investigated, although variation was observed in fatty acid production
  (Chapter 3).

* DNA barcoding genes proposed for diatoms (both nuclear and plastid), shared
  identical sequences for all *T. pseudonana* strains studied (Chapter 4).

* Whole genome approach (AFLP) differentiated between six axenic strains of *T.
  pseudonana* (Chapter 4), grouping them in three clusters which seem to be related to the
  strains biogeographies.

* A range of cryopreservation protocols were assessed for the long-term maintenance
  of this model diatom, however, although some of the strains regenerated healthy cultures
  after treatment, the time taken to regenerate indicated that an optimal cryopreservation
  protocol has yet to be developed, and that different protocols may be needed for the
  successful cryopreservation of the different strains.
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<tr>
<td>Ax</td>
<td>Absorbance at Xnm</td>
</tr>
<tr>
<td>AFLP</td>
<td>Amplified fragment length polymorphism</td>
</tr>
<tr>
<td>ANACC</td>
<td>Australian National Algae Culture Collection</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BRC</td>
<td>Biological Resource Centres</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>CCAP</td>
<td>Culture Collection of Algae and Protozoa</td>
</tr>
<tr>
<td>CCMP</td>
<td>Provasoli-Guillard National Center for Culture of Marine Phytoplankton</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>cox</td>
<td>Cytochrome c oxidase</td>
</tr>
<tr>
<td>CS</td>
<td>CSIRO (Australian National Algae Culture Collection)</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential interference contrast</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulphoxide</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EPA</td>
<td>Eicosapentaenoic acid</td>
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<td>Ethidium bromide</td>
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<td>Fatty acid</td>
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<td>FAMEs</td>
<td>Fatty Acid Methyl Esters</td>
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<td>FDA</td>
<td>Fluorescein diacetate</td>
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<tr>
<td>g</td>
<td>Gravitational force</td>
</tr>
<tr>
<td>GC-FID</td>
<td>Gas chromatograph-flame ionization detector</td>
</tr>
<tr>
<td>h.</td>
<td>Hour</td>
</tr>
<tr>
<td>ITS</td>
<td>Internal transcribed spacer</td>
</tr>
<tr>
<td>kPa</td>
<td>Kilopascal</td>
</tr>
<tr>
<td>LN2</td>
<td>Liquid Nitrogen</td>
</tr>
<tr>
<td>LP$_5$</td>
<td>Labiate processes</td>
</tr>
<tr>
<td>LSU</td>
<td>rRNA large subunit ribosomal RNA</td>
</tr>
<tr>
<td>MALDI-TOF ICMS</td>
<td>Matrix Assisted Laser Desorption Ionisation – Time Of Flight Intact Cell Mass Spectrometry</td>
</tr>
<tr>
<td>min</td>
<td>Minute/s</td>
</tr>
<tr>
<td>ML</td>
<td>Maximum likelihood</td>
</tr>
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<td>MP</td>
<td>Maximum parsimony</td>
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<td>NHM</td>
<td>Natural History Museum</td>
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<td>Neighbour-joining</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PUFAs</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>r</td>
<td>Pearson correlation coefficient</td>
</tr>
<tr>
<td>RAPD</td>
<td>Random amplified polymorphic DNA</td>
</tr>
<tr>
<td>rbcL</td>
<td>Ribulose-1,5-bisphosphatase carboxylase oxygenase (RuBisCO) large subunit</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SAG</td>
<td>Culture Collection of Algae at Göttingen University</td>
</tr>
<tr>
<td>SAMS</td>
<td>Scottish Association for Marine Science</td>
</tr>
<tr>
<td>SDV</td>
<td>Silica deposition vesicle</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscope</td>
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<tr>
<td>sp.</td>
<td>Species</td>
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<tr>
<td>SPs</td>
<td>Strutted processes</td>
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<tr>
<td>SSU</td>
<td>Small subunit ribosomal DNA</td>
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<tr>
<td>TAGs</td>
<td>Triacylglycerols</td>
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<td>TBE</td>
<td>Tris-borate-EDTA</td>
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<td>T. pseudonana</td>
<td><em>Thalassiosira pseudonana</em></td>
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<td>T. punctigera</td>
<td><em>Thalassiosira punctigera</em></td>
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<td><em>Thalassiosira rotula</em></td>
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<td>T. tumida</td>
<td><em>Thalassiosira tumida</em></td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>W</td>
<td>Watt</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
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Para mamá, mi vida
To mum, my life
1 Chapter 1. General Introduction

1.1 Diatoms, valuable natural resources

Diatoms (Class Bacillariophyceae) are unicellular, photosynthetic, eukaryote microorganisms, believed to have “appeared” around 200 million years ago (Medlin et al. 1997) as a result of a secondary endosymbiosis between a red eukaryotic alga and a heterotrophic flagellate (Medlin et al. 1997, 2000). They are successful organisms of great ecological significance and thus, can be found globally in almost all water bodies, and are often one of the main components of the phytoplankton (Round et al. 1990). They comprise a diverse group of organisms and the number of species has been estimated to be more than $10^5$ (Mann & Droop, 1996).

The main feature characterizing diatoms is their silica (SiO$_2$) cell wall called a frustule (Round et al. 1990). The frustule is composed of two valves (thecae), an epitheca (larger) and a hypotheca (smaller), these fit together in a similar fashion to a Petri-dish and between them are girdle bands (Fig. 1.1, step 1). Diatom reproduction is mainly asexual, with cell division, involving the production of two daughter cells; each of them retains one of the two parental valves and “grows” the smaller valve (hypotheca). This process results in size reduction of diatom cells at every division (Fig. 1.1, steps 1-6). To regain size, diatoms form an auxospore through sexual reproduction; this auxospore will grow and develop to produce a new frustule, thus restoring the diatom to its original size (Fig. 1.1, steps 1-9). The factors that activate sexual reproduction are not yet well understood, but it has been suggested that favourable environmental factors such as temperature and light, can induce auxospore production (Karsten, 1899). Electron microscopic studies have revealed that diatom valves and girdle bands are formed in specialized intracellular compartments named silica deposition vesicles (SDV, Fig. 1.1, step 2) (Drum & Pankratz, 1964). Since the structure of the frustules is specific to the species, numerous studies have
been focused on elucidating the molecular mechanisms of silica formation, and diatoms have been chosen as model organisms for the study of silica biomineralization processes (Sumper & Brunner, 2006, Hildebrand, 2008, Kröger & Poulsen, 2008, Brunner et al. 2009).

Over recent years, developments in genomics have greatly increased the quantity and quality of molecular data available to the scientific community. In phycology there has been particular emphasis on the sequencing of “model” organisms (Gachon et al. 2007), as well as the use of molecular markers to elucidate relationships at Division, Class, Order, Genus, Species and subspecies levels (Brodie & Lewis, 2007, Evans et al. 2007, Alverson, 2008, Coleman, 2009, Moniz & Kaczmarska, 2009, 2010). To date, the genome sequences of two “model” diatoms have been published: *Thalassiosira pseudonana* (Armbrust et al. 2004) and *Phaeodactylum tricornutum* (Bowler et al. 2008). These genomes have the potential to facilitate the use of genomic data to investigate diatom cell processes and their future biotechnological exploitation.

Diatoms are exceptional organisms of great importance and interest across an extensive range of disciplines. They are known to be one of the main phytoplankton components and thus, of great importance in the food chain. Moreover, they play an essential role in biochemical/biogeochemical cycles, being responsible for approximately 40% of marine primary production (Falkowski et al. 1998).

Diatoms frustules remain intact after the death of the cell and have, as a result, accumulated on the sea floor over long periods of time, forming deposits of diatomaceous earth (Lizitzin, 1967). This is of great importance for paleontological and geological studies providing markers and facilitating the estimation of salinity and nutrient concentrations in different regions of the world over geologic time (Reavie & Edlund, 2010, Mackay et al. 2010). Furthermore, diatoms have been used as indicators, of water
quality (Schoeman, 1979, Guzkowska & Gasse, 1990) and environmental change (Douglas & Smol, 2010, Spaulding et al. 2010) amongst others. In addition, studies on diatoms cell wall development and structure, have influenced biotechnological applications such as silica biomineralization processes (Sumper & Brunner, 2006) and the production of nanostructures in general (Noll et al. 2002, Hildebrand, 2003). The study of the processes involved in the formation of diatoms cell wall’s intricate patterns have become very important for materials scientists due to the properties of these natural materials, and what it could represent extrapolated to synthetic materials (Brunner et al. 2009).
Fig. 1.1 Diatom cell cycle. (Adapted from Kröger, 2007)

Steps 1-6 Asexual reproduction, resolving in size reduction.
Step 1 Diatom frustule, Step 2 Formation of the silica deposition vesicles (SDV).
Steps 7-8 Sexual reproduction, auxospore formation and regain of original size.
1.2 *Thalassiosira pseudonana*

*T. pseudonana* is a centric (radially symmetrical) diatom widely distributed in nature (Lowe & Busch, 1975, Belcher & Swale, 1977, 1986, Hasle, 1978, Ake-Castillo et al. 1999). Although there are many publications on this taxon (e.g. Volkman et al. 1989, Armbrust et al. 2004, Tonon et al. 2004, Alverson et al. 2007, Dassow et al. 2008, Hildebrand et al. 2009), only ten isolates have been deposited in the major culture collections worldwide (Table 1.1). Most culture collections maintain different isolates of the same species to ensure the conservation of a range of phenotypic and genotypic characteristics normally found within specific taxa (De Martino et al. 2007).

*T. pseudonana* plays an important role in aquaculture as a food organism; this has stimulated numerous studies on its lipid and fatty acid composition revealing that it contains high levels of lipids in the form of triacylglycerols (TAGs), making them candidates for aquaculture feeds and as a potential feedstock for biodiesel (Volkman & Hallegraeff, 1988, Mansour et al. 2005, Yu et al. 2009). In addition, due to the intricate diatom silica patterns and their division rates, diatoms have been used as a source of nanostructured materials (Hildebrand, 2005, Kröger, 2007) and some of the molecules involved in silica formation have been recently identified (Sumper & Kröger, 2004). *T. pseudonana* has been chosen to study silica cell wall formation due to the relatively simple silica structure and the availability of molecular data for this species (Hildebrand et al. 2007, 2009). Furthermore, *T. pseudonana* has been employed as a model organism for marine environmental studies including: the investigation of the effects of toxicity of metals (Rijstenbil et al. 1994); studies on the impact of heavy metals (Davis et al. 2006) and more recently investigations related to the effects of organic pollutants (Carvalho et al. 2011).
Chapter 1
General introduction

*T. pseudonana* was the first diatom chosen for whole genome sequencing (Armbrust et al. 2004). It was selected for this study because of its wide geographical distribution (Lowe & Busch, 1975, Belcher & Swale, 1977, 1986, Hasle, 1978), because it is already used as a model organism for many studies (Hildebrand et al. 2007, 2009, Carvalho et al. 2011), and due to its relatively small genome size of 34 million base pairs (Armbrust et al. 2004).

The above highlights the importance of this diatom species and the necessity to undertake phenotypic and genetic characterization of the different isolates of this model organism.

![Fig. 1.2. Scanning electron micrograph of *T. pseudonana* CCMP1335.](image)

A. Girdle view  
B. Valve view
Table 1.1 List of the strains used in this study.

<table>
<thead>
<tr>
<th>Strain ID</th>
<th>Geographical origin</th>
<th>Habitat</th>
<th>Ocean</th>
<th>Isolator</th>
<th>Year of isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCMP1007</td>
<td>37.9500N 79.9400W Chincoteague, Virginia USA</td>
<td>Neritic</td>
<td>North Atlantic</td>
<td>unknown</td>
<td>unknown</td>
</tr>
<tr>
<td>CCMP1011</td>
<td>17.7900N 64.8200W St. Croix, US Virgin Islands (approx.)</td>
<td>Neritic</td>
<td>North Atlantic</td>
<td>K. Haines</td>
<td>unknown</td>
</tr>
<tr>
<td>CCMP1012</td>
<td>31.9900S 115.8333E Swan River Estuary, Perth, Western Australia</td>
<td>Estuarine</td>
<td>Caribbean Sea</td>
<td>R. Guillard and R. Davis</td>
<td>1965</td>
</tr>
<tr>
<td>CCMP1013</td>
<td>53.2833N 03.8333W Conwy, Gwynedd, Wales, UK</td>
<td>Estuarine</td>
<td>North Atlantic</td>
<td>E. Paasche</td>
<td>1973</td>
</tr>
<tr>
<td>CCMP1014</td>
<td>28.000N 155.0000W North Pacific Gyre (very approx.)</td>
<td>Open ocean</td>
<td>North Pacific</td>
<td>J. Jordan</td>
<td>1971</td>
</tr>
<tr>
<td>CCMP1015</td>
<td>48.5400N 123.0100W San Juan Island WA USA (approx.)</td>
<td>Estuarine</td>
<td>North Pacific, Strait of Georgia</td>
<td>unknown</td>
<td>before 1985</td>
</tr>
<tr>
<td>SAG1020-1b</td>
<td>River Werra near Witzenhausen, Germany</td>
<td>Brackish</td>
<td></td>
<td>O. Pringsheim</td>
<td>1959</td>
</tr>
<tr>
<td>CS-20</td>
<td>31.9900S 115.8333E Swan River Estuary, Perth, Western Australia</td>
<td>Estuarine</td>
<td>Indian</td>
<td>unknown</td>
<td>before 1972¹</td>
</tr>
</tbody>
</table>


¹ Ian Jameson personal communication
1.3 Importance of intraspecific characterization of microalgae

Microalgae play an important role in ecological processes, as well as biotechnological applications and have been chosen as model organisms for a large number of studies (see section 1.2). Studies on these applications have an absolute requirement for phenotypically and genotypically characterized algal strains. The main aim of culture collections is to supply well characterized and healthy cultures to users worldwide; these are effectively “biological standards” on which the science is built (Day & Stacey, 2008). It is therefore critical to be able to discriminate between different strains of the same species in order to ensure that the information provided to the users is accurate, and that the same strains are employed in sequential experiments in different labs. Furthermore, an intra-species characterization will increase the possibility to investigate different strains of a given species for phenotypic variations or even polymorphisms in a specific gene, thus, allowing us to identify the most suitable strain for a particular study.

1.3.1 Intraspecific phenotypic characterization

1.3.1.1 Morphological characterization

For some taxa, several accessions of the same algal species are held in culture collections worldwide, for example 55 strains of the Chlorophyte *Chlorella vulgaris* are available from the major culture collections (Müller et al. 2005). Although morphological identification is required for the deposition of new isolates in all the major collections, no “exhaustive” morphological characterization is undertaken when strains are accessed due to lack of resources and time limitations. Intraspecific phenotypic characterization has proven to be of great importance as a means to define the strains available in the collections (Müller et al. 2005, De Martino et al. 2007, Vesela et al. 2009) and provides researchers worldwide with a phenotypic overview of the characteristics of the strains available for a particular species. This is particularly important for diatoms, where size
reduction over generations, results in the loss of morphological characters. Ideally when a new diatom strain is accessed into a culture collection, phenotypic characterization including micrographs should be performed. This would allow any subsequent morphological changes to be monitored and/or compared.

1.3.1.2 Chemotaxonomy

Classification of taxa according to the biochemical composition has been used in microalgae, especially in studies where other types of taxonomy are problematic (Marshall et al. 2002). Phytoplankton have been successfully characterized on the basis of photosynthetic pigments (Garibotti et al. 2003) and fatty acid composition (Viso & Marty, 1993, Marshall et al. 2002). However, one of the main constraints on the use of a chemotaxonomic approach is that many of the biochemical compounds vary with culture conditions and the stage the organism is in its life cycle. For example, Schlüter et al. (2000) observed that cell pigment ratios varied depending on growth conditions. Furthermore, lipid profiles may also vary with culture conditions (Fisher & Schwarzenbach, 1978, Dunstan et al. 1993) and total lipid content is generally higher in the stationary phase of diatom species (Dunstan et al. 1994). It is therefore critical to carefully design these types of experiments, establishing identical culture conditions, and harvesting cells at the same stage in the life cycle. Despite these constraints, chemotaxonomy offers an alternative approach to the identification of microalgae.

1.3.2 Intraspecific genetic characterization

Originally diatom taxonomy was primarily based on light microscope observations of the morphology of their cell wall (frustule). The use of electron microscope resulted in a significant advance for taxonomy, since it revealed additional characters not observable by light microscopy, and facilitated the description of many new diatom species (Round et al.
1990). However, recent advances in molecular techniques and tools have demonstrated that diatom diversity is even greater than originally thought (Mann et al. 2003, Amato et al. 2007). These new techniques have demonstrated that many species, whose descriptions were based on morphological characters, were actually comprised of a number of cryptic species, with identical, or almost identical morphologies, but differing in genotype and reproductively isolated (Amato et al. 2007, Mann et al. 2008). Furthermore, diatoms cell division involves the size reduction of the cell (Fig. 1.1, steps 1-6), which in many cultured isolates translates in the total loss of shape and morphological characters over time, making phenotypic characterization impossible.

Developments in molecular techniques have identified a variety of molecular markers, including a fragment of the mitochondrial gene citochrome c oxidase (\textit{cox1}), the large subunit of RUBISCO (\textit{rbcL}), the small subunit of the ribosomal DNA (SSU) and the internal transcribed spacer regions (ITS1+ ITS2), capable of distinguishing between species (Evans et al. 2007, Moniz & Kaczmarska, 2010, Hamsher et al. 2011). Furthermore, the use of DNA fingerprinting techniques that produce multi-locus banding patterns may be employed to characterize geographic isolates and closely related species (John et al. 2004). A variety of fingerprinting techniques have been successfully used in microalgal studies, including: Random Amplified Polymorphic DNA (RAPD), (Williams et al. 1990, Barker et al. 1994, Baillie et al. 2000); Amplified Fragment Length Polymorphism (AFLP), (Vos et al. 1995, John et al. 2004, Müller et al. 2005, De Martino et al. 2007) and microsatellites (Rynearson & Armbrust, 2000, Evans et al. 2009). In particular AFLP, is highly sensitive and reproducible, it is a whole genome approach and detects DNA polymorphisms in different genomic regions without the requirements for previous sequence knowledge of the organism investigated. It has the potential to differentiate between very closely related organisms (Müller et al. 2005) and the study of their biogeographies (John et al. 2004, Evans et al. 2008).
1.4 Phytoplankton biogeographies

One currently controversial view on phytoplankton biogeography is that microbial species are cosmopolitan and therefore their speciation rates are low (Finlay & Clark, 1999, Finlay, 2002). This concept has been based on morphologically described species, and more recently, with the development of molecular techniques, studies have revealed that some of these morphologically described species were, actually, a number of cryptic species (i.e. morphologically identical, or almost identical, but genetically distinct) (John et al. 2004, Sarno et al. 2005, 2007, Amato et al. 2007, Evans et al. 2008, Kooistra et al. 2008), confirming that speciation rates are higher than originally believed. It is clear that morphological studies may not always be able to resolve species identification, but a combined approach with molecular techniques has been able to reveal cryptic species (Sarno et al. 2005, Amato et al. 2007, De Martino et al. 2007, Evans et al. 2008, Kooistra et al. 2008), and in some cases demonstrate biogeographic patterns (Casteleyn et al. 2008, Kooistra et al. 2008).

1.5 Aims of the project

The aim of this project was to characterize T. pseudonana isolates obtained from various culture collections using a polyphasic approach. The main objective was to assess whether there was measurable intraspecific phenotypic and genotypic variation and/or distinctive biogeographic distribution patterns between the different isolates. This was important to characterize the main strains of this model organism available in culture collections worldwide, allowing researchers to identify the most suitable strain for a particular study. Furthermore, the study of genetic diversity of phytoplankton populations should allow us to resolve some of the “mysteries” surrounding phytoplankton dispersal, speciation and biogeographies aiding us in the better understanding of the microbial world.
Finally, we tested a range of cryopreservation protocols to elucidate if this model organism could be preserved in a stable way (reducing contamination risks, genetic drift and phenotypic change) and thus, improving the culture collections service to the user community, as well as the aquaculture industry that uses microalgae as a feed for larvae and juvenile shellfish.

