Mating interactions of the harmful dinoflagellate alexandrium tamarense from UK coastal waters

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MATING INTERACTIONS OF THE HARMFUL DINOFLAGELLATE *ALEXANDRIUM TAMARENSE* FROM UK COASTAL WATERS

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ABSTRACT

The *Alexandrium tamarense* species complex is a group of economically and ecologically important marine dinoflagellates. The complex is comprised of three morphospecies *A. tamarense*, *A. fundyense* and *A. catenella* grouped according to ribosomal DNA or 'ribotype'. There are five ribotype type groups (I-V) each consisting entirely of toxic or non-toxic isolates. Toxic isolates are associated with harmful algal blooms (HAB's) due to their ability to produce powerful neurotoxins, which are responsible for outbreaks of paralytic shellfish poisoning in areas of shellfish production.

Sexual reproduction in *A. tamarense* has important implications for the initiation and termination of HAB's associated with these species. Resistant, long-lived hypnozygotes are formed during blooms through gamete fusion and deposited in sediments. These hypnozygotes provide the source of inoculum of motile vegetative *A. tamarense* cells in temperate zones during subsequent spring/summer blooms. This study provides further insight into the mating interactions between toxic Group I and non-toxic Group III isolates primarily from UK coastal waters.

Study of the effect of temperature on the mating interactions of *A. tamarense* Group I and Group III in culture showed that temperature had a significant effect on both groups. Co-cultures of compatible Group III isolates showed a significant decrease ($p<0.05$) in hypnozygote yield at 15°C, compared to 20°C. In contrast the mating compatibility of co-cultures of Group I isolates showed significant increase ($p<0.05$) at 15°C, compared to 20°C. Similar to other studies, compatible Group I and Group

III isolates formed non-viable hybrid hypnozygotes in co-culture. Comparison of the average vigour of inbred Group I crosses and outbred Group I/III crosses suggest that Group I isolates are more likely to out-breed with a compatible Group III isolate. A finding that may have significance in areas where the two groups co-occur.

Preliminary data suggesting the presence of both Group I and Group III ribotypes in some isolates has been generated from a nested single cell PCR/qPCR protocol using group specific primers. These data were compared to a dual probe whole cell fluorescent in situ hybridisation (whole cell FISH) assay of isolates. Whole cell FISH showed no dual expression of ribosomal RNA. This suggests that some *A. tamarense* Group I and Group III isolates may have rDNA pseudogenes corresponding to different ribotypes. If correct this could have implications for the overestimation of *A. tamarense* group diversity in natural populations when using rDNA sequences for identification.

DECLARATION

I declare that the work presented here was carried out in accordance with the Guidelines and Regulations of the University of Westminster.

This thesis is entirely my own work and that where any material could be construed as the work of others, it is fully cited and referenced, and/or with appropriate acknowledgement given.

Until the outcome of the current application to the University of Westminster, the work will not be submitted for any such qualification at another university or similar institution.

Signed: Klaire Neale **Date:** 01/06/2014

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AV	Average Vigour
bp	Base pair
CI	Compatibility index
dH_2O	Deionised H_2O
DNA	Deoxyribonucleic acid
g	Gram
HAB	Harmful algal bloom
ITS	Internal transcribed spacer
L	Litre
LSU rDNA	Large sub-unit ribosomal deoxyribonucleic acid
m	Metre
M	Mole
Mb	Megabase
mL	Millilitre
mm	Millimeter
mp	Mega pixel
ng	Nanogram
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
RC	Reproductive compatibility
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SSU rDNA	Small sub-unit deoxyribonucleic acid
µg	Microgram
µL	Microlitre
µm	Micrometer

CHAPTER 1

INTRODUCTION

1.1 Introduction

Blooms of the armoured, planktonic, marine dinoflagellate *Alexandrium tamarense* are ecologically and economically important due to the ability of some strains to produce powerful biotoxins associated with paralytic shellfish poisoning (PSP). Such blooms pose a considerable and sustained threat to human health and shellfish production in coastal regions globally. Due to the threat to public health and the potential economic loss associated with restricted shellfish harvesting, much research has focused on developing a greater understanding of this species' phylogeny and life cycle events, including sexual reproduction, with a view to developing more efficient monitoring technologies and formulating novel mitigation strategies.

1.2 Plankton

Dinoflagellates have traditionally been defined as members of the phytoplankton. This can be attributed to their early classification alongside algae (Taylor 1987) resulting in predominantly botanical research that largely ignored the approximate 50% of dinoflagellates that are believed to be heterotrophic. Furthermore, according to Stoecker (1999) mixotrophy is widely displayed across all extant dinoflagellate orders. Thus many taxa are more appropriately assigned to the micro-zooplankton (Hoppenrath et al. 2009). It is probable that the generalisation of dinoflagellates as *phytoplankters* will be revised, as more about their complex nutritional requirements is uncovered.

1.2.1 Phytoplankton

Phytoplankton is the collective term for the free-floating, microscopic plants of lakes, seas and oceans. Phytoplankton species are the primary producers in aquatic environments and are particularly important in determining the productivity of coastal seas. By the end of the 1980's there were estimated to be 3444-4375 species of marine phytoplankton worldwide (Sournia et al. 1991). Around 300 of these species are deemed to be 'harmful' and can have a negative impact on the human use of marine ecosystem, goods and services (Sournia 1995). However only 60-80 of these are deemed harmful due to the production of biotoxins, physical damage to fish, hypoxia, anoxia etc. (Smayda 1997). Of these 60-80 harmful algal bloom or 'HAB' species, dinoflagellates that produce biotoxins are of particular concern because of potential for losses of aquaculture stock and human health implications. In fact dinoflagellates account for 75% of all known HAB species (Smayda 1997).

1.3 Dinoflagellates

Dinoflagellates are a morphologically diverse group of protists. They represent 40% of all described phytoplankton species (Simon et al. 2009), encompassing 14 orders and >2000 known extant species (Guiry 2012). The majority of described species are marine (90%), although species do also occur in freshwater and brackish environments. Species have variable life stages, alternating between haploid vegetative growth, gametogenesis and diploid sexual reproductive stages (Elbrachter 2003; Hoppenrath et al. 2009).

1.3.1 General morphology

The defining morphological feature of dinoflagellates is the arrangement of their flagella (Taylor 1987). All dinoflagellates possess two dissimilar flagella; a transverse flagellum that circumnavigates the cell from left to right in a wave formation, and a longitudinal flagella. There are two dinoflagellate cell types, desmokont and dinokont, distinguished from where their flagella arise (see fig. 1.1). In desmokont cells the flagella both arise from the apical pore at the apex of the cell however this type of flagella formation is only seen in species of Prorocentroids such as *Prorocentrum spp*. (Steidinger and Tangen 1997). The predominant conformation is the dinokont formation in which the flagella arise ventrally with the transverse flagellum associated with a groove called the cingulum that divides the cell into two halves, the epitheca and the hypotheca, and the longitudinal flagellum arising from the sulcus, a lateral groove on the ventral side of the cell (Steidinger and Tangen 1997).

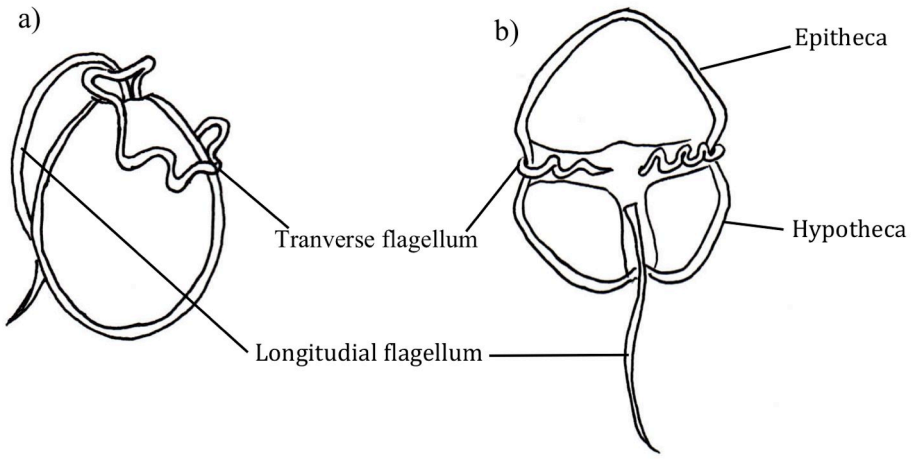


Fig 1.1 Distinction of desmokont (a) and dinokont (b) cell types (redrawn from Taylor 2005, with additional labelling added)

Another feature unique to the dinoflagellates is the enigmatic dinokaryon. This nuclear arrangement displays several strange characteristics that differ from the conventional nuclear arrangements of all other eukaryotes (Taylor 1987). The chromosomes of the dinokaryon are permanently condensed and lack basic histones (Steidinger and Tangen 1997; Taylor 1987). Furthermore, the genomes of dinoflagellates are often very large in comparison with other eukaryotes. Estimates range from 3000 – 215,000 Mb per haploid dinoflagellate genome compared to 3,180 Mb for humans (Hackett et al. 2005).

Other important cellular structures include amphiesmal vesicles located beneath the membrane that may, as in thecate taxa, or may not, as in athecate taxa, contain thecal plates that give an armour-like appearance to the cell surface and are routinely used in the morphological descriptions of thecate species (Steidinger and Tangen 1997).

Dinoflagellates also have conventional eukaryotic features such as chloroplasts and plastids, in photosynthetic taxa, mitochondria and membrane bound organelles e.g. Golgi apparatus (fig 1.2). For a thorough review of dinoflagellate morphology see Steidinger and Tangen (1997).