The overall hypothesis proposed for this study was that all the strains of *T. pseudonana* are distinct. The individual hypotheses were as follows:

* There is morphological plasticity within the taxon *T. pseudonana* (Chapter 2).

* Chemotaxonomic markers have the potential to differentiate between strains of *T. pseudonana* (Chapter 3).

* DNA barcoding genes can be employed to differentiate *T. pseudonana* strains (Chapter 4).

* Whole genome approaches are needed to differentiate between strains of *T. pseudonana* (Chapter 4).

* Genomic variation is expected between *T. pseudonana* strains according to biogeographic distribution patterns (Chapter 4).

* Cryopreservation ensures genotypic and phenotypic stability of *T. pseudonana* (Chapter 5).
The specific objectives established to test these hypotheses were:

* To characterize the morphology of the different strains using light and scanning electron microscopy (Chapter 2).

* To characterize fatty acid profiles for the axenic strains as a chemotaxonomic approach (Chapter 3).

* To sequence the molecular markers proposed for diatom barcode, including two nuclear (SSU and ITS), one plastid ($rbcL$) and one mitochondrial marker ($c_ox1$) to investigate intraspecific variation (Chapter 4).

* To further characterize the genotype of the axenic strains by using a fingerprinting approach that will screen the whole genome for variations (AFLP) (Chapter 4).

* To find a suitable cryopreservation method for the strains and compare phenotypic and genotypic stability pre and post-treatment (Chapter 5).
2 Chapter 2. Phenotypic characterization of *Thalassiosira pseudonana* isolates

2.1 Introduction

*Thalassiosira pseudonana* was first described as *Cyclotella nana* by Hustedt (1957) based on light microscopy. Due to the small size of this diatom as well as the lack of characters revealed by light microscopy, he subsequently revisited his description and, based on electron microscopical observations, proposed a taxonomic revision of the species (Hustedt, 1959). A few years later, electron micrographs confirmed Hustedt’s observations, revealing that the species was not a *Cyclotella*, but a *Thalassiosira* species (Guillard & Ryther, 1962). The formal taxonomic revision was published years later, and *Cyclotella nana* was renamed *Thalassiosira pseudonana* (Hasle & Heimdal, 1970). In recent years, Alverson, (Alverson et al. 2007) has shown that *T. pseudonana* is, in fact, genetically close to *Cyclotella*, and has suggested that it is now questionable whether Hasle and Heimdal should have removed it from *Cyclotella* in the first place. Hasle and Heimdal (1970) based their description on both, light and electron microscopy, observing a certain degree of variability among individual cells of the same culture with the following main characteristics: “specimens 4-9 µm in diameter and weakly silicified; most of the specimens with a central rosette of variable size close to the central process; some specimens with irregular radial ribs showing no areolae; others with equilateral, polygonal areolae in the valve centre, elongated at the middle and equilateral polygonal areolae in the marginal zone; and other specimens with well developed areolae over the entire valve surface. Number of marginal processes 8-17, with a rectangular or oval slit at about the same distance from the valve margin as the marginal processes” (Hasle and Heimdal, 1970). They observed considerable morphological variability among specimens, as previously observed in other clonal cultures of *Thalassiosira* (Hasle, 1976).
Phenotypic plasticity in culture has been investigated for a wide range of microorganisms (Ryan et al. 2002, Paasche, 2001, Lakeman et al. 2009), confirming that organisms in culture may be affected by selective pressures due to continued culture, showing phenotypic plasticity over time. Culture conditions may play a selective role, favouring a particular phenotype within the culture, and phenotypic plasticity may also be induced as a result of selective pressures in culture growth phases, such as stationary phase where the cell density is very high and so the competence among cells. Furthermore, genotypic changes, such as mutations, and genetic drift (Paasche, 2001, Lakeman et al. 2009) could also result in phenotypic plasticity. As different isolates of *T. pseudonana* have been maintained in culture collections for decades (Table 1.1) these warrant investigation.

The main aim of the work described in this chapter was to assess the level of phenotypic plasticity between different strains of the same centric diatom species, *T. pseudonana*, that are available from major culture collections, to test the hypothesis that there is morphological plasticity within the taxon *T. pseudonana*. The objective was to assess whether there was measurable intraspecific variation and/or distinct biogeographic distribution patterns between the different isolates. To test if slight differences in culture conditions and regimes will have an impact in the phenotype of this species, the morphology of the same strain held in two different culture collections for decades was compared. Furthermore, since cryopreservation is the technique chosen for long-term storage of stable cultures, the strain CCMP1335 was studied before and after cryopreservation to assess the success of the technique in preserving the morphology of this model organism.
Chapter 2
Phenotypic characterization of *Thalassiosira pseudonana* isolates

### 2.2 Materials and methods

#### 2.2.1 Strains analysed and culture conditions

Six *T. pseudonana* strains were analysed for phenotypic plasticity. Four of them were obtained from the Provasoli-Guillard National Centre for culture of Marine Phytoplankton (http://ccmp.bigelow.org/) CCMP1013, CCMP1014, CCMP1015 and CCMP1335. The other two CS-20 and CS-173 were supplied from the Australian National Algae Culture Collection (ANACC) for further details see Table 1.1. All the strains were grown in 100ml Erlenmeyer flasks containing 50ml sterile F/2 medium (Guillard, 1975) plus sodium metasilicate, referred here as F/2+Si. The culture conditions were standardised, incubated at 15°C under a 12/12h light/dark regime (irradiance: 100 µmol. photons.m⁻².s⁻¹). All the strains were tested for axenicity (axenic refers to the culture of an organism completely free from other organisms) on media containing nutrient agar. Cells were harvested in stationary phase; a small volume of each strain was removed for light microscopy examination, and 5ml used to prepare SEM specimens (see Section 2.2.2).

To investigate phenotypic stability after cryopreservation, one of the strains, *T. pseudonana* CCMP1335, was cryopreserved as described in Chapter 5, Section 5.2.3.1. This strain was thawed 24 h. after cryopreservation, inoculated in 10ml of fresh medium (F/2+Si) and incubated covered with aluminium foil, in darkness, at 20°C to reduce the effect of light-induced stresses or metabolic uncoupling. After 12 h., the aluminium foil was removed and the culture incubated for another 36 h. After this time, an aliquot of 5ml was removed and prepared for the SEM as described below.
2.2.2 Sample preparation for SEM

To prepare the samples for electron microscopy, a sub-sample from each of the six axenic cultures was acid washed following the method of Lundholm et al. (2002a), in order to remove all the organic material from the cells. About 5ml of each culture were poured into a 100ml Erlenmeyer conical flask; 2ml of 30% sulphuric acid (H$_2$SO$_4$) plus 10ml of saturated potassium permanganate (KMNO$_4$) were added. The mixture was left for 24 h., occasionally swirled, and after this time, 5-10ml of oxalic acid (COOH)$_2$ were added. Once the samples were clear, they were divided into centrifuge tubes and centrifuged for 20 min at 3000 rpm in a SIGMA 1-14 microcentrifuge (Sigma-Aldrich Ltd., Dorset, UK). The supernatant was decanted off and the samples washed in distilled water for a total of five cycles. Surplus cleaned diatoms were stored in glass vials, after exchanging water with absolute ethanol.

Cleaned diatom frustules were concentrated on 13mm cover glasses (Menzel-glaser), previously mounted on SEM aluminium stubs (Agar Scientific LTD, Essex, England). The stubs were coated using gold palladium; applied at a thickness of 5nm using a Cressington 208HR sputter coater and measured using a quartz crystal thickness monitor. Samples were examined with a Zeiss Ultra Plus Field Emission SEM (Carl Zeiss Ltd., UK), and digital photographs taken for measurements.

2.2.3 Morphometric measurements

Valve diameter, number and position of fultoportulae (strutted processes, SPs), number and position of rimoportulae (labiate processes, LPs), number of satellite pores in SPs and areola shape and patterns were determined for at least 30 valves using SEM images from each of the six *T. pseudonana* cultured isolates (Table 2.1). The main structures observed in the valve are represented in the diagram below (Fig. 2.1).
2.3 Results

2.3.1 Light microscopy

Live cells were examined using a Polyvar (Vienna, Austria) light microscope and photographs (Fig. 2.2) were taken using a Leica DFC320 camera and IM50 (Leica Microsystems, Heerbrugg, Switzerland) imaging systems. The cultured strains appeared as single cells, colonies were never observed (Fig. 2.2). Valves were circular varying in diameter 2-7 µm (Table 2.1) with 4 small chloroplasts.
Relatively few morphological features were recognizable with light microscopy due to the small size of this centric diatom and the weakly silicified valves, therefore finer detail required SEM.

### 2.3.2 Scanning electron microscopy

In general, all strains were weakly silicified (Fig. 2.4) as recorded by Hasle and Heimdal (1970). Valves were circular, varying in diameter among the strains, with a flat valve surface, and a pervalvar axis shorter than the cell diameter. The valve surface was perforated by radially arranged rows of small pores, the pattern being disrupted by the valve face SPs. External valve views revealed that the bases of both valve face and marginal SPs were surrounded by an external siliceous collar (Figs 2.3 a-c). Marginal SPs had tube-like openings, the external tube being longer than the internal one (Fig. 2.4 d).

#### 2.3.2.1 Thalassiosira pseudonana CCMP1013

Valves were 2.1 – 5.0 µm in diameter. Specimens mainly had irregular radial ribs, crossed by tangential ribs forming polygonal or elongated areolae on the external valve surface (Figs 2.3 a-c). The external valve surface had a marginal ring of SPs (6 to 16 per valve), and zero to three valve face SPs (Figs 2.3 a-e). If only one valve face SP was present, it was usually located midway between the valve centre and margin (Figs 2.3 c & d).

Internal valve views revealed the presence of one oval LP (Figs 2.3 d & e, long arrows), generally midway between two marginal SPs. Marginal SPs usually had three, sometimes four, satellite pores (Figs 2.3 d & e). In most cases the valve face SP had two satellite pores (mean of 2.28) (Table 2.1). In some specimens the number of satellite pores associated with the valve face SP varied from one to four (Figs 2.3 d & e).
Fig. 2.3. *Thalassiosira pseudonana* CCMP1013.

**Figs 2.3 a-c.** External valve views showing radially arranged areolae over the whole valve surface, marginal ring of SPs (long arrow) and valve face SPs (short arrow). Note the siliceous collar around both valve face and marginal SPs. **Figs 2.3 d & e.** Internal views of valves showing the oval LP, (long arrow) approximately midway between marginal SPs, and detail of SP satellite pores. Note the variation in satellite pore number of valve face SPs (Fig. e). **Fig. 2.3 f.** Incompletely silicified valve showing radial areola arrangement and three satellite pores per marginal SP. Scale bar, 1µm.
2.3.2.2 *Thalassiosira pseudonana* CCMP1014

Specimens varied in diameter from 2.9 - 5.3 µm. As described above, some specimens had an irregular pattern of radial ribs crossed by tangential ribs forming polygonal or elongated areolae on their external valve surface (Fig. 2.4b). Other specimens had irregular, radial ribs, but true areolation was absent (Fig. 2.4a). The external valve surface was characterized by a marginal ring of SPs (8 to 17 per valve), and usually one sub-central SP (Fig. 2.4a & b), but in sometime none to three valve face SPs (mean 1.03) (Table 2.1).

Internal valve views revealed the presence of one circular to oval LP situated between 2 marginal SPs, but generally closer to one than the other (Figs 2.4c, d & f). Valves usually had three, sometimes four, satellite pores per marginal SP (Figs 2.4d & e), and normally two satellite pores per valve face SP (Figs 2.4c-12), although some specimens showed a range, from one to four (mean 1.97).

2.3.2.3 *Thalassiosira pseudonana* CCMP1015

Valves were 2.8 - 4.6 µm in diameter. In most of the specimens the valve centre was occupied by irregular areolae with some elongated areolae on the valve mantle (Fig. 2.5b). A few specimens were more strongly silicified and had radial and tangential ribs forming polygonal areolae on their external valve surface (Fig. 2.5a). The external valve surface had a marginal ring of SPs (10 to 19 per valve), and usually one sub-central SP (Figs 2.5b-f), but occasionally up to four valve face SPs (mean 1.18).

Internal valve views revealed one circular, slightly oval LP (Figs 2.5d-f) midway between two marginal SPs. Marginal SPs had three satellite pores (Figs 2.5c-e) and in most samples valve face SPs had two satellite pores (Figs 2.5c-f), a few specimens had one to three (mean 2) (Table 2.1).
Fig. 2.4. *Thalassiosira pseudonana* CCMP1014.

**Figs 2.4 a & b.** External valve views showing the variation in areolation pattern and the basal siliceous collar around both, valve face (short arrow) and marginal SPs (long arrow). **Figs 2.4 d & f.** Internal views of the valve showing the LP (long arrow). Note the greater length of the marginal SPs to the exterior of the valve, and the radial pore arrangement. Detail of satellite pores in SPs. **Fig. 2.4 c.** Internal view showing the LP position (long arrow) generally closer to one marginal SP, and detail of the two satellite pores in valve face SP. **Fig. 2.4 e.** Incompletely silicified valve showing three (short arrow) and four satellite pores (long arrows) of marginal SPs and 2 of valve face SP (arrowhead). Scale bar, 1µm.
Chapter 2  
Phenotypic characterization of *Thalassiosira pseudonana* isolates

Fig. 2.5. *Thalassiosira pseudonana* CCMP1015.  
**Fig. 2.5 a.** Whole external valve surface covered by areolae. Marginal ring of SPs (long arrow), and valve face SP (short arrow) both with basal siliceous collars.  
**Fig. 2.5 b.** Weakly silicified specimen showing irregular areolae at valve centre (long arrow), and a ring of elongated areolae on the valve mantle (short arrow).  
**Figs 2.5 d-f.** Internal valve views showing the LP midway between marginal SPs (long arrow) and details valve face SPs with two satellite pores and marginal SPs with three.  
**Fig. 2.5 c.** External view of an incompletely silicified valve with SPs tubes forming. Scale bar, 1µm.
2.3.2.4 *Thalassiosira pseudonana* CCMP1335

In this strain the valve diameter varied from 2.2 - 5.6 µm. Most specimens had polygonal, sometimes elongated areolae, on their external valve surface (Figs 2.6 a-c). Other, less silicified, specimens had an irregular arrangement of areolae at the centre of the valve, and a ring of elongated areolae on the valve mantle. The external valve surface had a marginal ring of SPs (7 to 17 per valve) and, in most cases, one sub-central SP (Figs 2.6 a-d). On occasions up to four valve face SPs (mean 1.22) (Table 2.1) were observed.

Internal valve views revealed one circular, slightly oval LP (Figs 2.6 d & e), which was located midway between two marginal SPs. Marginal SPs had three satellite pores (Fig. 2.6 f) and, in most specimens, valve face SPs had two satellite pores, although a few cells showed between one and five satellite pores (mean 2.15) (Fig. 2.6 d-f).

2.3.2.5 *Thalassiosira pseudonana* CS-173

This strain, obtained from the ANACC, is a duplicate of CCMP1335, which has been maintained in the ANACC collection since 1984. The comparison of these identical strains, maintained in two different collections for decades, sought to reveal whether different culture regimes can influence phenotypic plasticity.

Valves were 3.1 - 5.6 µm in diameter. Some specimens had irregular radial ribs crossed by tangential ribs, forming polygonal or elongated areolae on the external valve surface (Figs 2.7 a & b); however, other specimens had irregular areolae at their valve centre and more elongated areolae on the valve mantle. External valve surface was characterized by a marginal ring of SPs (6 to 15 per valve), and all the specimens had only one valve face SP (Table 2.1).
Internal valve views revealed one circular, sometimes slightly elongated, LP (Figs 2.7 c &
e), usually located within the marginal ring of SPs. Marginal SPs had three satellite pores
(Figs 2.7 c-e), and in most specimens the valve face SPs had two satellite pores (Figs 2.7 d
& e). However, some specimens showed a range from one up to three satellite pores in SPs
(mean 1.91).

Fig. 2.6. Thalassiosira pseudonana CCMP1335.
Figs 2.6 a-c. Whole external valve surface covered by areolae. Note siliceous collars (long arrows) around both, marginal and valve face SPs. Figs 2.6 d & e. Internal views of valves showing the LP (long arrows) and detail of SP satellite pores. Fig. 2.6 f. External view of partially silicified valve with detail of the three satellite pores around developing marginal SPs. Scale bar, 1µm.
Fig. 2.7. *Thalassiosira pseudonana* CS-173.

Figs 2.7 a & b. Whole external valve surface covered by areolae. Siliceous collars (long arrows) around bases of SPs. **Fig. 2.7 d.** Partially silicified valve, showing areolae only forming a ring on the valve mantle. Detail of valve face SP with two satellite pores and marginal SPs with three. **Figs 2.7 c & e.** Internal valve views showing the LP (long arrows) and detail of SP satellite pores. Scale bar, 1µm.
2.3.2.6 *Thalassiosira pseudonana* CS-20

Valves were 2.2 - 5.6 µm in diameter. All the specimens were very weakly silicified, with the valve centre occupied by irregular areolae, and the valve mantle by a ring of areolae (Figs 2.8 a & b). No specimen had areolae over the whole valve surface. External valve surface exhibited a marginal ring of SPs (7 to 15 per valve), and a very variable number of valve face SPs, from zero to five (mean 2.11) (Figs 2.8 c-e).

Internal valve views revealed the presence of one oval LP (Figs 2.8 c & d) usually located slightly outside the marginal ring of SPs, towards the centre. Marginal SPs had three satellite pores (Figs 2.8 c & e). In most cases the valve face SPs had two satellite pores (Figs 2.8 c & d), however there were one to four satellite pores associated with the valve face SP (mean 1.83).

2.3.2.7 *Thalassiosira pseudonana* CCMP1335 Post-cryopreservation

Samples varied from 2.8 – 7.0 µm diameter. Some specimens had irregular radial ribs crossed by tangential ribs, forming polygonal or elongated areolae on the external valve surface (Fig. 2.9 a.). Other specimens had a ring of areolae on the valve mantle (Fig. 2.9 b.). A marginal ring of SPs (6 to 14 per valve), and in most samples one subcentral SP were observed (Figs 2.9 b). Occasionally zero or two valve face SPs (mean 0.90) were observed on the external valve surface. All these observations corresponded with those before cryopreservation (section 2.3.2.4).

Internal valve views revealed one circular to oval LP (Figs 2.9 c & e), with the same shape and position as before the treatment (Figs 2.6 d & e), usually midway between two marginal SPs. Marginal SPs had three satellite pores (Fig. 2.9 d) and valve face SPs mainly had two satellite pores (Figs 2.9 c & d), a few specimens had one or three (mean 1.64) (Table 2.1).
Chapter 2
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Fig. 2.8. *Thalassiosira pseudonana* CS-20.
Figs 2.8 a & b. Weakly silicified specimens showing irregular areolae at valve centre and a ring of areolae on valve mantle. Note the siliceous collar (long arrows) around the basal part of SPs. Figs 2.8 c & d. Internal views of valves showing the oval LP (long arrows) and detail marginal SPs with three satellite pores. Fig. 2.8 e. External view of an incompletely silicified valve, with developing SPs with 3 satellite pores. Scale bar, 1µm.
Fig. 2.9. *Thalassiosira pseudonana* CCMP1335 post-cryopreservation.  
**Fig. 2.9 a.** Whole external valve surface covered by areolae. Note siliceous collar (long arrows) around both marginal and valve face SPs.  
**Fig. 2.9 b.** Specimen showing a ring of areolae on valve mantle.  
**Figs 2.9 c & e.** Internal views of valves showing the LP (long arrows).  
**Fig. 2.9 d.** External view of partially silicified valve with detail of developing marginal SPs with three satellite pores and valve face Sps with 2. Scale bar, 1µm.
Table 2.1 Morphometric data of six strains of *T. pseudonana*.

<table>
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<tr>
<th></th>
<th>CCMP1013</th>
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<th>CCMP1015</th>
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<th>CCMP1335 Post-cryo</th>
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<th>CS-20</th>
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<tr>
<td>Diameter (µm)</td>
<td>3.2 (0.68)</td>
<td>3.8 (0.55)</td>
<td>3.6 (0.39)</td>
<td>3.9 (0.63)</td>
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<td>2.1-5</td>
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<td>Fultoportulae (marginal)</td>
<td>9.29 (2.26)</td>
<td>11.59 (2.00)</td>
<td>10.08 (2.06)</td>
<td>10.64 (1.90)</td>
<td>9.53 (1.87)</td>
<td>9.33 (2.23)</td>
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<td>Fultoportulae (valve face)</td>
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<td>1.03 (0.45)</td>
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<td>Satellite pores (marginal fultoportulae)</td>
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<td>2.87 (0.34)</td>
<td>3 (0)</td>
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<tr>
<td>Satellite pores (valve face fultoportulae)</td>
<td>2.28 (0.83)</td>
<td>1.97 (0.72)</td>
<td>2.0 (0.47)</td>
<td>2.15 (0.49)</td>
<td>1.64 (0.50)</td>
<td>1.90 (0.54)</td>
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<td>Rimoportulae</td>
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Values given for mean with standard deviation in parentheses, followed by range and number of measurements.
Chapter 2
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The relationship between diameter and number of marginal fultoportulae was tested (Fig 2.10) using the Pearson product-moment correlation coefficient, or "Pearson's correlation" (r). Pearson correlation coefficient is obtained by dividing the covariance of the two variables (in this case diameter and number of marginal fultoportulae) by the product of their standard deviations. Correlation values indicated a relationship between diameter and marginal fultoportulae, although does not imply causality and thus, does not prove that one variable is causing the other. Strain CS-20 showed the lowest correlation among the strains.

**Fig. 2.10** Scatter plots showing correlation (r) between diameter and number of marginal fultoportulae.
2.4 Discussion

The aim of the work described in this chapter was to assess the level of phenotypic plasticity between different strains of the same centric diatom species, *T. pseudonana*, available from culture collections worldwide. The objective was to assess whether there was measurable intraspecific variation and/or distinctive biogeographic distribution patterns between the different isolates. *T. pseudonana* is widely distributed in nature, and a number of isolates from around the world have been deposited in major culture collections. This taxon was the first diatom to have its whole genome sequenced (Armbrust et al. 2004), thus opportunities have arisen to use the genomic information to investigate numerous aspects of diatom research and biotechnology. It is therefore very important to undertake a comprehensive phenotypic and genetic characterization of the main strains available in culture collections. This characterization exercise has previously been undertaken (De Martino et al. 2007) for another diatom species, *Phaeodactylum tricornutum*, whose entire genome has also been sequenced (Bowler et al. 2008).

In general, observations of the strains corresponded with those reported by Hasle and Heimdal (1970). The SEM observations revealed variation in valve morphology between specimens of the same strain (Fig. 2.3). The level of intra-strain phenotypic plasticity causes significant challenges to the phenotypic characterization at the intra-specific level. One very important consideration is that, in a growing culture, cells can be at different stages of the cell cycle and valve formation, and thus, the silica patterns will reflect those stages of the cell cycle. There are two main stages in *T. pseudonana* valve formation (Hildebrand et al. 2006); the first stage involves the formation of the base layer, as radial ribs of silica with a smooth appearance, and the initial formation of the marginal fultoportulæ. During the next stage, additional silica deposition generates raised ribs and
further developed fultoportulae. This needs to be taken into account when characterizing the different strains.

Examination of the cell wall revealed variation in silica patterns, but in most of the strains, specimens were observed at different stages of silicification. The variation in pattern could be attributed to the different stages of valve formation. The six strains analysed showed fairly similar morphology (Figs 2.3-2.8). No conclusive variations were found among the strains, with the exception of *T. pseudonana* strain CS-20, where slight variation indicated some phenotypic plasticity.

Previous studies on diatom intraspecific variability and biogeographic distribution have reported mixed results. Studies on *P. tricornutum* (De Martino et al. 2007) demonstrated very high intraspecific morphological variability, easily recognizable by light microscopy, although the correlation between morphology, biogeographic distribution and phylogeny was limited (De Martino et al. 2007). The morphological variability between the strains was readily seen (De Martino et al. 2007), as in the case of *Pseudo-nitzschia pungens*, where the combination of a few morphological characters (observed by light microscopy) was enough to distinguish between strains (Casteleyn et al. 2008). In the present study, due to the very small size of *T. pseudonana*, the weakly silicified valves, and the lack of LM characters, a thorough SEM examination was necessary simply to reveal the main characters. Initial investigations employing the JEOL JSM-6390LV SEM at the Scottish Association for Marine Science (SAMS) were inconclusive, because this SEM has insufficient resolution to clearly identify structural features on the valve. A visit to the Natural History Museum (NHM) where a Zeiss Ultra Plus Field Emission SEM (Carl Zeiss Ltd., UK) was used, allowed higher magnification SEM micrographs to be taken, and these form the basis of the data generated in this study. No conclusive variation was found.
between the strains, with the exception of *T. pseudonana* CS-20, which showed certain variation as described in the section below (Section 2.4.1).

### 2.4.1 Comparison of isolates from different geographic origins

No morphological variation was found between isolates from the Atlantic and Pacific oceans (Table 1.1). Variation in diameter and number of fultoportulae was observed across all the isolates, even between specimens from the same strain (Table 2.1).

However one strain, *T. pseudonana* CS-20, isolated from the Swan River Estuary, Perth, Western Australia (Table 1.1) was slightly different. The Swan River estuary is characterized by seasonally changing physical and chemical factors; temperature, salinity, nutrients and rainfall being the principal controlling factors. This strain has been maintained in the Australian National Algae Culture Collection (ANACC) since 1972 and was originally identified by G. Hallegraeff, who observed some deviation from the original description of *T. pseudonana* (Jameson personal communication). In this study 40 specimens of this particular strain were studied. All were very weakly silicified and none had areolae over their whole valves (Figs 2.8 a & b). The absence of radial and tangential ribs to form areolae could be due to the cells being at a different stage in the silicification process (Hildebrand et al. 2006). However, it was noted that for all other strains investigated, valves were observed at different stages of silicification (Figs 2.5 a-c). This suggests that the degree of silicification of valves of this particular strain, CS-20, are in fact lower than in the other strains. In addition, all the other strains had round, or oval, rimoportulae situated within the marginal ring of fultoportulae (Figs 2.5 e & f). In *T. pseudonana* CS-20, an oval rimoportula was usually located outside the marginal ring of fultoportulae, towards the centre of the valve (Figs 2.8 c & d). These observations might indicate a certain variation in morphological pattern that could imply phenotypic plasticity.
Since all these strains have been maintained in culture on artificial media for decades, environmentally controlled phenotypic characteristics could have “faded” over time and generations. This apparently irreversible phenotypic shift has been reported for a range of algae and colourless protists (Day et al. 2007). It is also possible that variation between specimens of the same strain is related to the pressure of continuous subculturing over time. Variation in *T. pseudonana* CS-20 could be due to the environment, but an effect of culture regimes and conditions cannot be discounted. It has been reported that organisms in culture may be affected by selective pressures due to continued culturing, and that culture conditions may play a selective role, favouring a particular phenotype within the culture, leading to phenotypic drift in culture (Ryan et al. 2002, Paasche, 2001, Lakeman et al. 2009). Furthermore, genotypic changes such as mutations and genetic drift (Paasche, 2001, Lakeman et al. 2009) could also produce phenotypic plasticity. However, *T. pseudonana* CS-173, which had been acquired from the same culture collection and maintained in the same medium under an identical regime, did not show the variations seen in CS-20. Furthermore, CS-173 is a duplicate of the model organism CCMP1335. These identical strains have been held in different collections for decades, but they have not revealed any phenotypic plasticity between them, or with the rest of the strains. This suggests that, compared to the other strains, *T. pseudonana* CS-20 displays certain phenotypic plasticity, possibly related to the original environment. This will be discussed in more detail from a genotypic perspective in Chapter 4.

The original hypothesis proposed in this study that there is morphological plasticity within the taxon *T. pseudonana* has not been confirmed, since only one strain, *T. pseudonana* CS-20, appeared to display morphologc plasticity.
2.4.2 Comparison of *T. pseudonana* CCMP 1335 between pre and post cryopreservation

Maintenance of microalgal cultures in culture collections has historically been achieved mainly through the serial subculture of actively growing cultures (Day & Brand, 2005). In general, suboptimal conditions are chosen to extend transfer intervals and thus reduce costs and labour. However, continuous subculturing under such regimes over generations, as well as human error, have the potential to act as selective pressures for the microalgae, and could potentially result in genetic drift (Day & Brand, 2005), thus affecting their phenotypic and genotypic stability. Cryopreservation has been the method chosen by many in Biological Resource Centres (BRC, Day & Stacey, 2008) for long-term storage of cultures, reducing the risk of human error and genetic drift. However, phenotypic and genetic stability after cryopreservation has only been demonstrated for a limited number of microalgae (Hédoin et al. 2006, Müller et al. 2005, 2007). To date, this type of study has not been undertaken on *T. pseudonana*.

The strain chosen to test for phenotypic stability after cryopreservation was *T. pseudonana* CCMP1335, because it is the strain that was selected for entire genome sequencing (Armbrust et al. 2004), and therefore the choice for most other studies.

Comparisons of morphometric data before and after cryopreservation did not suggest any variation after the treatment. Silica patterns, valve diameter, number and position of fultoportulae (SPs), number and position of rimoportulae (LPS) as well as the number of SP satellite pores, were determined (Table 2.1). All the quantifiable characters observed before cryopreservation were directly comparable with those after cryopreservation (Table 2.1). Slight variation in number of fultoportulae and satellite pores was observed, but these were also observed between specimens in all the other strains. It can be concluded that the cryopreservation process did not affect phenotypic stability in this particular strain, and
therefore cryopreservation may be considered a valid method for long term storage of this valuable model organism.
Chapter 3
Chemotaxonomy

3 Chapter 3. Chemotaxonomy

3.1 Introduction

Various chemotaxonomic markers have been evaluated to assess their suitability for identifying/characterizing phytoplankton, and microalgae have been successfully characterized on the basis of their photosynthetic pigments (Mostaert et al. 1998, Garibotti et al. 2003) and fatty acid composition (Volkman et al. 1989, Viso & Marty, 1993, Marshall et al. 2002). Using this approach it is feasible to classify/identify some algae at higher taxonomic levels such as class (Zhukova & Aizdaicher, 1995), genus (Volkman et al. 1993), and in some cases at the strain level (Shaw et al. 1989).

Diatoms in particular, are one of the main components of marine phytoplankton (Round et al. 1990), and provide a major source of lipids for aquatic food-webs (Volkman & Hallegraeff, 1988, Volkman et al. 1989). One of the main biological functions of lipids is energy storage, and their synthesis by microalgae constitutes a great source of energy incorporated into marine animals through their diet. Furthermore some species of diatoms have attracted interest due to their production of polyunsaturated fatty acids (PUFAs). These lipids are known for their numerous health benefits in humans, and their importance for survival and growth during the larval stages of many marine animals (Volkman et al. 1989, Mansour et al. 2005). An additional driver that has created interest in algal lipids is the need to replace fossil fuels with renewable biofuels. This has stimulated a large number of screening and developmental projects aiming to find suitable candidates for biofuel production (Mutanda et al. 2011, Brennan & Owende, 2010). Under optimal conditions some diatoms species can produce up to 60% of their cellular mass as triacylglycerols (TAGs) (Yu et al. 2009). Coupled with their fast growth rate and the fact that their production does not represent competition for agricultural land used for crops production, diatoms may be considered suitable candidates for biofuel production.
In this study, the pigment and fatty acid profiles of several *T. pseudonana* strains were compared to elucidate if they share identical biochemical profiles, or if variation in biochemical composition could be observed between the strains studied. However, one of the main constraints on the use of a chemotaxonomic approach is that many of the biochemical compounds vary with culture conditions and the stage of the cell life cycle, for example cell pigment ratios may vary depending on growth conditions (Schlüter et al. 2000). Furthermore, lipid profiles may vary with culture conditions (Fisher & Schwarzenbach, 1978, Dunstan et al. 1993) and total lipid content is generally higher in the stationary phase of diatom species (Dunstan et al. 1994). It is critical in the design of these types of experiments, to establish identical culture conditions, and the harvesting of cells at the same stage in their life cycle.

Despite these constraints, in this chapter we tested the hypothesis that chemotaxonomic markers have the potential to differentiate between *T. pseudonana* strains.

### 3.2 Materials and methods

**3.2.1 Pigment analyses**

**3.2.1.1 Cell harvest**

Ten *T. pseudonana* strains were grown in 100ml Erlenmeyer flasks containing 50ml sterile F/2+Si medium (Guillard, 1975). The culture conditions were standardised (Chapter 2, Section 2.1.1.) to avoid profile variations due to culture conditions. Cell counts were performed using a haemocytometer to calculate cell biomass; a small volume of the cell suspension was transferred to the haemocytometer by capillary action and all cells within the 1mm² central squares counted using an Axiovert 200 light microscope (Carl Zeiss, Jena, Germany) prior to cell harvest.
All the cultures were harvested in stationary phase. Aliquots (40ml) of each culture (cells in F/2+Si medium) were filtered through a 47mm Whatman glass microfibre filter (GF/F, Whatman, GE Healthcare, UK). The filters were immediately wrapped in aluminium foil and stored at −80°C overnight.

3.2.1.2 Pigments extraction

Filters with the harvested cells were inserted in 15ml polypropylene centrifuge tubes and pigments were extracted in 7ml of 90% v/v buffered acetone by ultrasonication (1 min) in a Soniprep 150 probe sonicator (MSE, UK). The extracts were separated from the residue by centrifugation at 1500 g and 5°C for 10 min in an Eppendorf 5810R centrifuge (Eppendorf, UK). The supernatant extracts were then removed by pipetting and loaded into 1 cm optical glass cuvettes (Thermo Fisher), ready for the analyses.

3.2.1.3 Spectrophotometer analyses

Absorption spectra of the extracts were measured using a Nicolet evolution 300 spectrophotometer (Thermo electron, UK). Acetone (90% v/v) was used as a blank. Pigments were detected in the range of 350-800 nm, and were analysed using Vision Pro software (Thermo electron, UK). Pigments profiles were compared and chlorophyll a and c levels calculated according to the equations of Jeffrey and Humphrey (1975) for chromophyte algae.

3.2.2 Fatty acid analyses

3.2.2.1 Cell harvest

All strains investigated were grown in 250ml Erlenmeyer flasks containing 150ml sterile F/2+Si medium (Guillard, 1975) under standard culture conditions (Chapter 2, Section 2.1.1). As above, cultures were harvested in early stationary phase. All the strains were
tested for axenicity as described in Chapter 2, Section 2.2.1 and only the axenic strains were included in the subsequent analyses.

Cultures (150ml) were centrifuged for 10 min at 3000 rpm in a SIGMA 1-14 microcentrifuge (Sigma-Aldrich Ltd., Dorset, UK), and most of the supernatant was discarded by decanting. The remaining supernatant was used to resuspend the cell pellet which was then transferred to a 2ml Eppendorf tube, frozen in liquid nitrogen (-196°C) and freeze-dried overnight using an Alpha 1-2 LD plus freeze dryer (CHRIST, Germany). The dry weight of the strains was measured using an Ohaus Adventurer balance (Ohaus, USA), and biomass levels determined by measuring absorbance using a fluorometer (Turner designs, California).

3.2.2.2 Fatty acid extraction

Fatty acid extraction was performed using the Speed Extractor E-916 (BUCHI) following the manufacturer’s protocol. The Speed extractor was set up at 100 °C and 100 Bar of pressure using a solvent mixture of 2:1 Dichloromethane: Methanol, and a known volume of an internal standard was added (0.25mg/ml 15:0 triacylglycerol in hexane). After the extraction, 250µl MilliQ water (1:20 the volume of Chloroform:methanol) was added, and the mixture was transferred to small Corning tubes, then flushed with nitrogen. The content of the tubes was then filtered using a Pasteur pipette filled with glass wool and collected into another small tube. 0.88% potassium chloride (KCl) solution was added (as 25% of existing volume), mixed, and the upper aqueous layer discarded. The lower layer was then transferred to a clean tube and dried down under nitrogen stream for 30 min.
3.2.2.3 Trans-esterification

Trans-esterification to give Fatty Acid Methyl Esters, FAMEs, was performed. An aliquot (1.5ml) of toluene and 3ml of 1% sulphuric acid in methanol were added, mixed thoroughly and flushed with nitrogen. The mixture was then incubated at 100°C for 2 h. in a sealed Pyrex tube. On cooling, 2ml of 5% KCl and 2ml hexane: ether (1:1 v/v) with 0.01% Butylated hydroxytoluene (BHT) were added, gently agitated, and the upper layer was transferred into a small clean tube. The hexane/ether extraction was repeated by adding just 2ml hexane/ether again. To the combined hexane extracts, 2ml of 2% (w/v) potassium hydrogen carbonate (NaHCO₃) were added, mixed, and the top layer collected and placed in a small clean tube to evaporate under nitrogen. The dry extracts were resuspended in 1ml hexane and purification was performed.

3.2.2.4 Purification of FAMES

Equal volumes of Silica gel 70-230 (mesh 60 Å, for column chromatography, Sigma-Aldrich) and hexane (e.g. ~50ml each in a Duran bottle) were mixed together. A standard size Pasteur pipette was plugged with glass wool and positioned vertically to act as a silica gel column. The silica/hexane mixture was added to form a column leaving space to add
the sample. The FAMEs sample was loaded on to the column and eluted with 10ml of hexane: ether (95%:5% v/v). The final sample (after having been eluted through the column) was dried down under nitrogen and transferred to capped tapered vials for gas chromatograph-flame ionization detector (GC-FID) using hexane. The vials were then flushed with nitrogen and stored at -20°C prior to loading onto the gas chromatograph-flame ionization detector (GC-FID).

3.2.2.5 GC-FID analyses

GC analyses were carried out on a Shimadzu GC-2014 gas chromatograph with flame ionization detector and a Shimadzu AOC-20 autosampler (Shimadzu, Milan, Italy). A ZB – wax capillary column was used and helium was chosen as the carrier gas. FAMEs were separated based on chain length and saturation. The run time was 30 min, the temperature profile started at 160°C increasing up to 240°C over 20 min, then remaining at 240°C for 10 min. Inlet pressure was 166 kPA, column flow of 1.55ml/min, linear velocity of 40 cm/s, split ratio of 100 and total flow of 159ml/min. Samples were injected in triplicate. In addition, FAMEs standard (FAMEs 37, Supelco) was run for reference, along with hexane blanks at the beginning and end of the samples. Data was acquired by GC Solution software (Shimadzu).

3.3 Results

3.3.1 Pigment profiles

The chromatograms obtained were virtually identical for all the strains tested (see Appendix 3). The spectrophotometer chromatogram of pigment extracts from T. pseudonana strain CCMP1335 is shown in Fig. 3.2. The main pigments identified were: chlorophyll $a$ and chlorophyll $c$ ($c_1$ and $c_2$).
Fig. 3.2. Spectrophotometer absorbance chromatogram (350-380 nm) of pigments extracted from *T. pseudonana* CCMP1335.

Chlorophyll *a* constituted the main pigment in all the strains studied; it was found slightly higher in *T. pseudonana* CCMP1012 and CS-20, both Australian isolates, although the differences were not obviously significant (Table 3.1). However, chlorophyll *c* levels were highest for strain CCMP1012 at 2.4pg/cell, much higher (above 10%) than the other strains (Table 3.1). Due to time limitations, replicates were not performed, therefore no statistics could be performed to confirm these initial observations.
### Table 3.1. Chlorophyll levels in *T. pseudonana* strains\(^1\).

<table>
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<th>Strain</th>
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<th>Chlorophyll (c^2) (pg/cell)</th>
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<td>CCMP1007</td>
<td>1.7</td>
<td>0.5</td>
</tr>
<tr>
<td>CCMP1011</td>
<td>1.8</td>
<td>0.5</td>
</tr>
<tr>
<td>CCMP1012</td>
<td>2.5</td>
<td>2.4</td>
</tr>
<tr>
<td>CCMP1013</td>
<td>1.6</td>
<td>0.7</td>
</tr>
<tr>
<td>CCMP1014</td>
<td>1.1</td>
<td>0.67</td>
</tr>
<tr>
<td>CCMP1015</td>
<td>2.2</td>
<td>0.3</td>
</tr>
<tr>
<td>CCMP1335</td>
<td>1.9</td>
<td>0.5</td>
</tr>
<tr>
<td>CS-20</td>
<td>2.5</td>
<td>0.5</td>
</tr>
<tr>
<td>CS-173</td>
<td>1.6</td>
<td>0.4</td>
</tr>
<tr>
<td>SAG1020-1b</td>
<td>1.3</td>
<td>0.4</td>
</tr>
</tbody>
</table>

\(^1\) Obtained using the equations of Jeffrey and Humphrey (1975) for chromophyte algae and 90% acetone solvent. Units pg/cell.