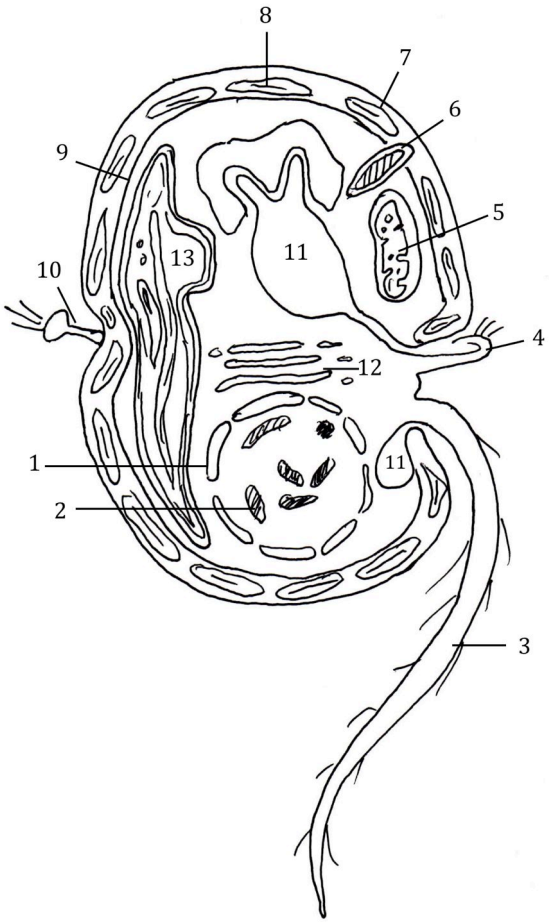


Fig. 1.2 Lateral view of a dinokont dinoflagellate cell. 1. Nucleus, 2. Condensed chromosomes, 3. Longitudinal flagellum, 4. Tranverse flagellum, 5. Mitochondrion, 6. Trichocyst, 7. Amphiesmal vesicle, 8. Thecal plate, 9. Pellicle layer, 10. Striated strand of transverse flagellum, 11. Pusules, 12. Golgi, 13. Plastid/chloroplast Redrawn from Taylor (1987).

1.3.2 Life cycles

The life cycles of dinoflagellates are poorly understood. It is estimated that complete life cycles are only known for 1% of the >2000 extant species (Elbrachter 2003). There are some general features of life cycle fitting most, but probably not all, species. All dinoflagellate vegetative cells are believed to be haploid and divide, with rare exception, by binary fission (Elbrachter 2003; Hoppenrath et al. 2009). Sexual reproduction is initiated by the formation of gametes. Gametes may be isogamous (equal size) or anisogmaous (unequal size) and may be heterogamous (morphologically similar to vegetative cells) or hologamous (morphologically different from vegetative cells) (Elbrachter 2003). Species may be heterothallic (unable to reproduce clonally) or homothallic (able to reproduce clonally). Different strains of the same species may be heterothallic or homothallic (Elbrachter 2003).

The fusion of gametes forms a motile diploid planozygote characterised by two longitudinal flagella (Hoppenrath et al. 2009). The fate of the planozygote is dependent on species and may include immediate meiosis and a return to haplont vegetative cells, or the formation of a non-flagellated resting cyst or hypnozygote followed by meiosis and a return to haplont vegetative growth following a period of dormancy (Elbrachter 2003; Hoppenrath et al. 2009).

1.3.3 Dinoflagellates and harmful algal blooms

Phytoplankton blooms are a natural phenomenon and are essential to the productivity of aquatic environments. Marine coastal blooms in temperate regions typically occur in spring and summer, when nutrients and light favour the proliferation of phytoplankton (Gowen et al. 2009). With dinoflagellates constituting a large

proportion of all phytoplankton species, second only to the diatoms, they have considerable significance as primary producers. However, as noted previously, some dinoflagellate species have been deemed harmful due to their potential for negative economic and ecological impact.

It is important to distinguish between high biomass and low biomass 'blooms' of dinoflagellate HAB species. While the term bloom seems to imply large numbers, or dominantion of the phytoplankton by a single species, this is rarely the case. In fact most biotoxin producing HAB species are considered background species, often constituting a minor component of the phytoplankton population (Anderson 1998).

High biomass blooms of non-toxic dinoflagellate species, previously referred to as 'red tides' due to discolouration of the water column, are usually not harmful. Although, they may interfere with human recreational use of the aquatic environment (Gowen et al. 2009). There are several species associated with noxious high biomass blooms including *Gonyaulax polygramma* (Pitcher et al. 2008), *Gymnodinium mikimotoi* (Kimura et al. 1999), *Noctiluca scintillans* and *Scrippsiella trochoidea* (Gárate-Lizárraga et al. 2009). Blooms of these species can become so dense that they result in fish and invertebrate mortality through hypoxia or anoxia (Hallegraeff 2003).

Low biomass blooms of biotoxin producing dinoflagellates, while less visually dramatic, pose a considerably larger threat to aquaculture and human health. There are several biotoxins produced by dinoflagellates that are associated with different types of shellfish and fish poisoning. These biotoxins are transmitted through the

food chain by their accumulation in filter feeding shellfish, such as mussels, oysters and clams and some fish (Haellegraeff 2003).

Biotoxins produced by dinoflagellates and associated with shellfish and fish poisoning in humans include; Okadaic acid the causative agent of diarrhetic shellfish poisoning (DSP) produced by several *Dinophysis* species and *Prorocentrum lima* (Quilliam 2003). Brevetoxins the causative agents of neurotoxic shellfish poisoning (NSP) produced by several *Karenia* species. Ciguatoxins the causative of ciguatera produced by *Gambierdiscus toxicus*. And PSP toxins the causative agent of paralytic shellfish poisoning (PSP) produced by several *Alexandrium* species, *Gymnodinium catenatum* and *Pyrodinium bahamese* (Hallegraeff 2003).

Of the dinoflagellate biotoxins, PSP toxins have the widest geographic range and are potentially the most severe. Indeed, the PSP toxin saxitoxin is listed as a schedule 1 chemical intoxicant by the Organisation for the Prohibition of Chemical Weapons (OPCW) (Chemical Weapons Convention, Sept 1998, The Hage, Netherlands). Furthermore, it's association with several *Alexandrium* species, including *A.tamarense*, makes it of particular interest to the subject of this thesis.

1.3.3.1 Paralytic Shellfish Poisoning

In parallel with other HAB's (Hallegraeff 1993) reported incidence of PSP has increased markedly over the last few decades (see fig. 1.3). There are several schools of thought used to explain these apparent increases including;

- Global increase of aquaculture and shellfish farming (Hallegraeff 2010).

- An increased awareness of toxic dinoflagellate species, alongside increased monitoring programmes (Hallegraeff 2003).
- The distribution of dinoflagellate cysts in ballast water (Bolch and de Salas 2007; Hallegraff 1998),
- Eutrophication (Heisler et al. 2008).

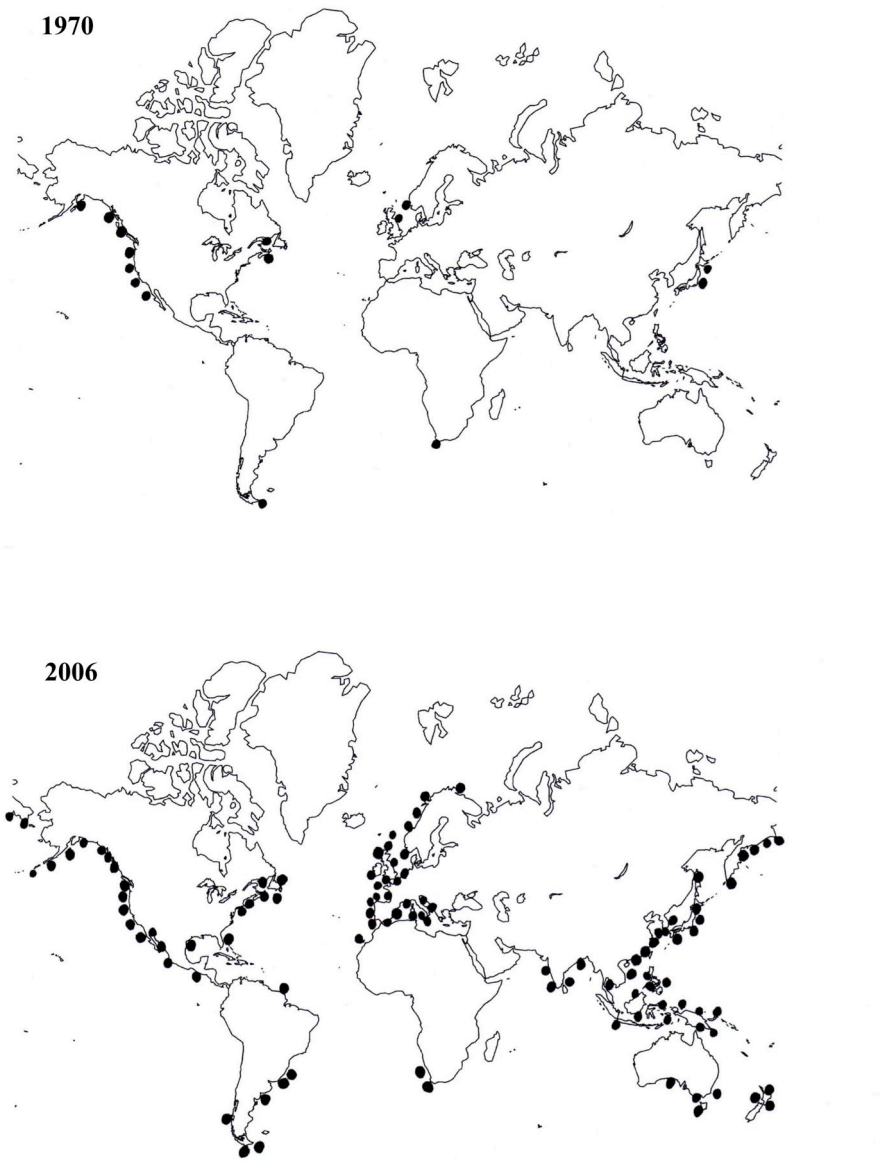


Fig 1.3 Increased global incidence of reported PSP toxicity between 1970 and 2006. Redrawn from http://www.whoi.edu/redtide/regions/world-distribution. Global distribution maps of other HAB toxins and fishkills are also available.