\(^2\)Chlorophylls \(c_1 + c_2\)

### 3.3.2 Fatty acid composition

The fatty acid composition of six axenic *T. pseudonana* strains was investigated to see if there was any intraspecific variation in composition (Fig. 3.3 A-F). Data for total fatty acid composition are shown in Table 3.2. There was very little differentiation between fatty acid profiles of all *T. pseudonana* strains tested.

The principle fatty acids present were: 14:0, 16:0, 16:1 (n-7) and 20:5 (n-3), followed by: 16:3 (n-4), 16:4 (n-1), 18:4 (n-3) and 22:6 (n-3) (Table 3.2, Fig. 3.3 A-F). The main polyunsaturated fatty acid (PUFA) identified was the eicosapentaenoic acid (EPA) 20:5 (n-3) (13.73-18.10%), followed by 16:3 (n-4) (6.77-10.13%), 18:4 (n-3) (2.77-6.24%), 22:6 (n-3) (2.81-5.11%) and 16:4 (n-1) (1.88-5.30%), all other PUFAs identified were at lower levels (Table 3.2).
Fig. 3.3. A-C. Chromatograms produced by the GC-FID for the axenic *T. pseudonana* strains.

Fatty acid profiles for the strains showed a very close production pattern. The same fatty acids were identified for all the strains, with small variations in fatty acid production. The main peaks corresponded to 16:0, 16:1 (n-7) and 20:5 (n-3) fatty acids.
Fig. 3.3. D-F. Chromatograms produced by the GC-FID for the axenic *T. pseudonana* strains.

Fatty acid profiles for the strains showed a very close production pattern. The same fatty acids were identified for all the strains, with small variations in the production. The main peaks as above corresponded to 16:0, 16:1 (n-7) and 20:5 (n-3) fatty acids.
### Table 3.2. Fatty acid composition of *T. pseudonana* axenic strains (as % total fatty acids).

<table>
<thead>
<tr>
<th>Fatty Acid name</th>
<th>Fatty Acid</th>
<th>CCMP 1013</th>
<th>CCMP 1014</th>
<th>CCMP 1015</th>
<th>CCMP 1335</th>
<th>CS-173</th>
<th>CS-20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic acid</td>
<td>14:0</td>
<td>15.28</td>
<td>12.26</td>
<td>11.46</td>
<td>12.57</td>
<td>13.80</td>
<td>12.31</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>16:0</td>
<td>18.58</td>
<td>26.07</td>
<td>18.99</td>
<td>24.40</td>
<td>21.18</td>
<td>23.77</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>16:1 (n-7)</td>
<td>21.63</td>
<td>16.86</td>
<td>17.94</td>
<td>19.46</td>
<td>15.73</td>
<td>26.14</td>
</tr>
<tr>
<td>6,9-hexadecadienoic acid</td>
<td>16:2 (n-7)</td>
<td>1.37</td>
<td>1.79</td>
<td>4.78</td>
<td>2.80</td>
<td>2.19</td>
<td>1.30</td>
</tr>
<tr>
<td>9,12-hexadecadienoic acid</td>
<td>16:2 (n-4)</td>
<td>2.24</td>
<td>2.20</td>
<td>2.58</td>
<td>2.98</td>
<td>2.80</td>
<td>2.23</td>
</tr>
<tr>
<td>6,9,12-hexadecatrienoic acid</td>
<td>16:3 (n-4)</td>
<td>10.13</td>
<td>6.77</td>
<td>8.41</td>
<td>8.35</td>
<td>8.30</td>
<td>7.77</td>
</tr>
<tr>
<td>6,9,12,15-hexadecatetraenoic acid</td>
<td>16:4 (n-1)</td>
<td>3.19</td>
<td>2.97</td>
<td>3.99</td>
<td>4.31</td>
<td>5.30</td>
<td>1.88</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>18:0</td>
<td>1.62</td>
<td>1.918</td>
<td>1.77</td>
<td>0.80</td>
<td>1.31</td>
<td>0.91</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>18:1 (n-9)</td>
<td>3.61</td>
<td>1.45</td>
<td>0.93</td>
<td>0.49</td>
<td>0.62</td>
<td>0.64</td>
</tr>
<tr>
<td>Vaccenic acid</td>
<td>18:1 (n-7)</td>
<td>0.25</td>
<td>0.38</td>
<td>0.17</td>
<td>0.28</td>
<td>0.29</td>
<td>0.29</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>18:2 (n-6)</td>
<td>0.20</td>
<td>0.14</td>
<td>0.23</td>
<td>0.13</td>
<td>0.10</td>
<td>0.22</td>
</tr>
<tr>
<td>Gamma-linolenic acid</td>
<td>18:3 (n-6)</td>
<td>0.19</td>
<td>0.12</td>
<td>0.23</td>
<td>0.07</td>
<td>0.12</td>
<td>0.10</td>
</tr>
<tr>
<td>α-Linolenic acid</td>
<td>18:3 (n-3)</td>
<td>0.25</td>
<td>0.43</td>
<td>0.10</td>
<td>0.23</td>
<td>0.39</td>
<td>0.57</td>
</tr>
<tr>
<td>Stearidonic acid</td>
<td>18:4 (n-3)</td>
<td>2.77</td>
<td>6.24</td>
<td>4.72</td>
<td>4.76</td>
<td>5.55</td>
<td>4.68</td>
</tr>
<tr>
<td>Arachidic acid</td>
<td>20:0</td>
<td>0.42</td>
<td>0.47</td>
<td>0.50</td>
<td>0.66</td>
<td>0.60</td>
<td>0.35</td>
</tr>
<tr>
<td>Eicosapentaenoic acid</td>
<td>20:5 (n-3)</td>
<td>15.60</td>
<td>15.62</td>
<td>18.10</td>
<td>13.74</td>
<td>17.73</td>
<td>14.0</td>
</tr>
<tr>
<td>Docosahexaenoic acid</td>
<td>22:6 (n-3)</td>
<td>2.65</td>
<td>4.3</td>
<td>5.11</td>
<td>3.96</td>
<td>4.0</td>
<td>2.81</td>
</tr>
</tbody>
</table>

#### Fig. 3.4. Comparison of fatty acid percentage area.
In addition, the total fatty acid percentage yield (percentage of oil in relation to dry weight) was calculated for each strain (Table 3.3). The total yield showed minor variations between the strains, with *T. pseudonana* CCMP1015 showing the higher oil production at 7.62% (Table 3.3).

**Table 3.3.** Percentage Oil Yield in *T. pseudonana* strains.

<table>
<thead>
<tr>
<th>Strain Number</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCMP 1013</td>
<td>6.20</td>
</tr>
<tr>
<td>CCMP 1014</td>
<td>6.05</td>
</tr>
<tr>
<td>CCMP 1015</td>
<td>7.62</td>
</tr>
<tr>
<td>CCMP 1335</td>
<td>6.77</td>
</tr>
<tr>
<td>CS-20</td>
<td>5.57</td>
</tr>
<tr>
<td>CS-173</td>
<td>5.36</td>
</tr>
</tbody>
</table>

*Non replicated chemotaxonomic pilot study*

### 3.4 Discussion

#### 3.4.1 Chemotaxonomic value of pigment composition at an intra-species level

The potential of using pigment composition as chemotaxonomic markers at the intra-species level was tested in this chapter. Spectrophotometer chromatograms obtained from pigment extracts were identical for all *T. pseudonana* strains studied (Fig. 3.2 and Appendix 3). Previous studies have reported that pigment composition can be a reliable chemotaxonomic marker of phytoplankton taxonomy (Wright et al. 1996, Schlüter & Møhlenberg, 2003). However, it has also been previously reported that pigment composition is conserved at species level and below (Carreto et al. 2001, Volkman et al. 1993, Brown & Jeffrey, 1992). Although, due to time limitations, biological replicates were not performed, the levels of chlorophyll *c* obtained in this study for the Australian isolate CCMP1012 were apparently significantly higher than the other strains. This "opens
the door" for a more in depth investigation, using biological replicates and a higher resolution method for pigment analyses, such as high-performance liquid chromatography (HPLC). A high resolution HPLC method would permit analyses of chlorophylls and carotenoids, as proposed and recommended by UNESCO (Wright & Jeffrey, 1997). This type of analysis would allow us to differentiate the carotenoids and to confirm if significant variation can be observed between the strains.

3.4.2 Chemotaxonomic value of fatty acid composition at an intra-species level

The fatty acid profile of the six axenic *T. pseudonana* isolates was uniform (Figs 3.3 A-F). However, some variation was observed for fatty acid levels despite the identical culture conditions and cells age.

The main fatty acids observed in this study were 14:0, 16:0, 16:1 (n-7) and 20:5 (n-3), these have previously been reported for other diatoms (Orcutt & Patterson, 1975, Volkman & Hallegraeff, 1988). High levels of 16:3 (n-4), 18:4 (n-3), 22:6 (n-3) and 16:4 (n-1) were also observed, and Fisher and Schwarzenbach (1978) obtained a very similar profile for *T. pseudonana* strain 3H (CCMP1335). In addition, Volkman and Hallegraeff (1988) studied strains CCMP1335 and CS-20; the results obtained here corresponded with their levels and fatty acid profiles, with a difference in the production of 14:0 FA. They reported that 14:0 FA levels were much lower in strain CS-20 than in strain CCMP1335; however, in this study, both these strains (and all the others) produced very similar amounts of 14:0 FA, confirming the studies by Fisher and Schwarzenbach (1978).

Culture regimens and stage in the life cycle have been reported to result in significant variations in fatty acid profiles (Fisher & Schwarzenbach, 1978; Marshall et al. 2002). Since all the cultures studied were grown under identical conditions and cells were harvested at the same time, it can be presumed that variations obtained in fatty acid profiles
indicated intraspecific variation between the strains (Table 3.2). Marshall et al. (2002) observed a similar variation in fatty acid production within strains of the raphidophyte *Chattonella* from different geographic locations. Shaw et al. (1989) reported that fatty acids of the diatom *Skeletonema costatum* differed between clones isolated from oceanic, coastal, or estuarine environments, but in the present study, differences in fatty acid production do not seem to correlate with their origin, as the results obtained for the estuarine strains (CCMP1013, CCMP1335, CS-173 and CS-20) were not closer together than the other strains studied (Table 3.2).

Due to the variation in data reported in previous studies for *T. pseudonana*, Volkman and Hallegraeff (1988) highlighted the importance of culture conditions, accurate taxonomic identification at the strain level, and the necessity of clearly stating strain designation in this type of studies.

This chapter was a pilot study to investigate the hypothesis that employing chemotaxonomic markers such as chlorophylls, or lipids profiles has the potential to distinguish between different strains of the diatom *T. pseudonana*. The results obtained were inconclusive and it was interesting that the two duplicate strains: CCMP1335 and CS-173, maintained in different culture collections for decades, showed as much variation in fatty acid production as “non-identical” strains (Table 3.2). This could potentially be due to the selective nature of different culture regimes employed in the different collections. However, it may equally be due to natural variability within the diatom cells.

The differences observed warrant further investigation in a more defined, better replicated study. The recent development of a technique to synchronise *T. pseudonana* growth (Hildebrand et al. 2007) may represent the answer to then be able to work with cultures at the precise same stage of their life cycle. Furthermore, the potential of using alternative proteomic markers employing Matrix Assisted Laser Desorption Ionisation – Time Of
Flight Intact Cell Mass Spectrometry (MALDI-TOF ICMS) is being investigated (Vargha et al. 2006, Carbonelle et al. 2011). MALDI-TOF ICMS is a sensitive, relatively novel approach that has gained popularity as a tool for microbial identification. The technique produces a profile of proteins which is then used as taxon specific fingerprints, and archived in a database. The advantages of this novel approach for microbial identification are, among others, simple sample preparation and a short timeframe for analysis. Up to date this technique has mainly been employed in bacteria and fungi (Vargha et al. 2006, Carbonnelle et al. 2011). These analyses will be very interesting from a taxonomic point of view to establish differences between closely related organisms, and will be very useful for culture collections worldwide in the characterization of their holdings, allowing researchers to use the most appropriate strain for a particular study.
Chapter 4
Genotypic characterization of geographic isolates among multiple strains of *Thalassiosira pseudonana*

4 Chapter 4. Genotypic characterization of geographic isolates among multiple strains of *T. pseudonana*.

4.1 Introduction

Over the past 12 years, there has been an increase in interest in phytoplankton biogeography focusing in particular on “whether or not” these organisms are cosmopolitan (Finlay & Clark, 1999, Coleman, 2002, Finlay, 2002, Medlin, 2007). On the basis of morphotypes many taxa appear to be cosmopolitan (Finlay, 2002), but genetic characterization of different isolates from the same widespread species should be able to help to reveal cryptic species (Sarno et al. 2005, Amato et al. 2007, Evans et al. 2008), and even uncover biogeographic patterns (John et al. 2004, Kooistra et al. 2008). Hence the importance of this study, which aims to investigate if biogeographic patterns can be detected amongst these different isolates, combining both, molecular markers proposed for diatom DNA barcoding and a DNA fingerprinting technique.

Multiple isolates of *T. pseudonana* have been maintained in different culture collections for many decades (Table 1.1). Furthermore the publication of this diatom’s genome (Armbrust et al. 2004) has led to a dramatic increase in the number of studies covering a diverse range of aspects of the biology of this centric diatom (Volkman & Hallegraeff, 1988, Hildebrand, 2005, Mansour et al. 2005, Davies et al. 2006, Kröger, 2007). In addition biotechnological applications have been developed, including the important role it has in mariculture as a food for larval stages of crustaceans, fish, and for bivalves and zooplankton (Volkman et al. 1989). Additionally, fatty acid analyses have demonstrated that *T. pseudonana* represents a good candidate to discover genes involved in the production and storage of polyunsaturated fatty acids (PUFAs, Tonon et al. 2004) and this alga is a model in the rapidly evolving topic of diatom nanotechnology (Hildebrand, 2005, Kröger, 2007).
One might anticipate genomic diversity within the multiple strains of *T. pseudonana* due to their different geographical origins. Similarly, genetic variability may have been induced in cultures by the selective nature of culture regimes employed by the different culture collections (Day & Brand, 2005).

Due to limited morphological features capable of being employed to discriminate between strains of some diatoms, their identification can be very difficult. In recent years, studies have proved that a fragment of a single gene (effectively a genetic barcode) could be used to identify species from a wide range of taxa, helping to unravel cryptic species (Amato et al. 2007, Evans et al. 2007, 2008, Mann et al. 2008, Coleman, 2009, Theriot et al. 2009) and in some cases even distinguishing among isolates of the same species (Alverson et al. 2007). The ongoing debate about DNA barcodes, and which is the optimal gene for diatom barcoding, continues and, to date, a few different markers have been investigated for this purpose. A 650bp fragment of the mitochondrial gene cytochrome c oxidase (*cox1*, Herbert et al. 2003) was successfully used to distinguish among closely related diatom species (Evans et al. 2007), but was later proved to be less universally applicable than other markers when used in more diverse taxa by Moniz and Kaczmarska (2009). They proposed the use of the 5.8S gene and the second internal transcribed spacer region (5.8S + ITS2) of the nuclear genome as a DNA barcode for diatoms (Moniz & Kaczmarska, 2009, 2010).

The small subunit of the ribosomal DNA (SSU) has also been investigated for diatom barcoding (Evans et al. 2007, Moniz & Kaczmarska 2009), as was the large subunit of RuBisCO (*rbcL*) (Alverson et al. 2007, Evans et al. 2007). However, these molecular markers are not always sensitive enough to assess genomic variation amongst strains of a single species of diatom, and other molecular analyses may be required. In the last decade, the use of amplified fragment length polymorphism (AFLP, Vos et al. 1995) to distinguish between geographic isolates and closely related species has become relatively widely used (Werner et al. 2001, De Bruin et al. 2004, John et al. 2004, Müller et al. 2005, De Martino
et al. 2007). AFLP is a highly sensitive and reproducible fingerprinting technique based on the simultaneous detection of DNA polymorphisms in different genomic regions, without previous sequence knowledge of the organism studied. AFLP involves the digestion of genomic DNA with two different restriction enzymes, ligation of complementary double stranded adaptors to the ends of the restriction fragments and the selective PCR amplification of some of these adapted restriction fragments. The selective amplified fragments are separated by electrophoresis and their visualization depends on the electrophoresis system employed (Vos et al. 1995).

Three hypotheses were tested in this chapter: the feasibility of barcode genes to characterize intraspecific variation of *T. pseudonana*, the necessity of whole genome approaches to differentiate between strains of *T. pseudonana* and the genomic variation expected according to biogeographic distribution patterns. To investigate this, two nuclear (SSU and ITS), one plastid (*rbcL*) and one mitochondrial (*cox1*) molecular markers that are widely used in assessing relationships at the species level or below (Sarno et al. 2005, Evans et al. 2007, Moniz & Kaczmarska, 2010) were assessed. Furthermore, the AFLP technique (that uses the whole genome) was investigated to reveal if it could resolve genomic differences at the intra-species level and/or reveal biogeographic patterns. The outcome of these analyses will not only facilitate the genetic characterization of the accessions of this model organism available in culture collections worldwide, but it also has the potential to reveal if these isolates are genetically distinct, or they are ubiquitous.

### 4.2 Material and Methods

#### 4.2.1 Strains analysed

Ten strains of *T. pseudonana* provided by three of the major culture collections, including two duplicates held in two different collections, were used for SSU, ITS, *rbcL* and *cox1* sequence analyses. These are the main genes currently used for the DNA barcoding of
plant species, and their capacity to distinguish the different *Thalassiosira* strains was investigated. All the strains were tested for axenicity on media containing nutrient agar and were grown as described in Chapter 2, Section 2.2.1. Of the ten strains, six were axenic, (two being duplicate strains), and those were further tested for genetic diversity using a whole genome approach, AFLP (Vos et al. 1995).

### 4.2.2 DNA extraction and sequencing

Two millilitres of each *T. pseudonana* strain were centrifuged at 3500 r.p.m in a SIGMA 1-14 microcentrifuge (Sigma-Aldrich Ltd., Dorset, UK) to harvest enough cells for extraction. The cells were frozen in liquid nitrogen, ground with pestle and mortar and the frozen powder was transferred to a microcentrifuge tube containing Plant DNAZOL Reagent (Gibco BRL, Grand Island, NY, USA) (approximately 0.3ml Plant DNAZOL for 0.1 g of plant tissue). Genomic DNA was extracted using the protocol provided by the manufacturer. Briefly, the solution was thoroughly mixed by gentle inversion and incubated at 25°C with shaking for 5 min. 0.3ml chloroform was added, mixed, and a further 5 min incubation step carried out at 25°C with shaking. The extracts were centrifuged at 12,000 × g for 10 min and the supernatant was transferred to a new tube. The supernatant was mixed with 0.225ml of 100% ethanol by inverting the tube a few times and stored at room temperature for 5 min. To precipitate DNA, the mixture was centrifuged at 5,000 × g for 4 min, and the supernatant was removed, leaving behind the pelleted DNA in the tube. This was washed using a Plant DNAZOL-ethanol wash which was prepared by mixing 1 volume of Plant DNAZOL with 0.75 volume of 100% ethanol. Plant DNAZOL-ethanol wash solution (0.3ml) was mixed with the pellet by vortexing and the pellet incubated in the wash solution for 5 min, the samples were then centrifuged at 5,000 × g for 4 min. The wash solution was removed after centrifugation, and the DNA pellet was washed again by vigorous mixing with 0.3ml of 75% ethanol followed by
centrifugation at 5,000 \times g for 4 min. The ethanol was removed and the DNA pellet air-dried and dissolved in 70µl TE buffer (pH 8.0) by repeated pipetting.

For AFLP analyses, genomic DNA was extracted twice for each strain on different days in order to identify any potential variation in AFLP patterns due to differences in the extraction process (Müller et al. 2007).

4.2.2.1 Current barcoding genes

4.2.2.1.1 Nuclear markers: ITS and SSU

In this study the SSU and ITS rDNA of 10 *T. pseudonana* strains were sequenced. In addition, four *Thalassiosira* species: *T. rotula*, *T. punctigera*, *T. tumida* and *T. sp* which are closely related to *T. pseudonana*, were sequenced as an out-group. The SSU and ITS rDNA were amplified (Luo et al. 2006) from the extracted genomic DNA using the Taq PCR Mastermix Kit (Qiagen, Hilden, Germany) with the primers EAF3 and ITS055R (Pröschold et al. 2001), these sequences are shown in Table 4.1. The PCR products were visualised by agarose gel electrophoresis and purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) prior to being sent for DNA sequencing to The GenePool Sequencing Facility (The University of Edinburgh, UK) using an ABI 3730 (Applied Biosystems) capillary sequencer. The sequences generated were assembled and manually aligned using the sequence editor MacVector 8.1 (Accelrys Inc.). To determine which evolutionary model fitted best for the analyses, the values of 56 different models were estimated using the program Modeltest 3.7 (Posada & Crandall, 1998, Posada & Buckley, 2004). Phylogenetic trees were created using maximum likelihood method (ML, employing the Tamura-Nei model (Tamura & Nei, 1993); chosen as the best model according to the Akaike Information Criterion by Modeltest) via the PAUP 4.0b10 program (Swofford, 2002). To test the confidence of the tree topology, bootstrap analyses were calculated by maximum likelihood (ML, 100 replicates) criteria.
4.2.2.1.2 Plastid marker: \textit{rbcL}

In addition to the nuclear markers, the plastid gene that encodes the large subunit of RuBisCO, \textit{rbcL}, was sequenced. DNA (Section 4.2.2) was amplified using the Taq PCR Mastermix Kit (Qiagen, Hilden, Germany) with the primers DPrbcL1 and DPrbcL7 (Jones et al. 2005), the sequences are shown in Table 4.1. The PCR conditions were an initial denaturing phase for 3 min (94°C), followed by 30 cycles of 94°C for 10 sec, 50°C for 1 min and 68°C for 3 min, with a final extension of 68°C for 7 min. The PCR products were visualised by agarose gel electrophoresis and purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) prior to being sent for DNA sequencing to The GenePool Sequencing Facility using an ABI 3730 (Applied Biosystems) capillary sequencer. The sequences generated were manually aligned using MacVector 8.1 (Accelrys Inc.) and were used to build a phylogenetic tree using PAUP 4.0b10 (Swofford, 2002). As described in Section 4.2.2.1.1, the phylogenetic trees were determined by ML method (using the Tamura-Nei model) using PAUP 4.0b10 (Swofford, 2002) and the confidence of the tree topology was tested by bootstrap analyses (100 replicates).

4.2.2.1.3 Mitochondrial marker: \textit{cox1}

Two strategies were used to amplify the \textit{cox1} from the Thalassiosira species DNA. The first involved amplifying the genomic DNA using the Taq PCR Mastermix Kit (Qiagen, Hilden, Germany) with the primers GAZF2 (Saunders, 2005) and KEdtmR (Evans et al. 2007), the sequences are shown in Table 4.1. The PCR protocol comprised an initial denaturation step at 94°C for 4 min followed by 12 touch-down cycles involving: denaturation at 94°C for 30 sec, annealing at 65-54°C for 1 min and extension at 72°C for 1 min; followed by another 25 cycles of denaturation at 94°C for 30 sec, annealing at 53°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. The PCR products were run on an agarose gel.
The second strategy involved using Takara Ex Taq (Takara Bio Inc., Shiga, Japan) using the PCR conditions described above. The PCR product was then cloned using the TOPO TA Cloning Kit (Invitrogen) following the manufacturer’s instructions. Growing colonies were picked and a PCR cycle was performed. The products were run on an agarose gel and then purified. A restriction-digestion reaction was performed to confirm that the inserted fragment was the correct size. The products were sent for sequencing to the GenePool Sequencing Facility.
Table 4.1 Oligonucleotide primers used to amplify and sequence SSU, ITS, rbcL and Coxl fragments from *T. pseudonana*.

<table>
<thead>
<tr>
<th>Name</th>
<th>Region</th>
<th>Sequence (5’ to 3’)</th>
<th>Original reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPrbcL1</td>
<td>rbcL</td>
<td>AAG GAG AAA THA ATG TCT</td>
<td>Jones et al. 2005</td>
</tr>
<tr>
<td>DPrbcL7</td>
<td>rbcL</td>
<td>AAR CAA CCT TGT GTA AGT CTC</td>
<td>Jones et al. 2005</td>
</tr>
<tr>
<td>14R</td>
<td>rbcL</td>
<td>GAA TAC GCA TAT CTT CTA AAC G</td>
<td>Jones et al. 2005</td>
</tr>
<tr>
<td>15R</td>
<td>rbcL</td>
<td>ACA CCA GAC ATA CGC ATC CA</td>
<td>Jones et al. 2005</td>
</tr>
<tr>
<td>16F</td>
<td>rbcL</td>
<td>TTA GAA GAT ATG CGT ATT</td>
<td>Jones et al. 2005</td>
</tr>
<tr>
<td>17R</td>
<td>rbcL</td>
<td>TGA CCA ATT GTA CCA CC</td>
<td>Jones et al. 2005</td>
</tr>
<tr>
<td>18R</td>
<td>rbcL</td>
<td>AAT CAG CTC TAT CTG TAG</td>
<td>Jones et al. 2005</td>
</tr>
<tr>
<td>EAF3</td>
<td>SSU</td>
<td>TCG ACA ATC TGG TTG ATC CTG CCA G</td>
<td>Pröschold et al. 2001</td>
</tr>
<tr>
<td>ITS055R</td>
<td>LSU</td>
<td>CTC CTT GGT CGG TGT TTC AAG ACG GG</td>
<td>Pröschold et al. 2001</td>
</tr>
<tr>
<td>E528F</td>
<td>SSU</td>
<td>TGC CAG CAG CYG CGG TAA TTC CAG C</td>
<td>Marin et al. 2003</td>
</tr>
<tr>
<td>920F</td>
<td>SSU</td>
<td>GAA ACT TAA AKG AAT TG</td>
<td>Marin et al. 2003</td>
</tr>
<tr>
<td>BR</td>
<td>SSU</td>
<td>TTG ATC CTT CTG CAG GTT CAC TTA C</td>
<td>Marin et al. 2003</td>
</tr>
<tr>
<td>920R</td>
<td>SSU</td>
<td>ATT CCT TTR AGT TTC</td>
<td>Marin et al. 2003</td>
</tr>
<tr>
<td>536R</td>
<td>SSU</td>
<td>GWA TTA CCG CGG CKG CTG</td>
<td>Marin et al. 2003</td>
</tr>
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<td>GF</td>
<td>ITS</td>
<td>GGG ATC GT TGC CGT AGG TGA ACC TGC</td>
<td>Coleman et al. 1994</td>
</tr>
<tr>
<td>GR</td>
<td>ITS</td>
<td>GGG ATC CAT ATG CTG AAG TTC AGC GGG T</td>
<td>Coleman et al. 1994</td>
</tr>
<tr>
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<td>COXI</td>
<td>CAA CCA YAA AGA TAT WGG TAC</td>
<td>Saunders 2005</td>
</tr>
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<td>COXI</td>
<td>AAA CTT CWG GRT GAC CAA AAA</td>
<td>Evans et al. 2007</td>
</tr>
<tr>
<td>coxl Th.F</td>
<td>COXI</td>
<td>GCA ACC TAT AGT CCG CAA</td>
<td></td>
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<tr>
<td>coxl Th.R</td>
<td>COXI</td>
<td>GTC TTG GCA TAC CTG CAA</td>
<td></td>
</tr>
</tbody>
</table>
4.2.2.2 AFLP technique

Genomic DNA from six axenic strains was extracted using Plant DNAzol as described in Section 4.2.2. Two extractions were performed on different days in order to assess if variations in the AFLP analyses were due to differences in extraction processes. The purity of DNA was estimated using a spectrophotometer (NanoPhotometer 2.0, Implen GmbH, Schatzbogen 52, Germany) only samples with ratio $A_{260}/A_{280}$ above 2 were processed.

Restriction and ligation of the genomic DNA was performed in a single reaction (Mannschreck et al. 2002) using the endonucleases $Mse$I and $Eco$RI. 5.5µl of genomic DNA were incubated following the protocol described in Müller et al. (2005) using the $Mse$I and $Eco$RI adaptors. The pre-selective amplification was modified as follows: 4µl of the diluted digestion products were used with primers $Mse$I+0 and $Eco$RI+0 (2.5 pmol each, primer sequence as in Vos et al. (1995), 10µl of Taq PCR Mastermix (Qiagen, Hilden, Germany) and 5µl double distilled water, in a total volume of 16µl. The parameters for the amplification were 5 min at 94°C, followed by 20 cycles of 20 sec at 94°C, 30 sec at 56°C and 120 sec at 72°C. Control of the pre-selective amplification was checked on a 1.5% agarose gel and depending on the amount of generated PCR products observed; these were then diluted prior to the selective amplification as described in Müller et al. (2005). In this next amplification, 4µl of the diluted pre-selective amplification products were used in a total reaction volume of 16µl with the following primer combinations: $Eco$RI+A (10 pmol), $Eco$RI+C (5 pmol) and $Mse$I+C (10 pmol). $Eco$RI+A was labelled with the fluorochrome 6-FAM and $Eco$RI+C with VIC (Applied Biosystems) and the selective amplification conditions were 5 min at 94°C, followed by 10 cycles of 20 sec at 94°C, 30 sec at 65°C with 1°C decrease after each cycle down to 56°C and 120 sec at 72°C, followed by 20 cycles of 20 sec at 94°C, 30 sec at 56°C and 120 sec at 72°C. The
AFLP reaction was completed twice with the first DNA extraction and for a third time with the second DNA extraction on different days to test the reproducibility of the technique.

4.2.2.3 AFLP fragments evaluation

4.2.2.3.1 Electrophoresis in agarose gel

The AFLP fragments were visualised using gel electrophoresis, a 4% gel was prepared using MetaSieve Agarose (Flowgen Bioscience Ltd., Nottingham, UK), a fine resolution agarose capable of providing separation of DNA fragments of between 100-1000bp. The agarose was prepared in 1x TBE buffer and poured in a chamber of 12x25cm. 8µl of the selective amplification plus 2µl of the loading dye were loaded and the gel was run for 10 h. at a voltage of 80V. The gel was stained for 90 min with ethidium bromide (EtBr) before being photographed.

4.2.2.3.2 Fragment evaluation with the Agilent 2100 bioanalyzer

The Agilent 2100 bioanalyzer (Agilent Technologies Deutschland, Waldbronn, Germany) is capable of sizing and quantifying PCR fragments based on capillary electrophoresis principles. The bioanalyzer analysed disposable chips (Fig. 4.1), each contain 16 wells interconnected via glass microchannels, which are filled with a gel-dye mix. Of the 16 wells, 12 were use for the samples, 3 are use for the gel-dye mix and one was used for a molecular weight ladder. DNA size separation is performed inside the microchannels. The movement of DNA through the microchannels is controlled by electrodes, which create electrophoretic forces, driving the DNA molecules. As the electrical voltage is applied, different sizes of DNA fragments labelled with fluorescent dye are separated according to their mass, with the smaller fragments migrating the fastest.

DNA 12000 LabChips were prepared and loaded with samples as recommended by the manufacturer. Gel-dye mix (9.0µl) was loaded at the bottom of the relevant well, left for
30 sec and pressure was then applied through a 1ml syringe forcing the gel-mix into the microchannels. The ladder and sample wells were loaded with 5µl of DNA marker plus 1µl of either sample or DNA ladder. Chips were then vortexed for 1 min at 2400 rpm in an IKA (IKA® Werke GmbH & Co. KG) vortex mixer. Immediately after vortexing the chips were inserted into the Agilent 2100 bioanalyzer for analysis using the Agilent 2100 expert software.

Fig. 4.1. Agilent DNA chip 12000
(A) The Agilent DNA chip 12000 used in the Agilent 2100 bioanalyzer, this chip is designed for the sizing and quantification of DNA fragments from 100 to 12000 bp.
(B) Internal view of the chip formed by interconnected microchannels used for separation of nucleic acid fragments based on their size, driven through electrophoretically. (from Lu et al. 2002)

4.2.2.3.3 Fragment evaluation with the ABI 3730 capillary sequencer

An ABI 3730 capillary sequencer (The GenePool Sequencing Facility, The University of Edinburgh, UK) was used to separate the fluorescently labelled AFLP fragments by electrophoresis. In order to be able to size the resultant fragments, the GeneScan 1200 LIZ size standard (20-1200bp range; Applied Biosystems, Foster City, CA, USA) was included in the samples. Sizing and detection of fragments was performed using the program GelQuest (SequentiX - Digital DNA processing, Klein Raden, Germany). This program performed an automated detection and sizing of fragments by using a set of predefined
fragments from the size standard, the recognized fragments are then used to construct a binary matrix for the presence/absence of fragments. When comparing the three replicates for each strain, only fragments present in at least two of the three replicates were counted. All the strains were further manually analysed, to avoid peak exclusion of low intensity peaks, and also to avoid recognition of “false” peaks due to a strong signal in a neighbouring channel or to background noise. The resulting binary matrix was exported in NEXUS format and analysed with PAUP 4.0b10 (Swofford 2002). A distance matrix was constructed using the restriction-site distance of Nei & Li (1979) and this was used for neighbour-joining (NJ) analysis. The confidence of the tree topology was tested by bootstrap analyses (2000 replicates). For maximum parsimony (MP) analyses, the binary matrix was equally weighted and the confidence of the tree topology was also inferred by bootstrap analyses (2000 replicates).

4.3 Results
4.3.1 Molecular phylogeny of *T. pseudonana* using nuclear, plastid and mitochondrial sequences

The phylogenetic analyses of nuclear SSU and ITS rDNA sequences presented in Fig. 4.2 revealed no variable sequence positions among the strains. All strains were completely identical in ITS1-5.8S-ITS2 and the SSU sequences, despite their distinct geographic origins. This was supported by bootstrap analyses. The same results were obtained in the phylogenetic analyses of the plastid *rbcL* sequences. As demonstrated in Fig. 4.3, all strains have identical sequences.

All attempts described below to amplify the mitochondrial *cox1* sequences failed. The following experiments to amplify the *cox1* were performed: variations in DNA concentration, PCR cycle and primers were made without success, new primers were then designed. The primer design was based on the genome sequence for this model diatom,
available from GenBank, the primers chosen were called \textit{cox}1 Th.F and \textit{cox}1 Th.R (sequences listed in Table 4.1). A new PCR was run with these primers, but once again, no fragments were visualized in the electrophoresis gel.

A new strategy involving the use of Takara Ex Taq (Takara Bio Inc., Shiga, Japan) produced a PCR product that was then cloned using the TOPO TA Cloning Kit (Invitrogen) following instructions from the manufacturer. The products obtained after amplification of the colonies were run on an agarose gel, where a product appeared to be present and this was then purified. A restriction-digestion reaction was performed to confirm that the fragment, product of the cloning, was approximately the size expected. The products were purified and sent for sequencing to the GenePool Sequencing Facility. The quality of the sequences obtained was very poor and were not possible to analyse. A new attempt was made to improve the sequencing data, but failed once again. Due to time restrictions it was not possible to progress further with these experiments, and the amplification of the mitochondrial marker \textit{cox}1 was not possible.
Fig. 4.2. Phylogeny of the strains of *T. pseudonana* based on ITS-SSU sequence comparisons. Phylogenetic tree inferred by maximum likelihood method, and bootstrap values from 100 replicates (using the Tamura-Nei model calculated as best model by Modeltest 3.7).
Fig. 4.3. Phylogeny of the strains of *T. pseudonana* based on *rbcL* sequences comparisons. Bootstrap values from 100 replicates calculated from Maximum likelihood method.
4.3.2 AFLP analyses

Since barcoding molecular markers were not able to establish differences between the strains of this particular diatom species, AFLP analysis was performed. This technique was also used to test its usefulness as a whole genome approach to characterise the different geographic isolates of *Thalassiosira pseudonana*, and its potential to reveal intra-species level relationships / strain differences in algae.

4.3.2.1 Visual analyses of fragments from the agarose gel

Products of the selective amplification were separated in 1% agarose gels (Appendix 4) and then visualised on the more sensitive MetaSieve Agarose gel (Flowgen Bioscience Ltd., Nottingham, UK), this high resolution agarose was stained with ethidium bromide (EtBr) for 90 min. A photograph of the gel was taken to visually analyze the fragments, and as showed in Appendix 5, the resolution of the fragments was not high enough to distinguish clear differences among the different strains.

4.3.2.2 Analyses of fragments with the Agilent 2100 bioanalyzer

The products obtained from the selective amplification were analyzed on an Agilent 2100 bioanalyzer. The bioanalyzer displays data as computer-generated virtual gels and electrophenograms. The gel generated revealed some differences in banding patterns between the strains (Fig. 4.4).
With this method, the visual comparison of fragments was easier due to the clarity of the image obtained. *T. pseudonana* CCMP1014 and CCMP1015 showed virtually identical banding patterns. A higher variation in banding patterns was observed in strain CS-20, and duplicate strains CCMP1335 and CS-173 seem to share an identical pattern, although the intensity of the bands was clearly stronger in CCMP1335 (Fig. 4.4).

To further analyse the data, the image files produced by the bioanalyzer were imported into GelQuest (SequentiX - Digital DNA processing, Klein Raden, Germany) and detection and sizing of the fragments performed. The automated evaluation of the image files revealed...
108 fragments and the resulting binary matrix was exported for phylogenetic analyses (Fig. 4.5). The method chosen for tree construction was a distance method that would calculate the divergences of each isolate related to the others, neighbour-joining, and to estimate the reliability of this phylogenetic tree, bootstrapping of 2000 replicates was used.

The unrooted tree demonstrated that the strains studied subdivided into three lineages according to their geographical origin (highlighted in different colours in Fig. 4.5), with the exception of CS-20 and CCMP1013, despite their different origins (Australia and United Kingdom respectively).
Fig. 4.5. Phylogeny of the axenic *T. pseudonana* strains based on AFLPs derived from image files produced by the bioanalyzer. Radial (unrooted) neighbour-joining tree obtained after 2000 bootstrap replicates. Genetic distances shown.
4.3.2.3 AFLP analyses with the ABI 3730 capillary sequencer

Initially, a small volume of the selective amplification (2µl) product was combined with formamide and sent for sequencing. No results were obtained although it was clear that products from the selective amplification were present in the MetaSieve agarose gel and in the bioanalyzer. This indicated that the problem was not with the AFLP reactions, but probably in the chemicals added. There was the possibility of free-radicals from the formamide reacting with the DNA in transit, so another batch of samples was sent, this time just the selective amplification products dried down. Once again, no results were obtained. Having eliminated the potential problem of the formamide reacting with the DNA, it was decided that the customised fluorescent primers could be faulty, since they are indispensable for the sequencer to be able to read the samples. The primers were tested using a spectrophotometer (NanoPhotometer 2.0, Implen GmbH, Germany) to check their fluorescence emission in comparison to that of unlabelled DNA; results demonstrated that no emission was produced at the wave length expected, indicating a fault in the customised primers. The primers were replaced and the selective amplification performed. This time 1µl of the ten times diluted selective amplification was combined with 1µl of the fifty times diluted GeneScan 1200 Liz Size Standard and sent for sequencing to the GenePool Sequencing Facility. Sequences were obtained and analysed using the GelQuest software.