1.3.3.1.1 PSP toxins

PSP toxins are comprised of a suite of ~24 powerful neurotoxins that block sodium channels in nerves, resulting in suppression of the excitation of neuronal impulses (Henderson et al. 1973). Of the PSP toxins saxitoxin (STX), neosaxitoxin (neoSTX) and gonyautoxins (GTX) are the most toxic (Luckas et al. 2003). Ingestion of shellfish contaminated with these toxins can result in PSP. The symptoms of PSP range from burning/tingling sensation of the lips, tongue and face, to numbness that inhibits voluntary movement and, in severe cases, paralysis, respiratory arrest and death (Halstead and Schantz 1984). It is estimated that there are ~2000 reported cases of PSP globally each year with a mortality rate of 15 % (Kellmann et al. 2008).

1.3.3.1.2 Economic and ecological effects of PSP

The economic impact of PSP poses to coastal communities is considerable, particularly in relation to restricted shellfish harvesting. A single outbreak in New England, Massachusetts is estimated to have cost the local economy $15 million (Anderson 2005). The sustained threat of recurrent outbreaks of PSP and other types of shellfish poisoning in some coastal regions has led to the implementation of coastal monitoring programmes, particularly in areas associated with shellfish farming. For example the UK monitoring programmes that fulfil the requirements of EU Regulation (EC) No 854/2004) for monitoring the occurrence of harmful phytoplankton species in shellfish cultivation and harvesting areas (OJEU 2004)[1] (Gowen et al. 2009). This on-going threat has also led to a substantial amount of

[1] OJEU (2004) Annex II Live bivalve molluscs. Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption

research into the dynamics of HAB's and possible mitigation strategies (Anderson 1997; Anderson et al. 2010).

The ecological impact of saxitoxin and PSP are less certain and it might be assumed its effects are limited to human use of the aquatic environment (Gowen et al. 2009). However, there have been reported cases of wildlife fatalities associated with PSP and unnatural mortalities are often associated with HAB's (Ramsdell et al. 2005). The most notable example is the death of 14 Humpback whales (*Megaptera novaeangliae*) washed up in Cape Cod, Massachusetts, in 1987, after ingesting contaminated mackerel (Anderson 1994). Furthermore, there have been reports of PSP toxins being found in the faeces and zooplankton prey of North Atlantic right whales (*Eubalaena glacialis*) in the Bay of Fundy, Canada (Doucette et al. 2006). The causative species in both Cape Cod and the Bay of Fundy was *Alexandrium fundyense,* a member of the *A. tamarense* species complex (Crespo et al. 2011; Martin et al. 1999)

1.4 *Alexandrium*

The genus *Alexandrium* as defined by Balech (1995) is comprised of 31 species, 11 of which are known to produce saxitoxins (Anderson et al. 2012). Other biotoxins produced by *Alexandrium* species include spirolides, which are potent neurotoxins associated, so far, only with *Alexandrium ostenfeldii* (Anderson et al. 2012; EFSA 2010) and goniodomins, which have been associated with fishkills (Anderson 2012). *Alexandrium* is the only known genera to produce such a variety of biotoxins.

1.4.1 Taxonomy of Alexandrium

According to Anderson et al. (2012) *Alexandrium* species have been particularly affected by the classification and re-classification of species as a result of morphological and molecular analysis. Sometimes resulting in re-classification from different genera (i.e. *Gonyaulax tamarensis – A. tamarense*), or the establishment of a species complex where morphological differences did not uphold under molecular scrutiny (i.e. the *A. tamarense* and *A. minutum* species complexes).

Until fairly recently the taxonomy of dinoflagellates was predominantly the domain of the morphologist with most orders, genera and species being classified by purely morphological standards (Willcox 1998). However the advent of molecular analysis has opened up new avenues of investigation into dinoflagellate phylogeny focusing primarily on genomic variability between species in what has been termed the 'genetic species concept' (Costas et al. 1995).

Morphological taxonomic systems generally divide dinoflagellates into two groups thecate and athecate. General features or characteristics, such as size, shape and flagella configuration are utilised for the identification of both groups (Taylor 1987). However the separation of dinoflagellates at the level of order, genus or species is usually made using finer morphological details such as the tabulation of thecal plates and pore location.

Under normal light microscopy, intact cells of *Alexandrium* are fairly indistinct and, unlike some other more elaborate dinoflagellates, it is not possible to identify between *Alexandrium* species under these conditions (see fig 1.4). The morphological taxonomy of *Alexandrium* species is therefore dependant largely on the tabulation patterns produced by their thecal plates along with features such as their size, the composition and arrangement of pores and the ability to form chains.

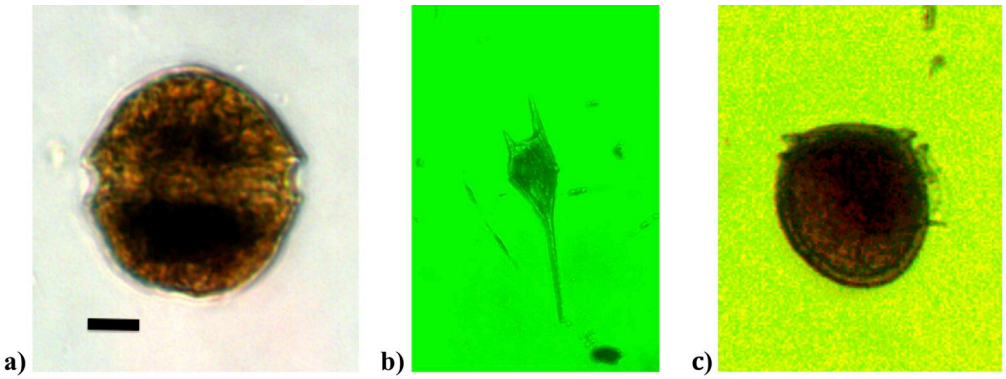


Fig. 1.4 Comparative light micrographs at x400 magnification of a) *Alexandrium spp.* b) *Ceratium furca* and c) *Dinophysis rotunda*. Scale bar =10 µm

In his 1995 monograph Balech used the Kofoid system to describe and define *Alexandrium* species. Developed by Charles Atwood Kofoid in the early 1900's (Taylor 1999), the Kofoid system relies on the attribution of each plate with a number and script notation (Taylor 1987, see table 1.1). The plates are numbered starting with the first plate of the apex on the ventral side being designated 1', subsequent plates are then numbered in an anticlockwise direction for example 2', 3', etc. Plate tabulation for all *Alexandrium* species is Po, 4', 6", 5''', 6C, 9-10S. Type species *A. minutum* (Halim) is shown in fig. 1.5.

Table 1.1 Designated terms of identification and notations used in the Kofoid system for thecate dinoflagellate groups such as Gonyaulacoids and Peridinioids (Produced from information in Taylor 1987).

Plate location	Systematic term	Notation
Epithecal plates		
Apex	*apicals*	'
Pole of apex	apical pore plate/complex	P_o or P_i
Anterior to cingulum	*precingulars*	''
Between *apicals* and *precingulars*	*anterior intercalaries*	^{a}
Lining girdle	*cingulars*	C
Hypothecal plates		
Posterior to cingulum	*postcingulars*	'''
Antapex	*antapicals*	''''
Between *antapicals* and *postcingulars*	*posterior intercalaries*	^{p}
Lining sulcus	*sulcals*	S^a, S^d, S^p, S^s etc.

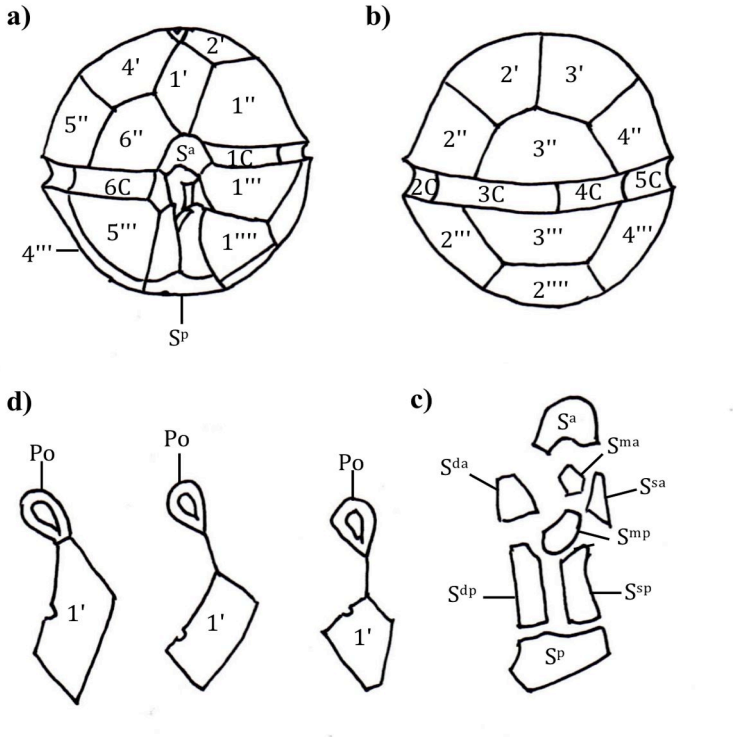


Fig. 1.5 Tabulation pattern and Kofoid annotation of *Alexandrium minutum* showing a) ventral view b) dorsal view c) sulcal plates of *A. minutum* (redrawn from Montresor et al. 2004) d) types of *Alexandrium spp*. apical pore complex (APC) (redrawn from Tomas 1997).