Automated evaluation was performed on three replicates of each of the six axenic strains. Ideally, a consensus of the three replicates would have been performed, but the software used for the automated evaluation did not contain this feature. The automated evaluation revealed differences between the strains including some differences between the replicates, especially between those of different DNA extractions. However, it was noticeable that the automated evaluation revealed a greater variation among samples due to differences in the intensity of the fragments. Those fragments of intensity lower than a certain threshold
value were not detected in automated evaluation, establishing a false variation in the AFLP pattern. A potential solution to this problem was to set threshold values lower, to allow recognition of low intensity fragments, the problem encountered then was “false” peak recognition due to a strong signal in neighbouring channels being detected as a peak or general background noise. To resolve these issues, after automated evaluation the AFLP phenograms were manually corrected to reduce the number of artifactual differences among the strains and also within the replicates.
Fig. 4.6. Electrophenograms of the AFLP patterns for each of the six strains with one primer combination: EcoRI+C/Msel+C. Fragments size range from 250 to 375 nucleotides. Examples of differences in fragments highlighted. Horizontal scales, size of fragments in nucleotides.
After the manual refinement, 547 fragments were recognized, and the resulting binary matrix was exported for phylogenetic analyses in PAUP 4.0b10 (Swofford, 2002). Phylogenetic trees were generated for both, character-based methods (parsimony, Fig. 4.7) and for distance-based methods (neighbour-joining, Fig. 4.8). The robustness of the trees was assessed by bootstrap analyses of 2000 replicates and percentage values determined. Both trees were very similar; however, the neighbour-joining tree seemed to group more closely together the sequences from the same DNA extraction.

Fig. 4.7. Radial (unrooted) maximum parsimony tree of the *T. pseudonana* strains based on AFLPs originated from automated evaluation with manual refinement. Bootstrap percentage values from 2000 replicates shown.
Fig. 4.8. Radial (unrooted) neighbour-joining tree obtained using the restriction-site distance of Nei & Li (1979). Bootstrap percentage values from 2000 replicates shown.
4.4 Discussion

The aim of this chapter was the genomic characterization of different strains of the centric diatom *T. pseudonana*. This is particularly important since this diatom has been chosen as a model organism for a great number of studies and biotechnological applications (e.g. Volkman et al. 1989, Armbrust et al. 2004, Tonon et al. 2004, Alverson et al. 2007, Dassow et al. 2008, Hildebrand et al. 2009). Furthermore, it has been proposed that all organisms below 1mm are cosmopolitan (Finlay & Clark, 1999, Finlay 2002), but this has been based on the morpho-species concept. We know from studies on cryptic diversity where species may be morphologically identical, or almost identical, but genetically distinct, that morphology on its own may not always be able to resolve species identification (Amato et al. 2007, Evans et al. 2007). However, molecular techniques, have been used to reveal cryptic species (Sarno et al. 2005, Amato et al. 2007, Evans et al. 2008), and in some cases even biogeographic patterns (John et al. 2004, Kooistra et al. 2008). It is therefore of great importance, to investigate if biogeographic distribution patterns could be detected among the different isolates of this diatom by combining molecular markers proposed for diatom DNA barcode, and a DNA fingerprinting technique.

In the present study, the amount of genetic diversity within ten *T. pseudonana* isolates was investigated using a barcoding approach, with a more detailed whole genome technique for the six axenic strains. For this purpose, three molecular markers previously proposed as barcode genes for diatoms were used, and as a whole genome approach, the AFLP technique was employed (Vos et al. 1995). This technique has been previously used to study genetic variation among other closely related algal species (De Bruin et al. 2004, John et al. 2004, Müller et al. 2005, De Martino et al. 2007).
In this study, all the strains had identical ITS and SSU sequences in the nuclear ribosomal DNA and also in the plastid gene that encodes for the large subunit of RuBisCO (rbcL) as shown in Figs 4.2 and 4.3. However, the AFLP patterns obtained showed genetic diversity and although the levels of intra-specific genetic diversity were low, they seem to correspond with biogeographic patterns (Figs 4.5, 4.7 & 4.8).

4.4.1 The use of current barcoding genes to establish genotypic variability at an intra-species level

The hypothesis that DNA barcode genes can be employed to differentiate *Thalassiosira pseudonana* strains was proven incorrect in this chapter. In this study, all ten *Thalassiosira pseudonana* strains had identical ITS and SSU sequences for the nuclear ribosomal DNA and also the plastid gene that encodes for the large subunit of RuBisCO (rbcL), despite their different geographic origins. No data was obtained from the mitochondrial gene (cox1), even after cloning of the fragment and the design of different primers, the resulting sequence was of a very poor quality and therefore not possible to analyse. As described in Evans et al. (2007), additional primers need to be developed for a universal *cox1* diatom barcoding system to be viable. These studies suggest a low universality of this mitochondrial marker, possibly due to the presence of introns (Armbrust et al. 2004), which implies that the mitochondrial gene may not be the best choice for barcoding of diatoms as it has been recently discussed in Hamsher et al. (2011 In press.).

These molecular markers were chosen for sequencing due to their previous success in identifying cryptic species in other organisms and comparison among closely related species (Alverson et al. 2007, Evans et al. 2007, Moniz & Kaczmarska 2009, 2010), but for this model organism no intra-species variation could be detected between the different isolates. Although no correlation between ITS identity and geographic origin has previously been reported for related green algal groups (Pröschold et al. 2005), a number
of studies have successfully used molecular markers to investigate biogeographic patterns and genetic variation between closely related *Skeletonema* isolates, (Kooistra et al. 2008). Furthermore, Casteleyn et al. (2008) successfully used the nuclear encoded internal transcribed spacers (ITS1-and ITS2) of the ribosomal operon to demonstrate genetic variation and geographic distribution of 193 isolates of the diatom *Pseudo-nitzschia pungens*. However, these molecular markers may not always be sensitive enough to reveal genetic variation among isolates of the same species as demonstrated in the present study, where no variable sequence positions in the ITS, SSU and *rbcL* barcodes were observed.

4.4.2 The use of a whole genome approach to establish genotypic variability at an intra-species level

AFLP analyses are generated for many loci across the whole genome (increasing the possibility to find those polymorphic). These analyses have been proven to resolve genetic variations when other markers have reached their limits (Werner et al. 2001, John et al. 2004, Müller et al. 2005) and so have the potential to reveal genetic diversity and differentiate between populations.

In this study AFLP was employed to examine the genetic diversity and biogeography of six isolates of *T. pseudonana*. To test if slight variations in culture regimes and conditions could have an effect in genetic stability, two duplicate strains originated from a single clone: CCMP1335 and CS-173 previously held in two different culture collections for decades were included in the study. Since a consensus of the three replicates was not possible with the software available, the three replicates for each isolate were included in the final analyses to test the reproducibility of the technique. By visual comparison, after automated evaluation, three clusters were distinguished from the ABI 3730 capillary sequencer analysis (Figs 4.7, 4.8). Patterns within the three replicates appeared grouped, and patterns within a cluster were more similar than those among the clusters. The three
clusters distinguished corresponded according to their origin (the North eastern Pacific Ocean, North western Atlantic Ocean and the third group comprised the North eastern Atlantic and the South eastern Indian Ocean). It is interesting to point out that the same clusters were obtained using the bioanalyzer (Fig. 4.5), which recognized only 108 fragments (compared with the 547 recognized by the ABI 3730 capillary sequencer) and those fragments were enough to be able to distinguish between these strains. The level of intraspecific genetic diversity between the strains was low, but nonetheless there seemed to be enough variation present to differentiate biogeographic patterns.

This fingerprinting approach discriminated between the isolates from different geographic origins, suggesting the importance of this technique for intra-species taxonomic analyses, especially when the molecular markers had reached their resolution limits. Furthermore, the results obtained using the bioanalyzer strengthened the overall results, confirming what previous studies had reported, that the bioanalyzer is a rapid, reliable and accurate instrument for sizing and quantifying multiple DNA fragments (Panaro et al. 2000, Nachamkin et al. 2001, Esteve-Zarzoso et al. 2010) reducing the need for sophisticated equipment.

These results proved the hypothesis that whole genome approaches are needed to differentiate between strains of *Thalassiosira pseudonana*, and that genomic variation is expected between the strains according to biogeographic distribution patterns. Furthermore, it highlights the importance of this technique to study planktonic microbial populations, their genetic variation and their biogeographies. The results gathered from this particular AFLP analyses, does not support the proposed concept of “everything is everywhere” (Finlay & Clark, 1999, Finlay, 2002). Although this study only employed a small number of strains, these six *Thalassiosira pseudonana* isolates exhibited detectable genetic variation; moreover, this variation was greater between different geographic areas than within the same area (Figs
The group containing isolates from Wales, UK, and Australia does pose the question of whether this wide distribution occurred naturally, or was due to human dispersal. A very similar distribution pattern has been previously documented for *Sellaphora capitata* (Evans et al. 2009) and for pseudocryptic species of *Navicula cryptocephala* (Pouličková et al. 2010), where they proposed the possibility that these species could have been introduced to Australia in the 1880s, when lakes were stocked with European fish (Crowl et al. 1992, Evans et al. 2009, Pouličková et al. 2010), as had been reported for other freshwater diatoms (Harper, 1994). Since the Australian strain CS-20 was isolated from an estuary sometime before 1972, there is the possibility that something similar could have happened. The transport of non-indigenous phytoplankton species by ships in ballast water was first suggested by Ostenfeld (1908) and it has since been well documented (Hallegraeff & Bolch, 1992, Ruiz et al. 1997, Burkholder et al. 2007). The Australian strain was isolated from the Swan River, an estuarine environment, suggested to be the one of the more difficult environments when trying to distinguish if a particular species is native or not (Ruiz et al. 1997), and Burkholder (2007) revealed that the most representative taxa of viable phytoplankton found in ballast tank water were diatoms and dinoflagellates, but especially centric diatoms. Although *T. pseudonana* has a widespread distribution, this study revealed certain genetic variation between the different isolates.

### 4.4.3 Duplicate strains

Two duplicate strains were included in the AFLP analyses, both strains clustered together; however, some genetic variation was observed between them and also within their replicates. Moreover, the variation between the replicates was, sometimes, bigger than the variation between the duplicates. The AFLP technique is very sensitive to DNA quality (Vos et al. 1995), if this is not high enough, the reproducibility of the technique can be
compromised, with the band intensity varying, altering the fingerprint profiles. In general, pure DNA has an expected $A_{260}/A_{280}$ ratio of >1.8 (Glasel, 1995). In this study the DNA quality was assessed using a spectrophotometer that revealed the quality as 2, suggesting a high purity and therefore, no alteration in AFLP profiles of these two strains was due to DNA quality. Another possible cause of variations in the AFLP profiles was DNA quantity, if this is not high enough, the resulting AFLP profile will demonstrate low reproducibility (Meudt & Clarke, 2007). The exact quantity of DNA was not estimated in this study and it may be speculated that this could be the reason for the AFLP profiles obtained for these two identical strains and their replicates. Contaminant DNA would affect banding patterns, generally introducing “false” peaks and so altering the desired AFLP profile (Dyer & Leonard, 2000, Müller et al. 2005), this could make the profiles of two identical strains exhibit distinct banding patterns. In this study only axenic strains were used and their axenicity was carefully monitored every two weeks by using nutrient agar and by microscopic examination. Additionally, upmost care was taken for the DNA extraction and the PCR reactions, which were performed following aseptic techniques on a clean bench. Furthermore, to confirm that no contaminant was present, two DNA extractions were performed on two different days and from different cultures. Bacterial contamination results in very significant AFLP differences (Müller et al. 2007). With all of these precautions, one more consideration was left, viral DNA contamination. Due to their small size, viruses are very difficult to detect in algal cultures, and there is no guarantee that the strains employed were virus free, but it is known that a viral contamination will generate a noticeable number of additional AFLP fragments (Müller, 2005), and the variation in AFLP patterns observed among the strains studied here, is unlikely to be due to viral infection. However, despite all the precautions some irregularities were observed between the duplicate strains, and although genetic variation could have occurred as a result of the selective nature of the culturing techniques and regimes employed, the
irregularities presented within the replicates suggested either a contamination problem or intrinsic variability. AFLP reproducibility can also be affected by a number of other parameters when analysing the data and to minimize error, a number of these can be optimised, such as: peak high thresholds (intensity above which a peak is scored) and minimum fragment size among others; but this optimization can be very challenging due to peak mobility and fragment intensity (Müller, 2005, Meudt & Clarke, 2007). In this study, a size standard was included in the samples to estimate fragment size (mobility) through automated evaluation, even though, visual inspection was required to correct or discard low-quality profiles. Reproducibility of the technique would, perhaps, have been more reliable with the possibility of performing a consensus out of the three replicates; moreover, the final analyses would have been easier, but this feature was not possible with the software available.

The results obtained in this chapter revealed that barcoding genes may reach their resolution at a species level, as demonstrated in this study where the ten *T. pseudonana* isolates shared identical ITS, SSU and *rbcL* sequences. In the present study a fingerprinting technique (AFLP) revealed some intra-species variation between the strains, and even discriminated on the basis of geographic origins. In future, when whole genome analyses are cheaper, these levels of resolution will become relatively routine and will assist in elucidating phytoplankton diversity and biogeographies.
Chapter 5. Cryopreservation of diatoms

5.1 Introduction

Cryopreservation facilitates the long-term storage of microalgae and can reduce the time, resources and cost for the maintenance of culture collections holdings. This approach can also reduce the risks of contamination, genetic drift, phenotypic change and loss by death (Day et al. 2005, 2007). Furthermore, cryopreservation has been applied in the aquaculture industry (Cañavate & Lubián, 1995, Rhodes et al. 2006), since microalgae are used as feed-stock for larvae and juvenile stages of shellfish there is a need to maintain healthy and stable stock-cultures. Over the last few decades a number of studies have focused on developing a universal protocol that would allow the cryopreservation of a wide range of microalgae, thus facilitating the long-term storage of these organisms (Cañavate & Lubián, 1994, 1995, Day, 1998). A range of protocols and techniques that have been applied to a variety of biological materials including algae can be found in the scientific literature (e.g. Polge et al. 1949, Karlsson & Toner, 1996, Andersen, 1996, Cañavate & Lubián, 1995, 1997, Day & DeVille, 1995, Day et al. 1997, Rhodes et al. 2006). A key aim of preserving microalgae at ultra-low temperatures is to permit the long-term storage of these organisms without a significant reduction in viability (Day et al. 1997). However, low temperatures can have dramatic effects on the cells (Karlsson & Toner, 1996), with the main causes of cell damage being due to ice formation, both intracellular and extracellular, and changes in osmotic potential experienced by the cells during cryodehydration (Mazur et al. 1972).

Cryopreservation and recovery protocols vary depending on the taxa studied, with “two-step” cooling processes involving controlled cooling from room temperature to a holding temperature, before immersion in liquid nitrogen (LN₂) being the most suitable approach to avoid cell damage in algae (Morris, 1978). Moreover, successful protocols usually involve the use of colligative chemical cryoprotectants, that penetrate inside the cell, maintain
“salts” in solution, thus avoiding the concentration of solutes in the interior of the cell (Mazur, 1970) and decreasing the freezing-point of the intracellular water (Franks, 1985). In a “two-step” slow cooling process, using a colligative cryoprotectant, extracellular ice formation occurs first, with the consequent movement of water from inside the cell to the outside, followed by the increase of intracellular solute concentration, which will be compensated by the presence of the cryoprotectant. If the cooling rate is slow, movement of water from inside the cell to the outside occurs and intracellular ice formation is normally avoided; but if the cooling rate is too fast, extracellular water will freeze and there is insufficient time for intracellular water to leave the cell, resulting in intracellular ice formation (Farrant, 1980). Temperature control, combined with the used of cryoprotectant, mitigates cryoinjuries in algae (Day et al. 1998a, b, Fleck et al. 1997, 1999). The most commonly employed cryoprotectants for microalgae are dimethyl sulphoxide (Me₂SO, DMSO) and methanol (Meryman, 1966, Fleck et al. 1996, Taylor & Fletcher, 1998). Both have been successfully used in a wide range of microalgae (McLellan, 1989, Cañavate & Lubián, 1994, 1995, Bodas et al. 1995, Day, 1998) and were originally chosen because of their fast penetration of the cell membrane, as well as their relative ease of removal after thawing. However, these cryoprotectants, can be toxic to the cells (Fleck, 1998), therefore the concentration and exposure time prior to treatment are crucial to the success of this technique.

There have been a few studies focussing on the effect of thawing temperatures, and it has been suggested that in some species such as *Tetraselmis chuii*, a rapid thaw correlates with higher viability (Cañavate & Lubián, 1997). Another consideration is the toxicity of most cryoprotectants and dilution of the sample in medium may be necessary straight after thawing (Day & Brand, 2005).
All of the above need to be considered when developing a cryopreservation protocol for an individual microalga. Furthermore, variation in susceptibility to cryoinjury within species, or even strains of the same species (Morris, 1976) may complicate protocol development further.

Initially, the aim of this chapter was to prove the hypothesis that cryopreservation ensures genotypic and phenotypic stability of *T. pseudonana* strains, and to find a suitable cryopreservation protocol for the long-term storage of *T. pseudonana* strains. Due to difficulties encountered finding an optimum cryopreservation protocol, the study of the stability after treatment was not possible, and the aim of this chapter became the development of an optimum cryopreservation protocol.

5.2 Materials and methods

5.2.1 Strains studied

In this chapter *T. pseudonana* strains CCMP1007, CCMP1011, CCMP1012, CCMP1013, CCMP1014, CCMP1015, CCMP1335, CS-20 and CS-173 were studied (see Table 1.1 for further details). All the strains were cultured as described in Chapter 2, Section 2.2.1. Early stationary phase cultures (two week old) were harvested by decanting medium from the top of the flask and resuspending the sedimented algae in 25ml of F/2+Si medium. This material was employed in all subsequent cryopreservation experiments. As detailed above (Chapter 2, Section 2.2.1) the axenic strains used in cryopreservation experiments were periodically tested in enriched medium containing nutrient agar for the presence/absence of contaminants.
5.2.2 Cryoprotectant toxicity test

The tolerance to different cryoprotectant concentrations was determined by preparing aliquots (0.5ml) of harvested cells for each *T. pseudonana* strain. Harvested cells were dispensed into cryovials (Greiner Bio-One GmbH, 72636 Frickenhausen, Germany). DMSO 99.9% (for molecular biology use, Sigma-Aldrich Ltd., UK), was filter-sterilized in sterile F/2+Si medium to a final concentration of 10%, 20% and 30% (v/v) using a 0.20μm sterile syringe filter (IWAKI, Japan). An aliquot (0.5ml) of the 10%, 20% and 30% (v/v) DMSO solution was added to the harvested cells (except for the control) to give a final DMSO concentration of 5%, 10% and 15% (v/v). This was then incubated at 20°C under standard conditions (see Chapter 2, Section 2.2.1 for details) for 24 h., and the samples were observed under a light microscope after 1.5 h. and 24 h. After 24 h. of incubation, the DMSO was removed by gentle centrifugation (5 min at 3000 rpm in a SIGMA 1-14 microcentrifuge (Sigma-Aldrich Ltd., Dorset, UK). The samples were then resuspended in 0.5ml of fresh media prior to inoculation into 9ml of fresh F/2+Si medium, and incubated under standard conditions (Chapter 2, Section 2.2.1) for three weeks.

5.2.3 Cryopreservation protocols

5.2.3.1 Standard CCAP algal cryopreservation protocol

Aliquots (0.5ml) of harvested *T. pseudonana* cells were dispensed into cryovials (Greiner Bio-One GmbH, 72636 Frickenhausen, Germany). DMSO 99.9% (Sigma-Aldrich Ltd., UK), was filter-sterilized as described above (Section 5.2.2). A 0.5ml aliquot of the 10% (v/v) DMSO solution was added to 0.5ml of the harvested cells to give a final DMSO concentration of 5% (v/v). This was then incubated at room temperature (~20°C) for 15 min prior to cryopreservation, to ensure that the cryoprotectant had entered the cells. The cryovials were then transferred to the cooling chamber of a Planer Kryo 360-3.3 (Planer,
UK) programmable freezer and cooled from room temperature at -1°C min\(^{-1}\) to -40°C as outlined below (Fig. 5.1).

![Diagram showing the two-step cooling protocol]

**Fig. 5.1.** Standard two-step cooling protocol used at CCAP.

After plunging into LN\(_2\), the samples were transferred to a cryostorage container and the location logged on the CCAP cryo-storage database.

### 5.2.3.1.1 Thawing and recovery procedure

After 24 h., samples were transferred in LN\(_2\) from the cryostorage facility to the lab. They were then thawed by direct immersion in a preheated water-bath and were removed as soon as all visible ice had melted. Three different thawing regimes were investigated using a water bath pre-set to 40°C, 30°C and 20°C.

Immediately after thawing, the samples were aseptically inoculated into test tubes containing 9ml of sterile F2+Si medium to dilute the concentration of the DMSO. The tubes were wrapped in aluminium foil to reduce the effect of light-induced stresses/metabolic uncoupling and incubated under standard conditions (see Chapter 2, Section 2.2.1 for details) for 24 h. before removal of the aluminium foil and subsequent viability testing.
5.2.3.2 Influence of cryoprotectant concentration and post-thaw removal on viability

Triplicate cryovials containing 0.5ml aliquots of *T. pseudonana* CCMP1335 were pre-treated with 5% DMSO, 10% DMSO or 15% DMSO (final concentrations). They were then cooled employing the Standard CCAP algal cryopreservation protocol as described in Section 5.2.3.1.

Samples were thawed and recovered as outlined above (Section 5.2.3.1.1) at 40°C. In addition, for aliquots that had been pre-treated with 5% DMSO, the cryoprotectant was removed after thawing by centrifugation for 5 min at 3000 rpm in a SIGMA 1-14 microcentrifuge (Sigma-Aldrich Ltd., Dorset, UK). These samples were resuspended in 0.5ml of fresh F/2+Si media prior to inoculation in the 9ml of fresh F/2+Si medium. In all cases samples were incubated as detailed above (Section 5.2.3.1.1).

5.2.3.3 Standard CCMP cryopreservation protocol to study the effect of different cryoprotectants and a different cooling protocol

*T. pseudonana* CCMP1335 and CS-20 were cryopreserved employing the standard protocol employed at the Provasoli-Guillard National Centre for culture of Marine Phytoplankton (CCMP) as detailed by Day and Brand (2005). Aliquots (0.5ml) of harvested cells were then cryoprotected using the following cryoprotectant solutions:

- DMSO 5% (v/v) final concentration.
- Sorbitol 5% (w/v) final concentration.
- DMSO 5% (v/v) + sorbitol 5% (w/v) final concentration.
After incubation at room temperature (~20°C) for 15 min, the cryovials were transferred to the Planer Kryo 360-3.3 programmable freezer and cooled as outlined below (Fig. 5.2).

**Fig. 5.2.** Standard CCMP two-step cooling protocol

Immediately after plunging into LN₂, the cryovials were transferred in LN₂ to a cryostorage container and their locations logged on the CCAP cryo-storage database. After 24 h., the strains were thawed at 40°C as described in the standard protocol (Section 5.2.3.1.1), and aseptically transferred into tubes containing 9ml of fresh, sterile F/2+Si medium. The tubes were then wrapped in aluminium foil and incubated under standard conditions for 24 h. before removal as outlined previously.

### 5.2.4 Post-thaw viability

The principal methods employed to assess post-thaw viability in this study were an assessment of cell growth by cell counting, or visual appearance of the culture, and vital staining, employing methods that demonstrated active metabolic/ enzymatic activity and the presence of an intact cell membrane (Day & DeVille, 1995).
5.2.4.1 Cell counts

Cell counts were performed on a haemocytometer, as described in Chapter 3, Section 3.2.1.1. using an Axiovert 200 light microscope (Carl Zeiss, Jena, Germany) prior to cryopreservation treatment, 24 h. after thawing, and after five days “recovery”.

5.2.4.2 Fluorescein diacetate

Fluorescein diacetate is a fluorochrome capable of producing fluorescent products after enzymatic modification. Viable cells, with an intact membrane and enzymatic activity, will fluoresce green, while non-viable cells are colourless or fluoresce red due to autofluorescence of chlorophyll (Rotman & Papermaster, 1966).

In this study, cells were stained with FDA (Sigma, final concentration 5µg/ml) for 15 min before examination under a Zeiss Axioskop 2 epifluorescence microscope (Carl Zeiss, Jena, Germany) fitted with 100W UV illumination and type 09 filter set (BP450-490, FT510, LP515) suitable for excitation between 450-490nm and emission at 515nm. Micrographs were taken with an AxioCam HRc camera (Carl Zeiss) using the AxioVision software, version 4.7.1 (Carl Zeiss). Viability assessment was estimated by calculating the percentage of FDA positives (live cells, stained in green) out of a total of 50 cells observed per sample.

5.2.4.3 Carboxyfluorescein diacetate succinimidyl ester

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a non-fluorescent, membrane permeable molecule. When inside a viable cell, due to enzymatic modification, CFSE becomes highly fluorescent; in addition, it losses permeability to the cell membrane, and it can remain detectable inside the cell for several days (Lyons, 2000).
CFSE in the form of a 5mM stock-solution in DMSO was freshly prepared and added to the samples to give a final concentration of 2µM. CFSE labelled cells were incubated at room temperature for 15 min before examination under an Axioskop 2 epifluorescence microscope and micrographs were taken as outlined above. Viability assessment was estimated as above, by calculating the percentage of CFSE positives (live cells, stained in green) out of a total of 50 cells observed per sample.

5.3 Results

5.3.1 Cryoprotectant toxicity test

No obvious cell lysis or changes in cell phenotype were observed after 1.5 or 24 h. exposure to DMSO (data not shown). The effect of prolonged exposure to cryoprotectants on their capability to divide was tested as described in Section 5.2.2, and results are shown in Table 5.1.

Table 5.1. T. pseudonana tolerance to cryoprotectant exposure.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control</th>
<th>Cryoprotectant concentration (DMSO) 5%</th>
<th>Cryoprotectant concentration (DMSO) 10%</th>
<th>Cryoprotectant concentration (DMSO) 15%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Survival and growth after 1 week</td>
<td>Survival and growth after 3 weeks</td>
<td></td>
</tr>
<tr>
<td>CCMP1007</td>
<td>+/+/+</td>
<td>+/+/+</td>
<td>+/+/+</td>
<td>+/+/+</td>
</tr>
<tr>
<td>CCMP1011</td>
<td>+/+/+</td>
<td>+/+/+</td>
<td>+/+/-</td>
<td>+/+/-</td>
</tr>
<tr>
<td>CCMP1012</td>
<td>+/+/+</td>
<td>+/+/+</td>
<td>+/+/+</td>
<td>+/+/+</td>
</tr>
<tr>
<td>CCMP1013</td>
<td>+/+/+</td>
<td>+/+/+</td>
<td>+/+/-</td>
<td>+/+/-</td>
</tr>
<tr>
<td>CCMP1014</td>
<td>+/+/+</td>
<td>+/+/+</td>
<td>+/-/-</td>
<td>+/-/-</td>
</tr>
<tr>
<td>CCMP1015</td>
<td>+/+/+</td>
<td>+/+/+</td>
<td>+/-/-</td>
<td>+/-/-</td>
</tr>
<tr>
<td>CCMP1335</td>
<td>+/+/+</td>
<td>+/+/+</td>
<td>+/+/-</td>
<td>+/+/-</td>
</tr>
<tr>
<td>CS-20</td>
<td>+/+/+</td>
<td>+/+/+</td>
<td>+/+/+</td>
<td>+/+/+</td>
</tr>
<tr>
<td>CS-173</td>
<td>+/+/+</td>
<td>+/+/+</td>
<td>+/+/+</td>
<td>+/+/+</td>
</tr>
</tbody>
</table>

+/+-/+ indicates post-thaw viability in three replicates
+/+-/+ indicates post-thaw viability in two replicates
+-/-/- indicates post-thaw viability in one replicate
-/-/- indicates no post-thaw viability observed
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Although all the strains treated regenerated in all the replicates by the end of week three (Table 5.1), it should be noted that initially most of the strains treated with the higher concentration (15%) seem to have died and after two weeks very little, or no growth was observed (Table 5.1).

5.3.2 Standard CCAP algal cryopreservation protocol

The CCAP standard cryopreservation protocol detailed in Section 5.2.3.1 was tested in nine strains, and post-thaw viability was calculated after thawing at 20°C, 30°C and 40°C (Table 5.2).

Viable and non-viable cells were easily differentiated microscopically on the bases of their fluorescence (Fig. 5.3). High viability levels (31-96) were observed 24 h. post-thaw for the nine strains examined using the standard CCAP algal cryopreservation protocol. Higher post-thaw viability levels were observed when thawing at 40°C (Table 5.2); however, five days after thawing, most cells appeared to be dead, i.e. cells changed colour and/or cell contents were lost leaving empty frustules. After two to three weeks incubation, only three strains (CCMP1007, CCMP1012 and CS-20) were recoverable (Table 5.2).

![Fig. 5.3. Epifluorescence microscope micrographs.
A. Live cells stained with CFSE.
B. Dead cells stained with CFSE, red fluorescence in the chloroplasts due to the chlorophyll. There is no green fluorescence in the cells, which indicates cell death.
C. Live cells stained with FDA.
D. Dead cells stained with FDA. Cells appear slightly green due to background fluorescence of the stained sample.](image-url)
Table 5.2. Effects of thawing temperature on the post-thaw viability of *T. pseudonana* strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Thawing temperature</th>
<th>% FDA Positive 24h</th>
<th>Regeneration of viable culture (3 weeks)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>40°C</td>
<td>30°C</td>
<td>20°C</td>
</tr>
<tr>
<td>CCMP1007</td>
<td>77 (3)</td>
<td>57 (10)</td>
<td>55 (20)</td>
</tr>
<tr>
<td>CCMP1011</td>
<td>69 (9)</td>
<td>47 (12)</td>
<td>55 (15)</td>
</tr>
<tr>
<td>CCMP1012</td>
<td>91 (11)</td>
<td>75 (15)</td>
<td>75 (10)</td>
</tr>
<tr>
<td>CCMP1013</td>
<td>85 (16)</td>
<td>60 (7)</td>
<td>73 (10)</td>
</tr>
<tr>
<td>CCMP1014</td>
<td>75 (5)</td>
<td>47 (16)</td>
<td>73 (21)</td>
</tr>
<tr>
<td>CCMP1015</td>
<td>57 (17)</td>
<td>38 (13)</td>
<td>47 (11)</td>
</tr>
<tr>
<td>CCMP1335</td>
<td>67 (2)</td>
<td>32 (14)</td>
<td>43 (29)</td>
</tr>
<tr>
<td>CS-20</td>
<td>96 (4)</td>
<td>73 (8)</td>
<td>84 (7)</td>
</tr>
<tr>
<td>CS-173</td>
<td>94 (7)</td>
<td>31 (15)</td>
<td>37 (11)</td>
</tr>
</tbody>
</table>

1Cooling rate was -1°C/min and the cryoprotectant used was 5% DMSO (final concentration). Results are mean for 3 observations of each of the three replicates. Standard deviations given in brackets. +/-/+ indicates post-thaw viability in three replicates. +/+/- indicates post-thaw viability in two replicates. +/-/- indicates post-thaw viability in one replicate. -/-/- indicates no post-thaw viability observed.

5.3.3 Influence of cryoprotectant concentration and post-thaw removal on viability

High levels of post-thaw viability were obtained after 24 h. with all three cryoprotectant concentrations tested (Fig. 5.4). However, higher initial post-thaw viability levels were observed with 10% DMSO (Fig. 5.4); furthermore, only cells treated with this concentration of cryoprotectant showed regeneration after two to three weeks (Table 5.3).
Table 5.3. Effect of cryoprotectant concentration on the capacity of *T. pseudonana* CCMP1335 to regenerate cultures.\(^1\)

<table>
<thead>
<tr>
<th>Strain</th>
<th>5% DMSO(^2)</th>
<th>10% DMSO(^2)</th>
<th>15% DMSO(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCMP1335</td>
<td>+/-/-</td>
<td>+/+/-</td>
<td>+/-/-</td>
</tr>
</tbody>
</table>

\(^1\)The cooling rate was -1°C/min and the thawing temperature 40°C. Results are mean of 3 observations of each of the three replicates. Standard deviations given in brackets.

\(^2\)Regeneration of viable culture after three weeks.

+/-/+ indicates post-thaw viability in three replicates

+/+/+ indicates post-thaw viability in two replicates

+/-/- indicates post-thaw viability in one replicate

/-/- indicates no post-thaw viability observed

The results obtained from the experiment testing the effect of cryoprotectant removal on post-thaw viability revealed a slightly higher initial post-thaw viability levels when the cryoprotectant was diluted, but not removed after thawing (Table 5.4). However, the differences in post-thaw viability were not significant, and in fact regeneration of normal cultures was only observed when the cryoprotectant was removed (Table 5.4).
Table 5.4. Effect of cryoprotectant removal on the post-thaw viability of *T. pseudonana* CCMP1335.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cryoprotectant dilution post-thaw</th>
<th>Regeneration of viable culture</th>
<th>Cryoprotectant removal post-thaw</th>
<th>Regeneration of viable culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCMP1335</td>
<td>59 (14)</td>
<td>+/-/-</td>
<td>52 (7)</td>
<td>+/-/-</td>
</tr>
</tbody>
</table>

1Cooling rate was -1°C/min; the cryoprotectant used was 5% DMSO (final concentration) and the thawing temperature 40°C. Results are mean for 3 observations of each of the three replicates. Standard deviations given in brackets.

+/+/+ indicates post-thaw viability in three replicates
+/+-/- indicates post-thaw viability in two replicates
+-/-/- indicates post-thaw viability in one replicate
-/-/- indicates no post-thaw viability observed

5.3.4 Standard CCMP cryopreservation protocol to study the effect of different cryoprotectants and a different cooling protocol

The CCMP standard cryopreservation protocol detailed in Section 5.2.3.3 was tested in the strains, and post-thaw viability determined (Table 5.5).

On the basis of vital staining, samples cryopreserved in the presence of 5% DMSO were initially assumed to have higher viabilities than the other cryoprotectants employed for both strains (Fig. 5.5). However, after three weeks no survival was observed (Table 5.5). Furthermore, no recovery was observed for *T. pseudonana* CCMP1335 treated with any of the cryoprotectants employed using this cooling protocol (Table 5.5). High levels of regeneration were observed in *T. pseudonana* CS-20 with the combined treatment of 5% DMSO and 5% Sorbitol (Table 5.5).
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Table 5.5. Effect of cryoprotectant choice and CCMP cryopreservation protocol on capacity of *T. pseudonan*a CCMP1335 and CS-20 to regenerate cultures

<table>
<thead>
<tr>
<th>Strain</th>
<th>5% DMSO²</th>
<th>5% DMSO+5% Sorbitol²</th>
<th>5% Sorbitol²</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCMP1335</td>
<td>+/-/-</td>
<td>+/-/-</td>
<td>+/-/-</td>
</tr>
<tr>
<td>CS-20</td>
<td>+/-/-</td>
<td>++/++</td>
<td>+/-/-</td>
</tr>
</tbody>
</table>

¹Cooling rate as described in Section 5.2.3.3, Fig. 5.2.
Thawing temperature 40°C.
²Regeneration of viable culture after three weeks.
Results are mean for 3 observations of each of the three replicates. Standard deviations given in brackets.
+/-/+ indicates post-thaw viability in three replicates
+/-/+ indicates post-thaw viability in two replicates
+/-/+ indicates post-thaw viability in one replicate
-/-/- indicates no post-thaw viability observed

Fig. 5.5. Effect of choice of cryoprotectant on viability 24 h. post-thaw of *T. pseudonana* CCMP1335.
5.4 Discussion

The initial aim of this chapter was to develop an optimal cryopreservation protocol for the model organism *T. pseudonana* that would then allow the genetic and phenotypic stability to be investigated pre- and post-cryopreservation. Cryopreservation has been proposed as a standard technique to assure the deliverables of service culture collections, specially the maintenance of phenotypic and genotypic stability of the organisms held (Müller et al. 2007).

Light microscopy observations did not reveal anomalies, i.e. the cells and chloroplast shape appeared normal in cultures regenerated after cryopreservation. The morphology of *T. pseudonana* was studied after cryopreservation using electron microscopy (Chapter 2, Sections 2.3.2.7 and 2.4.2). SEM micrographs revealed no structural anomalies 48 h. after thawing (Figs 2.9 a-e). However, is unlikely that the thawed cells had divided in 48 h.; therefore the observations post-thawed could have been on the original material and not on newly divided cells. Unfortunately, since the SEM observations were not performed "in house", but at the natural History Museum (NHM), at the time of the visit there were no other thawed samples available. If the conservation of the morphology was confirmed after division, it would be of particular importance for diatoms, due to their size reduction over generations and consequent loss of original phenotype (Chapter 1, Fig. 1.1). Unfortunately, the study of genotypic stability after cryopreservation was not possible within the time limitations of this project due to the challenges encountered in the design of an optimum protocol for the cryopreservation and recovery of this organism. Therefore, the aim of this chapter changed, to become a “quest” in search of the optimal protocol to cryopreserve the *T. pseudonana* strains studied.

The results obtained in this study confirmed the challenges faced in the successful cryopreservation of new organisms, and in particular diatoms. A large variety of factors
have been reported as the cause of cryoinjury (Day, 2004) and those investigated in the present study include: cryoprotectant choice and concentration, cooling rates and thawing temperatures.

Although most of the protocols trialled in this chapter were initially apparently successful (Tables 5.2-5.5), recovery of cultures was only possible for four organisms, after three to four weeks incubation, indicating that an optimum cryopreservation protocol has yet to be developed for this diatom species.

In following sections, the individual steps of cryopreservation protocols are discussed in the context of the apparent cryorecalcitrance of some of the *T. pseudonana* strains studied.

### 5.4.1 Cryoprotectant toxicity

In this study, the tolerance to DMSO concentrations was investigated by incubation of the cultures in the presence of three DMSO concentrations. After 24 h, the cultures seem to be stable, not affected by the cryoprotectant (Table 5.1). However, after prolonged exposure, the strains treated with the highest concentration (15% DMSO) seem to have died, and regeneration was only observed at the end of the third week (Table 5.1), suggesting that an extremely small number of cells had actually survived.

Cryoprotectant toxicity has been suggested by Pegg (1989) as one of the main factors responsible for cryoinjury and cell death. Fleck (1998) observed that the diatom *Cyclotella pseudostelligera* died after the treatment with certain cryoprotectants including DMSO, and when the incubation time in presence of the cryoprotectant was too long. In this study, differences in regeneration dependent on the cryoprotectant concentration were observed, with some of the strains dying after treatment with 15% DMSO, but slow regeneration was observed overtime and by week three normal cultures had grown. Although all the strains had regenerated after three weeks under all the three concentrations tested, it was decided
that 5% DMSO had the lowest toxicity to the cells, and therefore that concentration was employed in most subsequent studies.

5.4.2 Cooling protocols

Optimum cooling protocols are required for the successful cryopreservation of microalgae due to the conflict between rapid cooling resulting in intracellular ice formation and slow cooling related to cell damage due to osmotic forces (Mazur et al. 1972, Karlsson & Toner, 1996). The standard CCAP algal cryopreservation protocol (Section 5.2.3.1, Fig. 5.1) was tested on all nine *T. pseudonana* strains with 5% DMSO as cryoprotectant and high post-thaw viability levels observed for all the strains tested (Table 5.2). However, although initially successful, most of the cells appeared dead after a few days, and regeneration of only four of the strains: *T. pseudonana* CCMP1007, CCMP1012, CCMP1335 and CS-20 was observed (Tables 5.2-5.4). This cryopreservation protocol has been successfully used in CCAP in a wide range of organisms (over 1200 strains successfully cryopreserved, CCAP unpublished data). It has been previously reported that cryoprotectant choice and concentration can have significant effect on the success/failure of microalgae cryopreservation (McLellan, 1989, Fleck, 1998). To investigate this, the same protocol was applied to one strain, CCMP1335, using 5%, 10% and 15% DMSO (final concentrations). Post-thaw viability after 24 h. was higher with 10% DMSO at 70%, versus 62% with 5% DMSO and 68% with 15% DMSO (Table 5.3). Furthermore regeneration of the culture was only achieved using 10% DMSO concentration (Table 5.3). This could indicate that 10% DMSO is the most suitable cryoprotectant concentration, at least for this particular strain, in accordance with Morris (1981), who reported that a particular cooling rate may be linked to a particular cryoprotectant concentration for a specific algal strain.