1.4.1.1 Morphological versus molecular taxonomy

Tabulation, although still routinely used for the identification of thecate dinoflagellates, does have significant drawbacks. Not least of which is the amount of subjectivity that may be imparted by the researcher. For example, distinction between species according to standards set for the identification of *Alexandrium* species can be made on the determination of minute details such as a difference in the width of a single thecal plate, as in distinguishing *A. tamatum* from *A. minutum* (Montresor et al. 2004) or the presence or absence of a ventral pore as in the

A.tamarense and *A.minutum* complexes (Lilly et al. 2005). Researchers have questioned the use of such fine scale details in distinguishing *Alexandrium* species and have incorporated molecular phylogenetic studies alongside conventional morphological analysis to assess the validity of the use of some traits in species identification.

One such study, carried out by Lilly et al. (2005) found that several traits, such as the presence of a ventral pore and anterior sulcal plate width, used to distinguish *Alexandrium lusitanicum* as a species distinct from the *A. minutum* group were questionable due to their variability and the unknown effects environmental influence may play in their appearance. Morphological analysis of both species did not show any significant differences. As a consequence it was concluded that the ventral pore was a particularly variable characteristic, being present in some strains and not others, and was therefore not suitable for use in the taxonomy of the *A. minutum* group. Molecular phylogenetic analysis of domains 1 and 2 (D1-D2) of the large subunit rDNA (LSU rDNA) confirmed the suspicions of the researchers showing that the species formerly identified as *A. lusitanicum* consistently fell within the clade of the *A. minutum* group leading to their conclusion that the two were actually part of a single species complex and not distinct species as formerly proposed.

The conclusions of Lilly et al. (2005) mirrored the findings of very similar earlier studies carried by Costas et al. (1995) and Scholin et al. (1994) which also found no grounds for the distinction of *A. lusitanicum* and *A. minutum* as separate species

based on both morphological analysis and sequencing of the D9-D10 and D1-D2 regions of the LSU rDNA respectively.

1.5 The *Alexandrium tamarense* species complex

The *Alexandrium tamarense* species complex is comprised of the morphospecies *Alexandrium tamarense, Alexandrium fundyense* and *Alexandrium catenella*. In addition to morphological classification current knowledge dictates that members of the complex can be divided into five genetically distinct ribotype groups.

A. tamarense is probably the most widely distributed and economically important of all *Alexandrium* species (Anderson et al. 2012). Toxic strains of the *A. tamarense* species complex produce saxitoxin and its derivatives. These toxins are responsible for outbreaks of paralytic shellfish poisoning (PSP) in coastal regions across the globe during bloom periods (Hallegraeff 1993).

1.5.1 A. tamarense complex morphospecies

The delineation between morphospecies of the *A. tamarense* species complex is based largely on the identification of several fine-scale morphological differences. According to Balech (1995) *A. tamarense* are distinguished from *A. fundyense* and *A. catenella* based on the presence of a ventral pore on the 1st apical plate (1') or shared between the 1st and 4th apical plates (1', 4'). Neither *A. fundyense* nor *A. catenella* have a ventral pore and are distinguished from each other largely by the ability of *A.*

catenella to form long chains. Although the apical pore plate of *A. catenella* is slightly more angular than that of *A. fundyense* and has an attachment pore, these differences are extremely difficult to observe with certainty under normal light microscopy (Personal observation). Fig. 1.6 provides an illustration of major morphological differences between *A. tamarense* complex morphospecies.

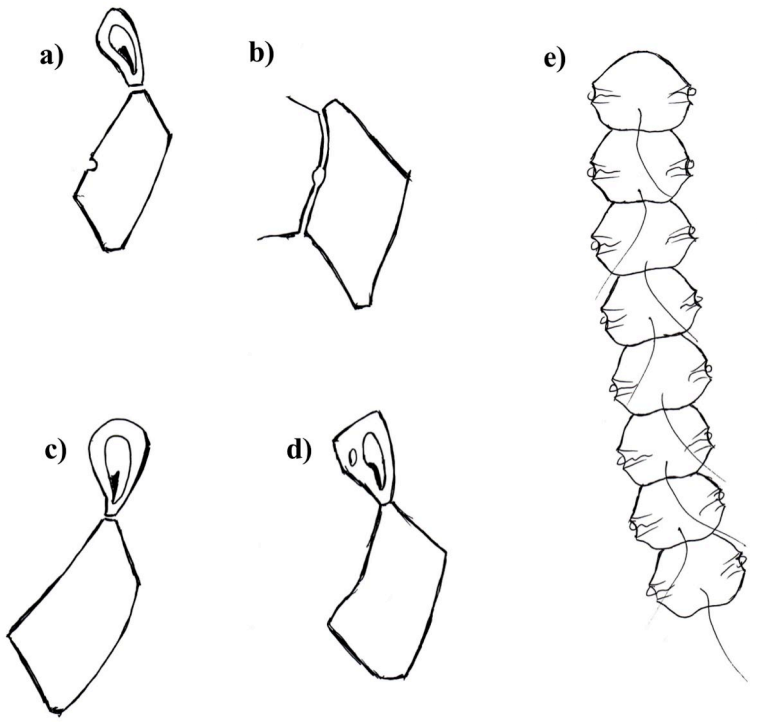


Fig 1.6 Critical morphological differences between morphospecies of the *A. tamarense* complex: a) Apical pore and 1' plate (with ventral pore) of *A. tamarense*. b) Ventral pore shared between 1' and 4' plates in *A.tamarense*. c) Apical pore and 1' plate (absence of ventral pore) of *A. fundyense*. d) Angular apical pore (with attachment pore) and 1' of *A. catenella*, virtually indistinguishable from *A. fundyense* (all adapted and redrawn from Balech, 1995), e) A chain of *A. catenella.*

1.5.2 A. tamarense ribotypes

Based on analysis of the D1-D2 LSU rDNA by Scholin et al. (1994), it was initially thought that *A. tamarense* complex groups were bound by geography and each ribotype was attributed a name according to the geographic region from which they originated e.g. North American, Western European, Temperate Asian. In 2007, Lilly et al. published a study of the D1-D2 LSU rDNA sequences from 110 strains of globally distributed *A.tamarense*, *A. fundyense* and *A. catenella.* Phylogenetic analysis of this data appeared to dispel the idea that ribotypes were geographically isolated, although it is arguable that some isolates have been dispersed, and it was proposed that a group numbering system (Groups I-V) replace the geographic nomenclature.

The phylogenetic tree presented by Lilly et al. (2007) did however uphold the separation between toxic and non-toxic members of the *A. tamarense* species complex, with each clade consisting entirely of toxic or non-toxic isolates. This distinction between toxic and non-toxic groups is of particular importance to *A. tamarense* as, unlike *A. fundyense* and *A. catenella* which are exclusively toxic, *A. tamarense* isolates may be toxic or non-toxic dependant on ribotype. It is also of note that *A. tamarense, A. fundyense* and *A. catenella* did not separate into distinct clades in either study suggesting that morphology is not a good indicator of evolutionary relatedness for these species (Scholin et al. 1995).

1.5.3 A. tamarense complex life cycle

The life cycles of several *Alexandrium* species have been well documented in the literature including *Alexandrium tamutum* (Figueroa et al. 2007), *A.minutum* (Garces 2004; Figueroa et al. 2007; Figueroa et al. 2011), *Alexandrium taylori* (Figueroa et al. 2006)*, Alexandrium peruvianum* (Figueroa et al. 2008) *and A. tamarense* (Anderson and Wall 1978; Anderson et al. 1984*;* Fritz et al. 1989*).* Of these, *A.tamarense* is probably the most extensively studied and yet there are still gaps within our understanding of how life cycle events such as the initiation of sexual reproduction in the natural environment and gamete recognition occur.

The current understanding of the life cycle of *A. tamarense* is that it follows a cyclical pattern with haploid vegetative cells being germinated from diploid hypnozygotes, formed through sexual reproduction during previous blooms that are then deposited in sediment. These hypnozygotes overwinter in the sediment for several months and excyst the following spring/summer when mandatory dormancy has passed (Anderson and Rengefors 2006; Genovesi et al. 2009; Wyatt and Jenkinson 1997) and external factors such as the availability of oxygen, temperature and light favour germination (Genovesi et al. 2009).

In brief, asexual reproduction of *A. tamarense* predominates during bloom periods as cells proliferate. Sexual reproduction occurs when haploid vegetative cells undergo gametogenesis, forming gametes. Compatible gametes fuse to form a diploid planozygote which remains motile for several days or weeks before falling to the

sediment, shedding its theca and emerging as a newly formed hypnozygote. Hypnozygotes undergo a period of maturation and mandatory dormancy, during which they are unable to excyst. Following the expiration of this dormancy period hypnozygotes may either excyst, if conditions are favourable, or remain quiescent under unfavourable conditions for many years. Excystment results in the emergence of a diploid planomeiocyte that undergoes meiosis to form four haploid daughter cells. The cycle of asexual division then begins again. In addition vegetative diploid cells can form short term, or pellicle, asexual cysts under adverse conditions. Fig 1.7 provides an illustration of the life cycle of *A.tamarense*.

At present, the mating interactions of *A. tamarense,* and other members of the species complex, and the mechanisms that determine gamete recognition and their mating compatibility are poorly understood. What is known is that sexual reproduction in *A. tamarense* is a selective process and successful mating interaction between gametes is dependent upon mating type compatibility with isolates being mostly heterothallic. Various models of mating compatibility have been proposed from simple male, female (+ & -) gamete formation (Anderson 1998), to systems involving four or more mating types (Brosnahan et al. 2010).

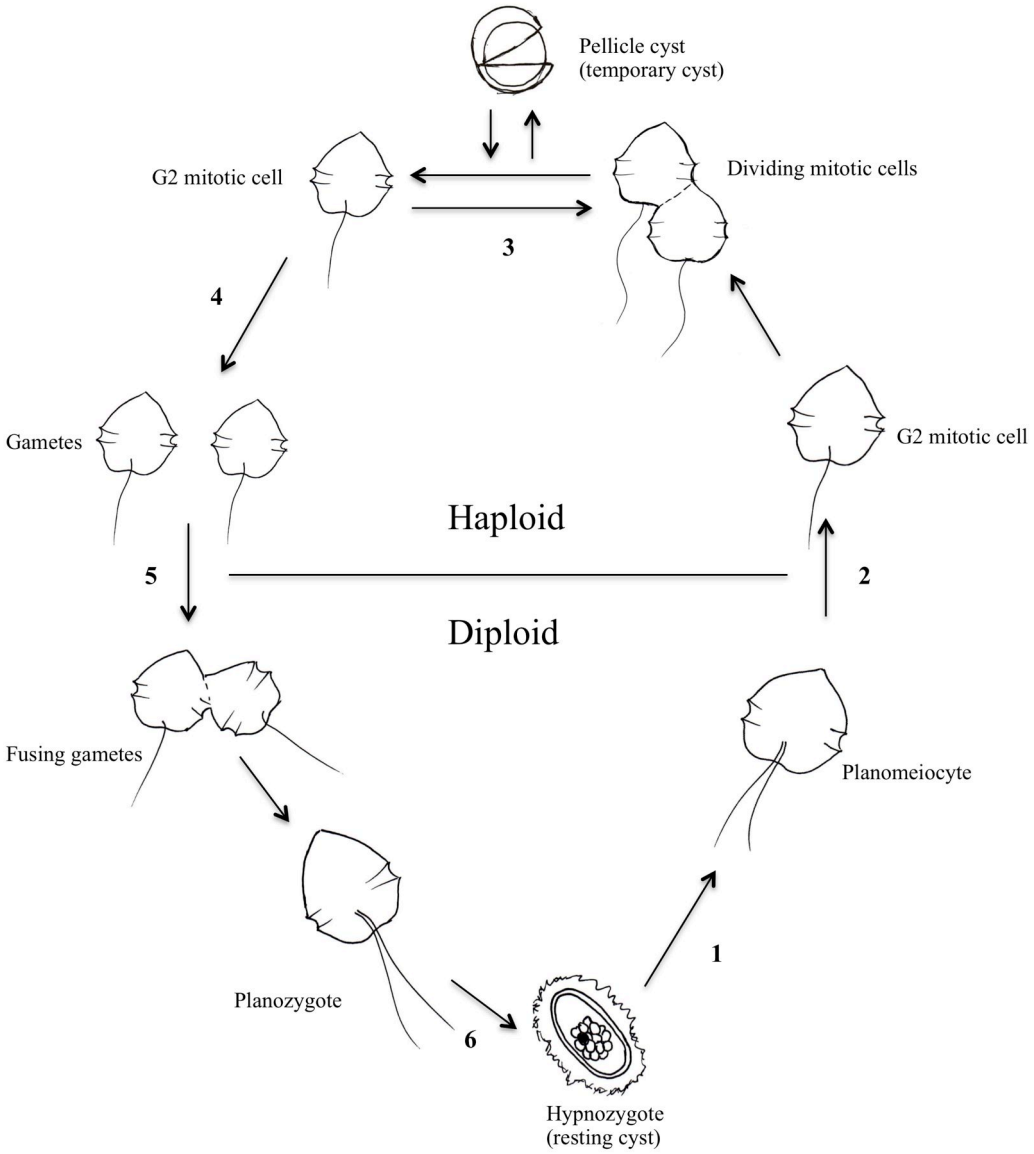


Fig 1.7 The *Alexandrium tamarense* life cycle showing: 1. Excystment, 2. Meiosis 3. Temporary encystment, 4. Gametogenesis, 5. Fusion, 6. Encystment (adapted and redrawn from Brosnahan et al. 2010).

1.5.3.1 Sexual reproduction between A.tamarense groups

Previous research investigating the mating interactions between toxic Group I isolates of *A. tamarense/fundyense* from the north east coast of the USA (Anderson et al. 1994) has shown that successful mating can occur between different

morphospecies within the complex, with viable progeny surviving in culture. Similar results were described by MacKenzie et al. (2004) in relation to sexual reproduction between toxic Group IV *A.tamarense/catenella*, isolated in New Zealand. However, these authors did report poor survival of progeny.

In contrast, mating interactions between toxic Group I and non-toxic Group III *A. catanella/fundyense/tamarense* (Brosnahan et al. 2010) showed that despite successful formation of hypnozygotes in the laboratory, there was complete lethality of hybrid progeny post excystment, following meiosis. It was hypothesised that hybrid mortality could be the result of an inability of the cells to return to a stable rDNA copy number following initial division. The data from this research has led to the question of whether the introduction of compatible non-toxic isolates could be used to mitigate blooms in coastal regions where there are recurrent outbreaks of PSP related to some members of the *A. tamarense* species complex (Brosnahan et al. 2010).

The logic behind the proposal of deliberate introduction of non-toxic isolates as a mitigation strategy appears to be the idea that a less abundant, or less dominant, group would more frequently interact with the dominant group than with it's conspecifics, resulting in non-viable hypnozygotes and eventually the lesser group being out-bred. While there have been calls from some to embrace and explore the idea of biological mitigation in marine environments more fully (Anderson 2004), such an idea still remains, at best, sketchy. Why would it be presumed that non-toxic isolates would be either more abundant or dominant as an invasive species? How do

you define compatible isolates at the level of a population when it is still unclear what drives mating compatibility in *A. tamarense* under laboratory conditions?

1.5.4 The A. tamarense complex in the UK

If there are answers to the questions posed above, they probably lie in coastal waters of the UK. Being the only known region globally to have both toxic Group I and non-toxic group III *A. tamarense* co-occurring, the UK is the model place to assess the mating interactions of the two groups. In fact, until the recent isolation of Group I and III *A. tamarense* from Belfast Lough, Northern Ireland (Brosnahan et al. 2010), the Orkney Islands (Collins et al. 2009), the Shetland Islands (Touzet et al. 2010) and the north east coast of Scotland (Toebe et al. 2013), the global geographical distribution of *A. tamarense* had indicated that there was no overlap between toxic and non-toxic groups. Previous to these discoveries it was perceived that groups were geographically isolated, that is, all *A. tamarense* isolated from a particular region belonged to the same group, with the exception of a geographic overlap of toxic Groups I and IV in eastern China (Lilly et al. 2007).

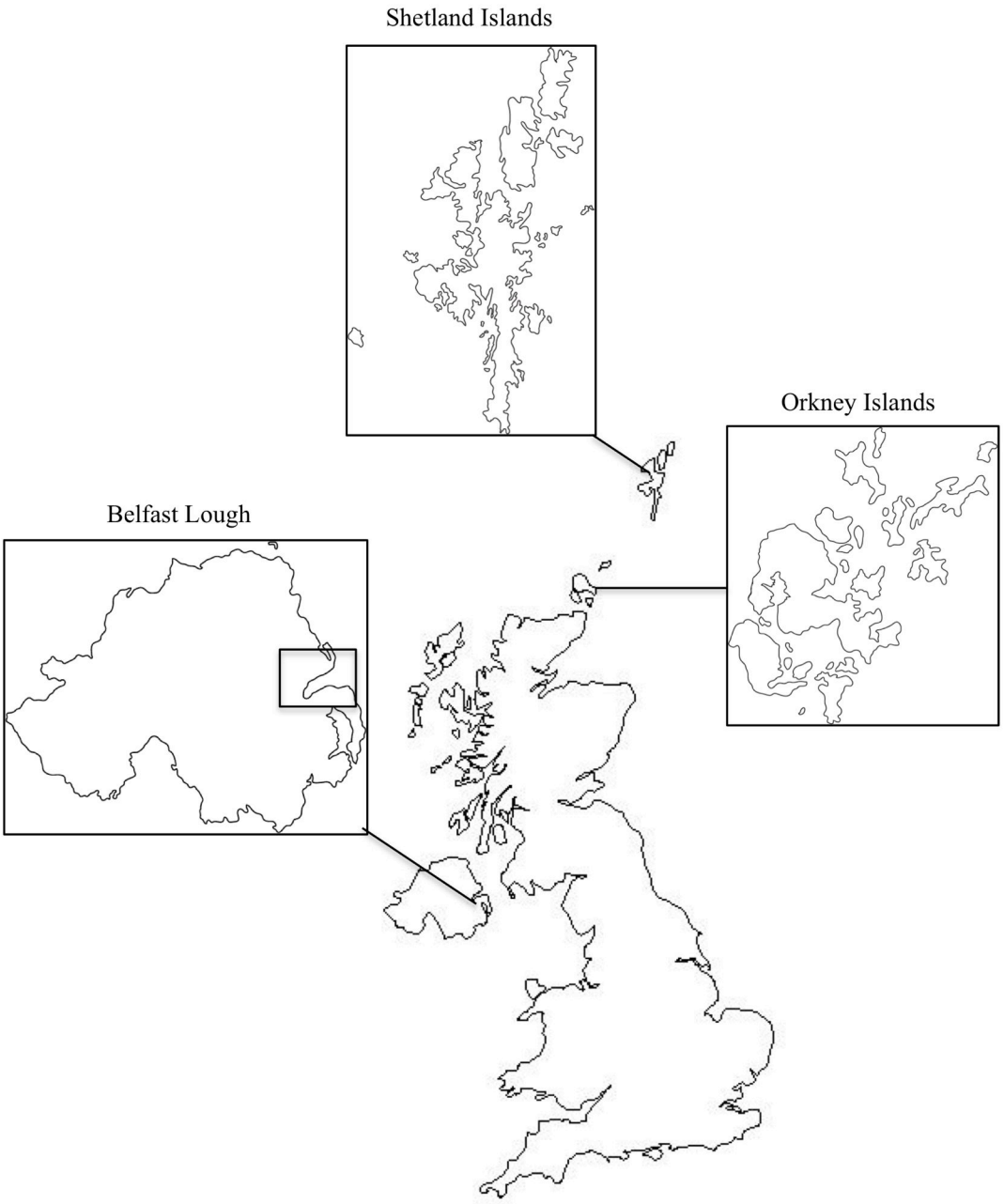


Fig 1.8 Geographic regions in the UK where *A. tamarense* Group I and III distribution overlap.