An additional cooling protocol was employed for two of the strains studied: *T. pseudonana* CCMP1335 and CS-20. This protocol (detailed in Section 5.2.3.3, Day & Brand, 2005) has
been successfully employed for marine strains in CCMP and its success in combination of
different cryoprotectants, was investigated. Furthermore, Hubálek (2003) reported that
some cryoprotectants worked better in combination e.g. glucose and sorbitol, and sugars
have previously been demonstrated to be successful cryoprotectants by reducing cell
membrane permeability to solutes, thus reducing injury due to solutes movement
(Santarius, 1996). In this study, 5% DMSO, 5% Sorbitol and the combination of 5%DMSO
and 5% Sorbitol were used as cryoprotectants (Table 5.5, Fig. 5.5). However, as in other
experiments, post-thaw viability 24 h. after thawing did not correspond with subsequent
recovery of the cultures (Table 5.5). The highest viability values observed after 24 h. were
for strain CCMP1335 treated with 5% DMSO, although subsequent regeneration of this
strain completely failed (Table 5.5). However, good recovery was observed for strain CS-
20 employing the combine treatment of 5% DMSO and 5% Sorbitol, despite of the initial
low post-thaw viability levels (Table 5.5).

There was no correlation observed in this study between vital staining and cell division/capacity to regenerate normal cultures. Vital staining has previously been demonstrated to correlate well with capacity to re-grow (Day et al. 2007). However, other studies have reported higher viability levels using vital staining versus capacity to form colonies/cell division (Fenwick & Day, 1992, Day & Fenwick, 1993, Fleck, 1998). It has previously been reported that some non-viable cells, retain an undamaged membrane and still remain enzymatically active, i.e. can stain FDA positive up to 24 h. post-thaw (Fleck, 1998). The high initial viability levels (in terms of vital staining) obtained in this study could be due to “false” positive staining. Alternatively, it could also mean that some of the strains, although initially viable, were unable to recover due to sub-lethal injuries that were subsequently not reparable.
5.4.3 Thawing protocols

There is relatively little published on the effects of thawing rate on post-thaw viability. Rapid warming has been adopted to improve viability levels (Morris, 1976, Cañavate & Lubián, 1997b) as it avoids possible cell damage, which may occur at slow thawing rates due to ice formation (Day & Deville, 1995). As a result, thawing in a preheated water bath at ~40°C has been adopted as standard (Day and Brand, 2005) as it has proven very successful for a wide range of microalgae at the CCAP and other algal collections.

In this study, thawing temperatures of 20°C, 30°C and 40°C were investigated, since it has been observed at CCMP that post-thaw viability levels of certain microalgae species apparently improved at lower thawing temperatures (Julie Sexton personal communication). In this study, higher post-thaw viability levels were obtained at 40°C (Table 5.1), which can be explained by the prevention of intracellular ice growth at higher warming rates (Morris, 1981). However, despite the initial high viability levels, on the basis of vital staining, obtained for all the strains (Table 5.2), the same *T. pseudonana* strains: CCMP1007, CCMP1012 and CS-20 recovered and regenerated cultures after two to three weeks using all the three thawing rates (Table 5.2). This indicates that thawing temperature may not represent a significant impact in viability levels for this diatom species.

It has also previously been reported that cryoprotectant removal after thawing may improve viability levels in some microalgae species (Day & Brand, 2005). The main method used for cryoprotectant removal is the dilution of the sample with fresh, sterile medium (Day & Brand, 2005). Although total removal of the cryoprotectant could be achieved by centrifuging, this method could cause stress in the already fragile post-treatment cells (Fleck, 1998). Removal of cryoprotectant by dilution and by gentle centrifugation was investigated in this study to assess if differences in viability levels could
be observed and slightly initial higher post-thaw viability levels were obtained with the dilution of the cryoprotectant after thawing (Table 5.4). However, after incubation for three weeks, recovery of culture was only observed for the culture in which the cryoprotectant had been removed. Further work is needed to substantiate this observation, but it suggests that even low levels of DMSO may be toxic to seriously damaged cells.

5.5 Conclusions

Although high post-thaw viability levels (on the basis of vital staining) were observed with some of the protocols 24 h. after treatment, successful regeneration of cultures was only observed for four of the strains: CCMP1007, CCMP1012, CCMP1335 and CS-20 with no consistency in the protocol used. In most cultures, cells died after ~48 h., and in those where recovery was observed this was observable three weeks later (Tables 5.2-5.5). Comparable recovery times have been reported for other cryopreservation studies on marine diatoms (McLellan, 1989, Rhodes et al. 2006) and have been accepted as successful. In this study the initial apparently high post-thaw viability obtained was always followed by cell death a few days later. This suggested that only a few cells survived after the treatment, that were able to divide and regenerate a normal culture over time.

The mechanisms of lethal cryoinjury in microalgae have previously been investigated using a number of techniques including cryomicroscopy (Morris et al. 1986, Fleck et al. 1997a), and in many cases the main cause of injury was reported to be associated with intracellular ice formation. Although in the present study cryomicroscopy was not employed, and thus, ice formation can not be confirmed, the slow cooling rates employed and intact morphology of the cells post-thaw suggested that this may not be the cause of cell injury in *T. pseudonana*. Morris et al. (1981) studied the mechanisms on freezing injury in *Chlamydomonas*, and reported that the cell injury observed was related to stress produced by shrinkage and rehydratation of cell membranes and that this injury induced
alterations in the membranes structure and function. Fleck (1998) observed that the cryoprotectant (Methanol) affected the photosynthetic capacity of *Vaucheria sessilis*, although after removal, the alga was able to recover. Damage to chloroplasts and their thylakoid membranes have been reported due to low temperatures in a number of plant systems (Benson, 1990, Heber et al. 1971, 1981) and although in this study the shape of the chloroplast was not affected by the process, ultrastructural studies were not performed. Furthermore, physiological damage due to metabolic uncoupling has also been reported as a common cause for cell injury (Fleck et al. 1999, 2000). In the present study the mechanisms of cryoinjury in *T. pseudonana* were not investigated and therefore, the causes can not be concluded. It can be presumed that some of the above injuries could have been responsible for the long incubation times needed for the recovery of cultures and the failure to recover for some of the strains. It could also be speculated that accumulative effects of some of these injuries, or the absence of recovery mechanisms in the cells could explain the results obtained.

Overall, the results obtained in this study suggest that the optimal cryopreservation protocol has yet to be developed for this particular species. Although post-thaw survival of some of the strains did occur, the time taken to regenerate new cultures clearly suggested that the number of live cells, able to divide post-treatment, was lower than the acceptable level for long-term maintenance in a culture collection. An ultrastructural investigation of the cells aided by cryomicroscopy and electron microscopy, may be able to unravel the mechanisms of cryoinjures suffered by *T. pseudonana* and may be able to reveal if these mechanisms are the same for all of the strains studied. Further investigations on cooling protocols, cryoprotectants, thawing protocols and cryoinjury are required to establish if an optimal protocol can be designed for the long-term cryopreservation of all strains of this model diatom.
Chapter 6  
General discussion

6  Chapter 6. General discussion

6.1  Polyphasic approach to algal characterization and taxonomy

Polyphasic approaches have been proven to be able to better distinguish and identify microalgal species (Pröschold et al. 2001, Kooistra et al. 2008, Sarno et al. 2005), so contributing to the increase of known microalgal biodiversity. Studies on diatoms include the work of Sarno et al. (2005) who investigated the taxonomy of *Skeletonema* using both morphological observations and DNA sequence data, revealing four new species. Beszteri et al. (2007) discovered, aided by molecular data, that *Cyclotella meneghiniana* is a complex of reproductively isolated species. Due to diatoms size reduction and loss of morphological characters over generations, polyphasic approaches have proven very successful in revealing greater diversity than originally thought (Mann et al. 2003, Amato et al. 2007).

In the present study, a polyphasic approach combining phenotypic (morphology and chemotaxonomic markers) and genotypic (DNA barcodes and AFLP) techniques was used to characterize strains of the model diatom *T. pseudonana*. Overall, the results obtained confirmed that a polyphasic approach was needed to distinguish *T. pseudonana* strains.

The aims/hypotheses of this study and their outcomes are summarized in following sections.

6.1.1  “There is morphological plasticity within the taxon *T. pseudonana*”

Six *T. pseudonana* strains were characterized using the classical taxonomic approach, morphology (Chapter 2). While light microscopy is a useful tool for morphologic identification, in the present study, due to the small size of this diatom and the weakly silicified frustules, finer detail was needed and scanning electron microscopy was used. SEM observations confirmed, in general, those reported by Hasle and Heimdal (1970). The
strains exhibited a very similar morphology: specimens were in general weakly silicified, 2.1-7µm in diameter; specimens mainly had irregular radial ribs, crossed by tangential ribs forming polygonal or elongated areolae on the external valve surface. Number of marginal SPs 6-19 with 2-4 satellite pores. Number of valve face SPs 0-6 with 1-4 satellite pores. Number of marginal SPs seemed to be correlated with diameter of the cell. Presence of one circular to oval LP situated between 2 marginal SPs, but generally closer to one. Variations in diameter and number of fultoportulae were observed, even among specimens of the same strain (Chapter 2). However, observations of the Australian strain (CS-20) revealed some variation in the level of frustule silicification, with the presence of a very weakly silicified frustule in all the specimens studied. In some specimens the valve centre was occupied by irregular areolae, and the valve mantle by a ring of areolae (Figs 2.8 a & b), but no specimen had areolae over the whole valve surface. In addition, it was observed that the rimoportulae was usually located slightly outside the marginal ring of SPs, towards the centre of the frustule (Chapter 2, Fig. 2.8).

These observations exposed a certain level of phenotypic plasticity in *T. pseudonana* CS-20, perhaps related to its original isolation location (Swan River estuary, Western Australia) and the seasonal changes in environmental factors. The morphological characterization of these strains will be useful for culture collections worldwide to better characterize their holdings. Further observations on a higher number of specimens of this particular strain will be able to certify if the morphological variations found in the present study constitute a constant pattern for *T. pseudonana* CS-20, confirming in that case the phenotypic plasticity of this strain. Furthermore, due to time limitations the strain *T. pseudonana* CCMP1012 was not morphologically characterized; however, this strain was isolated from the same location as CS-20 (Table 1.1) and it would be of great interest to morphologically characterize it and compare it with CS-20. This could assist in resolving
whether the variations observed are in fact related to their original isolation location and the environmental factors present.

The results obtained suggested some morphologic plasticity for the strain CS-20, but not for the other strains studied. Therefore, the initial hypothesis was not proved, and further analyses are needed to distinguish between \textit{T. pseudonana} strains, proving the need for a polyphasic approach when characterizing microalgae at the intra-species level.

### 6.1.2 “Chemotaxonomic markers have the potential to differentiate between strains of \textit{T. pseudonana}”

To test this hypothesis, two chemotaxonomic markers (chlorophylls and fatty acid profiles) were investigated. The results obtained were inconclusive; all the strains shared common pigments and fatty acids profiles, although some variation was obtained for qualitative production of fatty acids (Chapter 3, Figs 3.3 A-F) confirming the results reported from Volkman and Hallegraeff (1988). They studied strains CCMP1335 and CS-20 and also reported variation in the production of some fatty acids. Furthermore, Shaw et al. (1989) reported that fatty acids of the diatom \textit{Skeletonema costatum} were different among clones isolated from oceanic, coastal or estuarine environments although this was not the case in this study.

Further investigation is needed to ascertain if the variation observed in this study is related to phenotypic plasticity due to natural variability within \textit{T. pseudonana}, or related to other factors such as the selective nature of different culture regimes employed in the different culture collections. If the variation in FA production was proven to be due to natural variability within \textit{T. pseudonana}, it would add very interesting information to this study, not only proving the need for accurate taxonomic identification at an intra-species level of
the species chosen for any particular study, but also confirming that fatty acid profiles could be a useful tool from a chemotaxonomic perspective.

6.1.3 “Barcode genes can be employed for intra-species characterization of \textit{T. pseudonana}”

Phylogeny inferred from the sequences of two nuclear genes (ITS and SSU) and a plastid gene (\textit{rbcL}) previously suggested as molecular markers for diatom DNA barcoding (Sarno et al. 2005, Evans et al. 2007, Moniz and Kaczmarska, 2009, 2010), demonstrated that all the strains shared identical sequences (Chapter 4, Figs 4.2-4.3). The results obtained in the present study revealed that although previously successful in identifying cryptic species in other organisms and for comparison among closely related species (Alverson et al. 2007, Evans et al. 2007, Moniz & Kaczmarska 2009, 2010) the molecular markers used in the present study were not able to resolve genetic differences among \textit{T. pseudonana} strains, suggesting that they may reach their resolution limit at a species level and therefore disproving the proposed hypothesis. These findings may also indicate a low population variability of this diatom species. Another possibility is that the methods used for sampling and isolation of these strains are very standardized, which could mean that the same populations may have been selected every time, favoured by the established sampling and isolation techniques.

6.1.4 “Whole genome approaches are needed to differentiate between strains of \textit{T. pseudonana}”

Fingerprinting analyses using AFLP revealed three clusters, which seemed to correspond with the strains original geographic distribution: North eastern Pacific Ocean, North western Atlantic Ocean and the third group comprised the North eastern Atlantic and the South eastern Indian Ocean (Chapter 4, Figs 4.9-4.10). The third group poses the question
of whether this wide distribution occurred naturally, or due to human dispersal as discussed in Chapter 4, Section 4.4.2. The similar distribution patterns previously reported for *Sellaphora capitata* (Evans et al. 2009) and for pseudocryptic species of *Navicula cryptocephala* (Pouličková et al. 2010) were explained with the possibility that these species could have been introduced to Australia in the 1880s, when lakes were stocked with European fish (Crowl et al. 1992, Evans et al. 2009, Pouličková et al. 2010) and since the Australian strain CS-20 was isolated from an estuary sometime before 1972, this possibility also exists. At the same time, the transportation of non-indigenous phytoplankton species by ships in ballast-water discussed in Chapter 4, Section 4.4.2 and first suggested by Ostenfeld (1908) could also explain the results obtained.

On the whole, the genetic distances obtained between clusters were small, but this whole genome approach revealed a degree of variation between the strains that could not be uncovered using solely morphology, nor proposed barcoding genes and confirmed the hypothesis that a whole genome approach is needed to distinguish between *T. pseudonana* strains.

6.1.5 “Cryopreservation ensures phenotypic and genotypic stability of *T. pseudonana*”

This hypothesis was not proven due to the problems encountered designing an optimal cryopreservation protocol. Four strains were able to regenerate normal cultures after the treatment, but the long regeneration times needed suggested that the number of viable cells capable to divide post-treatment was lower than the acceptable level for long-term maintenance of *T. pseudonana* in a culture collection. As suggested in Chapter 5, Section 5.5, further investigation is needed to reveal the mechanisms of cryoinjuries suffered by this model organism and to establish if an optimal protocol can be designed for the long-term cryopreservation of all strains of this model diatom.
6.1.6 Future research

In the final section, future research building on the findings of the present study is discussed.

* One of the most important findings of the present study was based on the AFLP analyses, which indicated that *T. pseudonana* populations are genetically distinct (Chapter 4). Furthermore, although the level of genetic variation observed was low, it appeared to correlate with the population’s biogeographic origin. Clearly, all the strains investigated in this study were cultured strains, and they may be affected by selective pressures as a result of culturing in some cases for over four decades. Genotypic changes in cultures maintained *ex situ* such as mutations and genetic drift have previously been reported (Paasche, 2001, Lakeman et al. 2009). It would be of great interest to test this for *T. pseudonana* by obtaining new isolates and performing the same fingerprinting analyses. A time course could be followed over a period of years and alternative culture regimes (e.g. optimal and stressful) employed to ascertain if genetic drift is induced in culture.

* The low variability amongst populations may also be explained by standard sampling and isolation methods being employed. These could have a selective impact favouring the isolation of certain sub-populations. An alternative study employing a range of sampling and isolation techniques to isolate *T. pseudonana* could help explore this. Once isolated and established in standard cultures, further genetic analyses could be performed and the results compared with those of the present study. These experiments could reveal if more distinct genetic populations of *T. pseudonana* can be isolated using different sampling and isolation techniques.

* The chemotaxonomic pilot study (Chapter 3) suggested that this is worthy of further investigation. A more refined experiment analysing FA profiles, could confirm the
preliminary data obtained and the possible use of more powerful analytical tools could validate the approach. Plans have been developed and funding obtained to explore the potential of using a different approach MALDI-TOF ICMS (see Chapter 3, Section 3.4.2) to investigate if specific protein fingerprints can be obtained for the *T. pseudonana* strains.

* The initial aim of the cryopreservation chapter (Chapter 5) was to develop an optimal cryopreservation protocol for the model organism *T. pseudonana* that would allow the genetic and phenotypic stability to be investigated pre- and post-cryopreservation. This was not achieved due to the difficulties encountered in the design of an optimal cryopreservation protocol. Cryopreservation of diatoms is very important to preserve their phenotypic and genotypic stability, especially due to their size reduction and consequent loss of morphological characters (Chapter 1, Fig. 1.1). It is therefore of great importance to develop an optimal protocol that will allow not only the cryopreservation of the strains of this model organism, but also the study of their phenotypic and genotypic stability after cryopreservation.

Finally, the polyphasic approach employed in the present study opens new avenues to further investigate and characterize the strains of this model diatom. The use of this type of polyphasic approach would be useful in the study of phytoplankton diversity and populations, facilitating a better understanding of phytoplankton dispersal and biogeography and on the whole, a better understanding of the microbial world.
References

List of references


Belcher, J.H., Swale, E.M.F., 1986, Notes on some small *Thalassiosira* species (Bacillariophyceae) from the plankton of the lower Thames and other British estuaries identified by transmission electron microscopy. British Phycological Journal 21(2), 139-146.

 References


Carreto, J.I., Carignan, M.O., Montoya, N.G., 2001, Comparative studies on mycosporine-like amino acids, paralytic shellfish toxins and pigment profiles of the toxic dinoflagellates *Alexandrium tamarense*, *A. catenella* and *A. minutum*. Marine Ecology Progress Series 223, 49–60.


References


Garibotti, I., Vernet, M., Kozlowski, W., Ferrario, M., 2003a, Composition and biomass of phytoplankton assemblages in coastal Antarctic waters: a comparison of chemotaxonomic and microscopic analyses. Marine Ecology Progress Series 247, 27–42.


References


Hildebrand, M., 2003, Biological processing of nanostructured silica in diatoms. Progress in Organic Coatings 47, 256-266.


References


Kaczmarska, I., Beaton, M., Benoit, A.C., Medlin, L.K., 2006, Molecular phylogeny of selected members of the order Thalassiosirales (Bacillariophyta) and evolution of the fultoportula. Journal of Phycology 42, 121-138.


References


Lowe, R.L., Busch, D.E., 1975, Morphological observations on two species of the diatom genus Thalassiosira from fresh-water habitats in Ohio. Transactions of the American Microscopical Society 94(1), 118-123.


References


References


References


References


References


Cooling protocol profile originated by the computer controlled freezer after employing the CCAP cooling protocol.
Line on the left represents the protocol selected.
Line on the right represents the real progress in the controlled freezer.
Cooling protocol profile originated by the computer controlled freezer after employing the CCMP cooling protocol.

Line on the left represents the protocol selected.
Line on the right represents the real progress in the controlled freezer.
Appendix

Appendix 3

Pigments profiles for the *T. pseudonana* strains.

![Graphs of pigments profiles for CCMP1007, CCMP1011, and CCMP1012](image_url)
Appendix

CCMP1335

CS-20

CS-173
Appendix

![Graph of SAG-1020-1b](image-url)
Appendix

Appendix 4

AFLP profiles of six strains of *T. pseudonana* using a traditional agarose gel (1% w/v).

Appendix 5

AFLP profiles of *T. pseudonana* strains using a MetaSieve agarose gel.
L, ladder.
Left: AFLP profiles for the six strains from the 1st DNA extraction.
Right: AFLP profiles for the six strains from the 2nd DNA extraction.