1.6 Aims of the project

The aim of the project was to assess the mating interactions of toxic Group I and non-toxic Group III *A. tamarense* isolated from UK coastal waters, with particular

emphasis on Group I and III isolates from Belfast Lough and the Orkney Islands. One of the main objectives was to assess whether the two groups co-occur in the water column. Unfortunately, it was not possible to address this during the project due initially to methodological issues, and then further to field sampling coinciding with a diatom bloom where very few *A. tamarense* cells were present (see chapter 2). However, the issue of co-occurrence in the water column was addressed by Touzet et al. (2010), with the discovery of *A. tamarense* Group I and III cells co-occurring in the water column in Clift Sound and Vaila Sound in the Shetland Islands. Given the constraints of the project, in terms of time, budget and limited field sampling (Chapter 2), the following questions are addressed;

1) Frequency of mating between compatible isolates: Is there a difference in the frequency of mating between compatible outbred (Group I and III) and inbred (same group) *A. tamarense* isolates?

2) The inheritance of mating type between *A. tamarense* parents and progeny: Can mating compatibility be predicted?

3) Is the system of mating compatibility in *A. tamarense* influenced by ribosomal genes?

CHAPTER 2

FIELD SAMPLES AND *A. TAMARENSE* CULTURE ISOLATION

2.1 Introduction

In order to address the questions posed in chapter 1 (section 1.6) the project demanded that a culture collection of Group I and III *A. tamarense* isolates from Belfast Lough and the Orkney Islands be established. The isolation of cultures was a prerequisite to the studies presented in chapters 3, 4 and 5.

Some of the sample materials necessary for the isolation of *A. tamarense* from these regions were already held in the laboratory. For example there were sediment samples previously collected from Belfast Lough by other researchers. Additional sample material was collected during two field-sampling trips to the Orkney Islands in 2010 and 2011.

It was originally estimated that at least five distinct *A. tamarense* Group I and five Group III clonal cultures (isolated from single cells) would need to be isolated from both Belfast Lough and Orkney. This would have resulted in a total of ten Group I and ten Group III isolates from both locations. However, as the process of isolation progressed it became clear that it would not be possible to generate the required cultures from the two primary regions of interest and so the experiments were revised to include a broader geographic area focussing on sediment samples that were already available within the laboratory from regions were *A. tamarense* hypnozygotes had previously been recorded. What follows is an outline of the sampling methods used, collected samples, the method of culture isolation and their identification/genotyping.

2.2 Field samples

Field sampling in the Orkney Islands was undertaken in 2010 and 2011 in conjunction with the annual water column sampling for the MIDTAL project[1]. Numbering of stations (where applicable) is therefore consistent with those of the MIDTAL project. Collection sites sampled exclusively for this project in 2009, 2010 and 2011, in addition to the MIDTAL sites, are geographically named e.g. Stromness Pier.

Sediment (2010) and water column samples (2010 & 2011) were collected from a variety of locations to isolate and establish monoclonal *A. tamarense* cultures for the purpose of mating, excystment and genetic studies. Additional sediments collected

[1] MIDTAL is a cooperated project for the development of microarrays for the detection of toxic algae, covering several institutes over European coastal seas. Ten partners make up the consortium and include scientists from 7 European countries and the USA.

previous to 2010 from Belfast Lough and Weymouth Harbour (UK) and Bedford Basin (Canada) were also used for this purpose.

Fig. 2.1 Distribution of sample sites and *A. tamarense* groups I and III.

2.2.1 Sediment

Orkney Island sediment samples were collected from sites across the Orkney Island mainland and the island of Westray, in May 2010. There was a preference for locating and collecting fine/muddy sediments as previous studies (Dale 1976; Lewis 1988) have shown that dinoflagellate cysts behave like fine sediment particles and are, therefore, likely to be more abundant in this type of sediment. Samples were collected either offshore via a boat or onshore from accessible beaches.

2.2.1.1 Offshore sampling

Offshore sediment samples were collected via a boat, using an Ekman grab at a depth of ~5 - 10 m. The grab has a spring-loaded mechanism and was lowered in an open position to the sediment by hand. A weight fired along the taught line causes the mechanism to close collecting the surface layer of sediment (see fig. 2.2). This type of grab requires the location of soft sediment. The entire sample from the Ekman grab was collected.

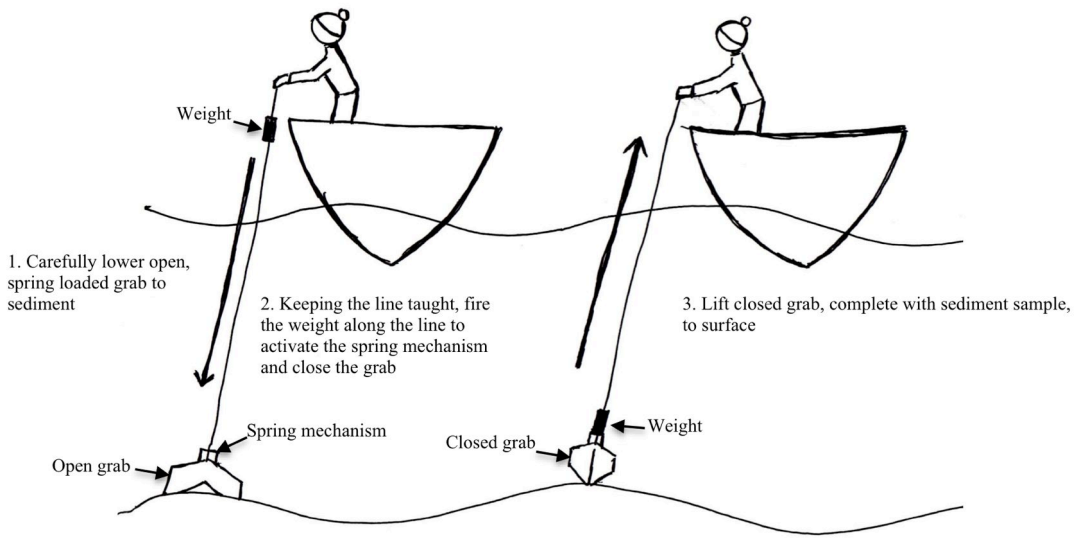


Fig 2.2 Ekman grab sediment sampling method

2.2.1.2 Shoreline sampling

Shoreline sediment samples were taken using a 60 mm diameter hand corer. Hand corers are clear polycarbonate tubes that are pushed several centimetres into the sediment. A bung placed in the open end of the tube creates a vacuum allowing the tube to be removed from the sediment without losing the sample. A plunger is then used to push the sediment core up and out of the tube. The top 5 cm only of shoreline samples was collected (see fig. 2.3).

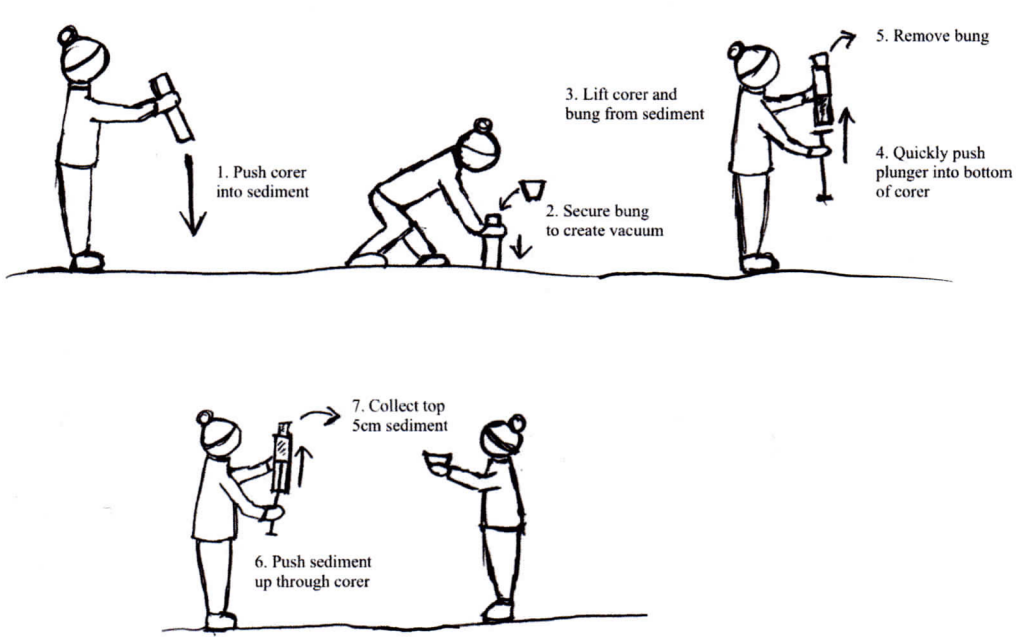


Fig 2.3 Hand corer sediment sampling method

2.2.1.3 Storage of sediment samples

All sediment samples were placed in airtight plastic containers, overlaid with seawater from the collection site and stored in the dark at 4°C until processed. Sediment samples were processed between 1-12 months after collection.

2.2.1.4 Additional sediments

Additional sediment samples from Belfast Lough, Weymouth Harbour (collected and donated by Linda Percy) and Bedford Basin (Canada) were used for the purpose of isolation and culture. Previous studies had shown these to contain Group I and III, Group III and Group I *A. tamarense* cysts respectively. These sediments had been stored in airtight containers, overlaid with seawater and stored in the dark at 4°C as previously. These sediments had been stored for between ~1 - 13 years.

Full details of sediment samples are listed in table 2.1. Figures 2.4 – 2.8 illustrate the geographic location and, where known, the distribution of sediment sample sites.

Table 2.1 Sediment samples, collection sites and dates. Sediments collected prior to 2010 were collected by others and have been used for isolation work.

Location	Site	Coordinates	Date	Sampling method
Orkney, mainland	Nr. Deerness	58°57.083'N 2°46.623'W	23.05.10	Hand corer
	Nr. Deerness	58°55.050'N 2°47.908'W	23.05.10	Hand corer
	Station 2, Bring Deeps	58°54.183'N 2°08.383'W	24.05.10	Grab sample
	Warbeth Beach	58°57.422'N 3°18.820'W	25.05.10	Hand corer
	Marwick Bay	59°5.804'N 3°20.820'W	25.05.10	Hand corer
	Helston, Wide Wall Bay	58°47.986'N 3°0.029'W	25.05.10	Hand corer
	Eastside	58°48.151'N 2°55.126'W	25.05.10	Hand corer
	Stromness Pier	58°57.851'N 3°17.668'W	26.05.10	Grab sample
	Waulkmill Bay	58°56.552'N 3°04.601'W	28.05.10	Hand corer
	Houton Bay	58°54.887'N 003°11.109'W	27.08.09	Grab sample
	Rysa Sound	58°52.010N 003°12.312'W	06.07.09	Grab sample
	Bay of Myre	58°54.854'N 003°03.755'W	06.07.09	Grab sample
	Finstown	58°00.176'N 003°06.266W	05.07.09	Hand corer
Orkney, Westray	North of Biggins	59°20.461'N 2°58.162'W	27.05.10	Hand corer
	Pierowall	59°19.446'N 2°58.480'W	27.05.10	Hand corer
	Bay of Tuquoy	59°17.333'N 2°57.891,W	27.05.10	Hand corer
	Rapness	59°15.103'N 2°51.508'W	27.05.10	Hand corer
Belfast Lough	A6	54º39.800'N 005º48.80'W	02.07.08	Sediment trap
	P9	Unknown	16.07.08	Sediment trap
	P9	Unknown	30.07.08	Sediment trap
Weymouth Harbour	---	Unknown	20.02.07	Unknown
Bedford Basin, Canada	---	Unknown	03.98	Unknown

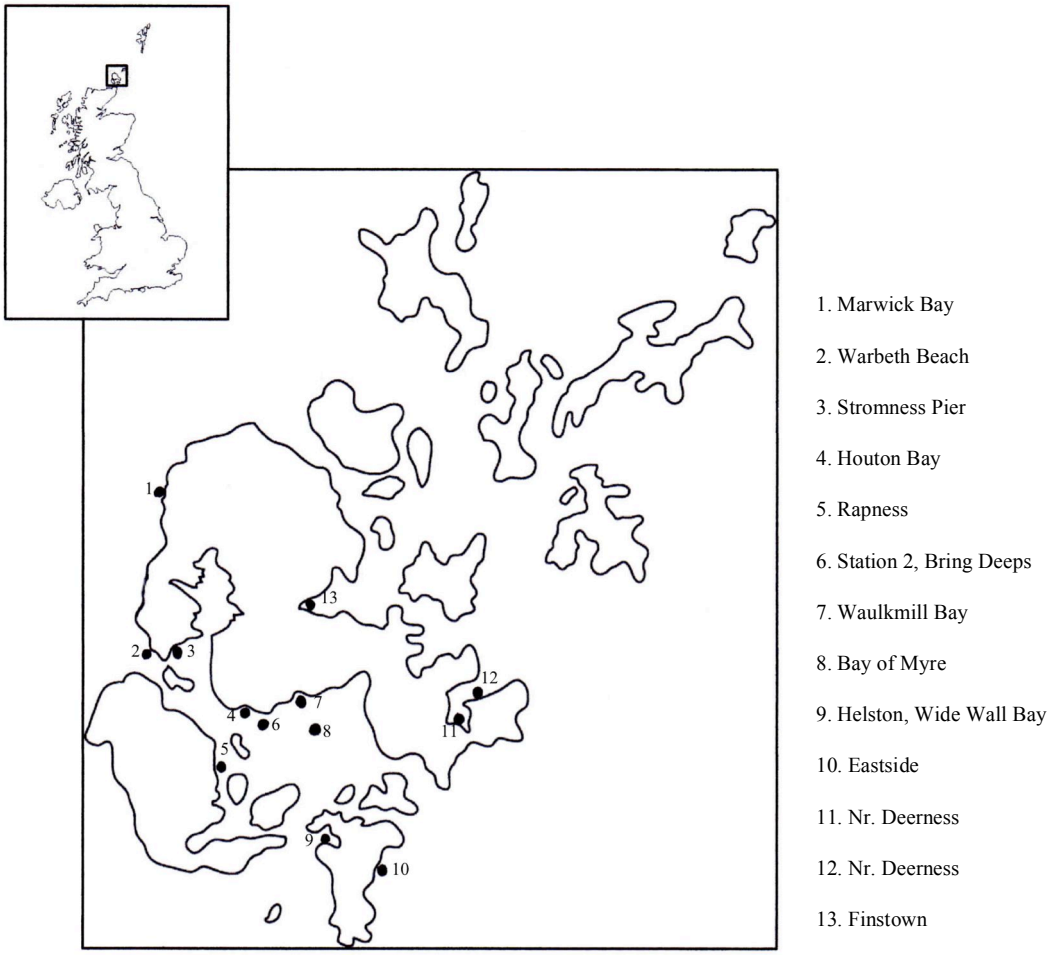


Fig 2.4 Distribution of mainland Orkney sediment sample sites for 2009 and 2010.

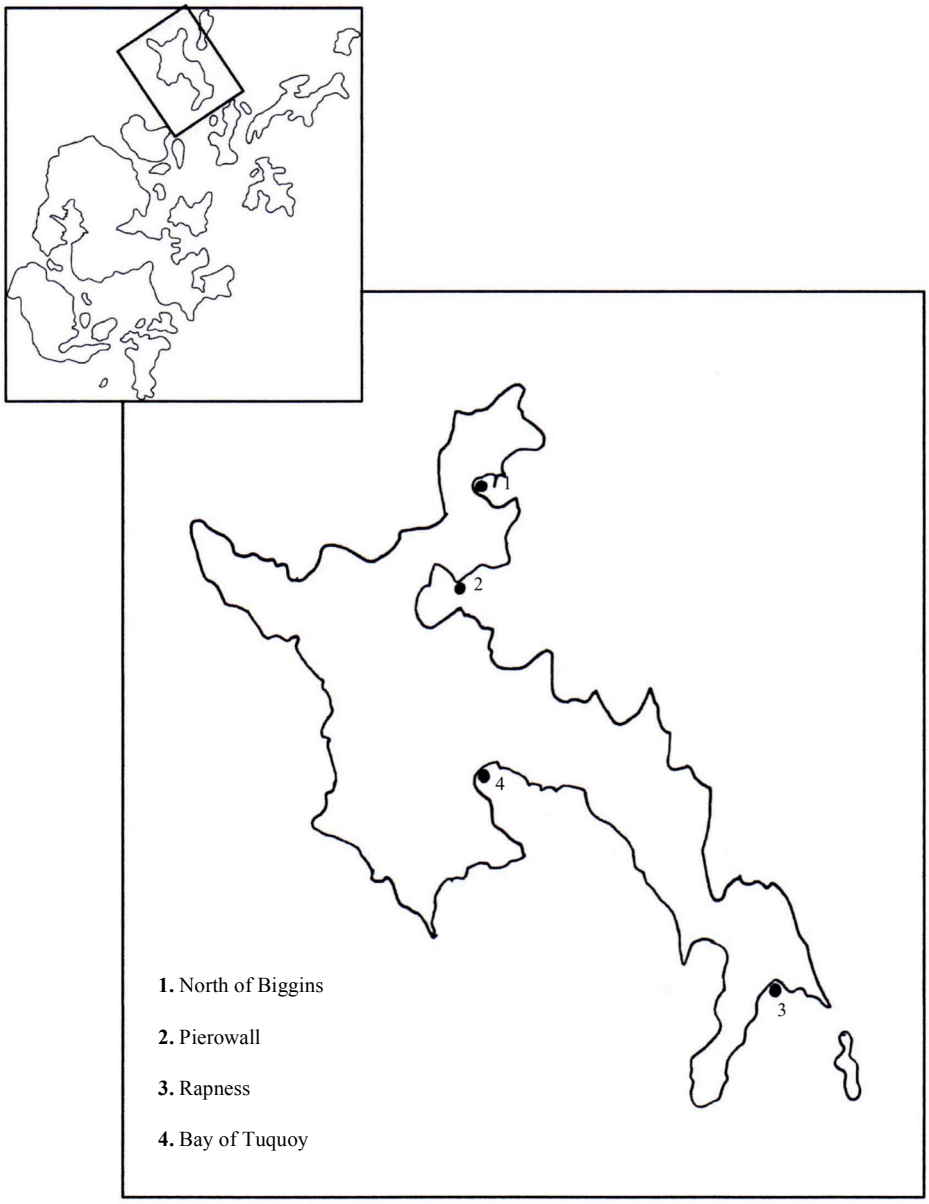


Fig 2.5. Distribution of sediment sample sites Westray, Orkney, 2010

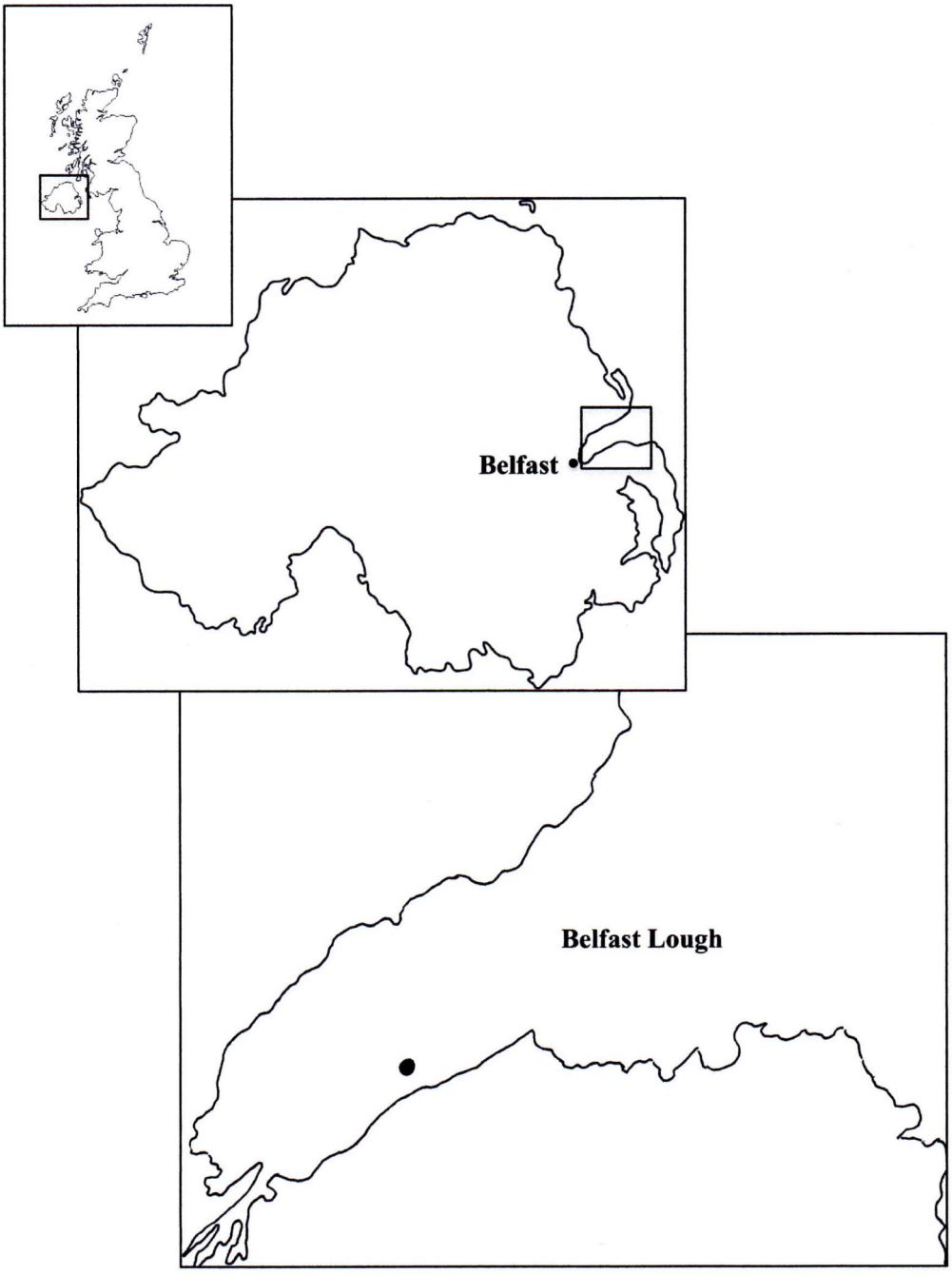


Fig 2.6 Location of Belfast Lough including sediment sample site A6.

Fig 2.7. Location of Weymouth Harbour.

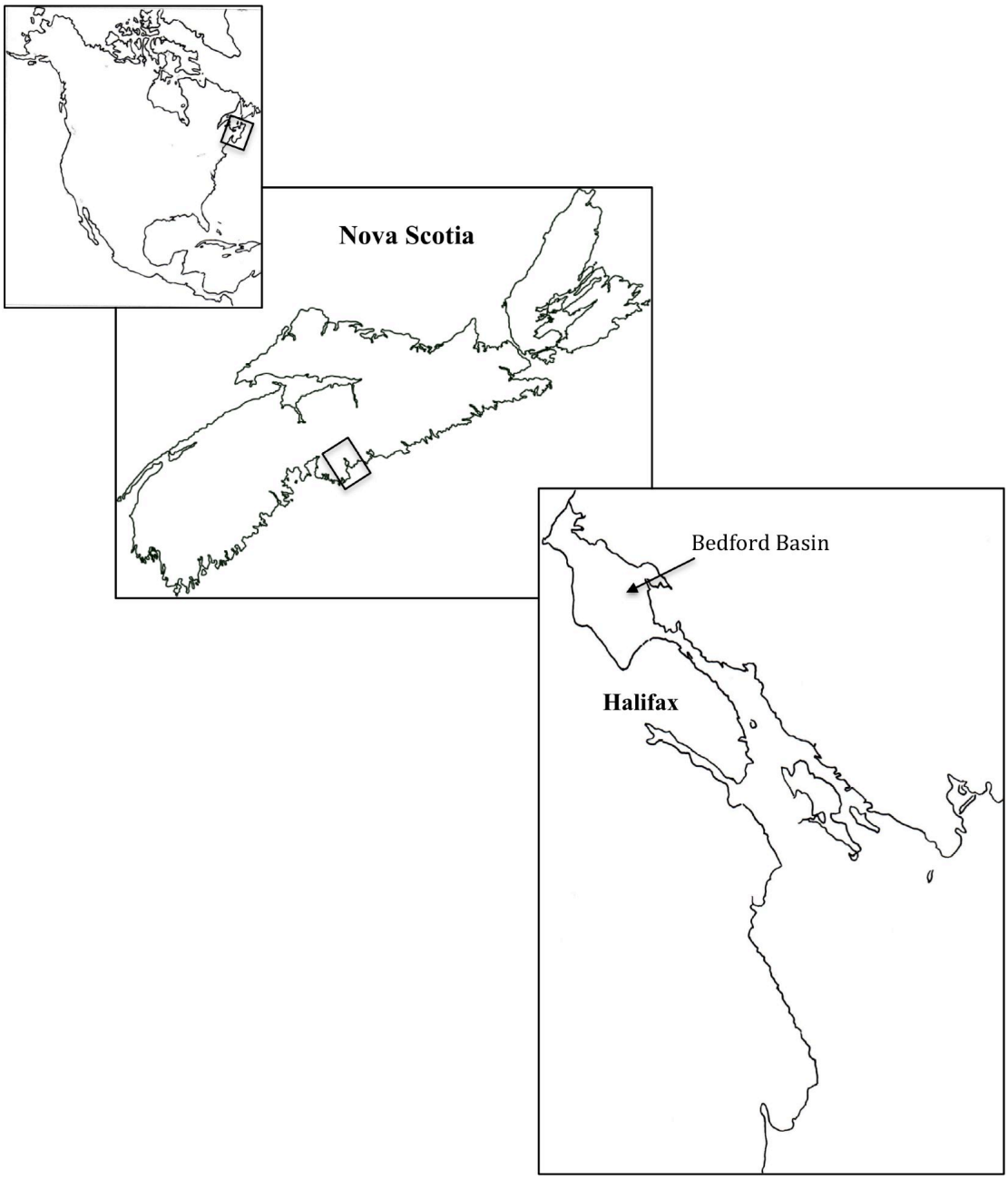


Fig 2.8 Location of Bedford Basin, Nova Scotia, Canada

2.2.2 Water column

Water column samples were collected from several stations in Orkney in May 2010 and June 2011 for the isolation and culture of *A. tamarense* for genetic analysis and mating experiments.

2.2.2.1 Integrated tube sampler

Water column samples were collected using a 5 m integrated tube sampler (ITS). This method of sampling provides a combined sample of the planktonic population across the depth of the tube. Samples were filtered on-board the boat through a 200 µm nylon sieve to remove large zooplankton and detritus. Pre-filtered samples were aliquoted into 2L bottles and placed in a cool box until processed. An additional 250 mL tube sample from each station was preserved in acidic Lugol's iodine (Throndsen 1978) for cell counting.

2.2.2.2 Collins bottle

A Collins water bottle was used to collect discrete samples from each station at a fixed depth of 2 m for the purpose of isolation. Samples were kept in 500 mL Thermos® flasks until processed.

2.2.2.3 Plankton net

Additional samples for isolation and culture of *A. tamarense* were taken using a 55 µm mesh plankton net. The concentrated net samples were taken as a precautionary

measure in case only very low numbers of *A. tamarense* were present in the water column at the time of sampling. Net samples were placed in lidded pots and kept cool until processed.

All water column samples used for isolation were examined at the field laboratory in Orkney and processed for isolation of cells within 1 week. Full details of water column samples are listed in table 2.2. Figure 2.9 illustrates the different methods.

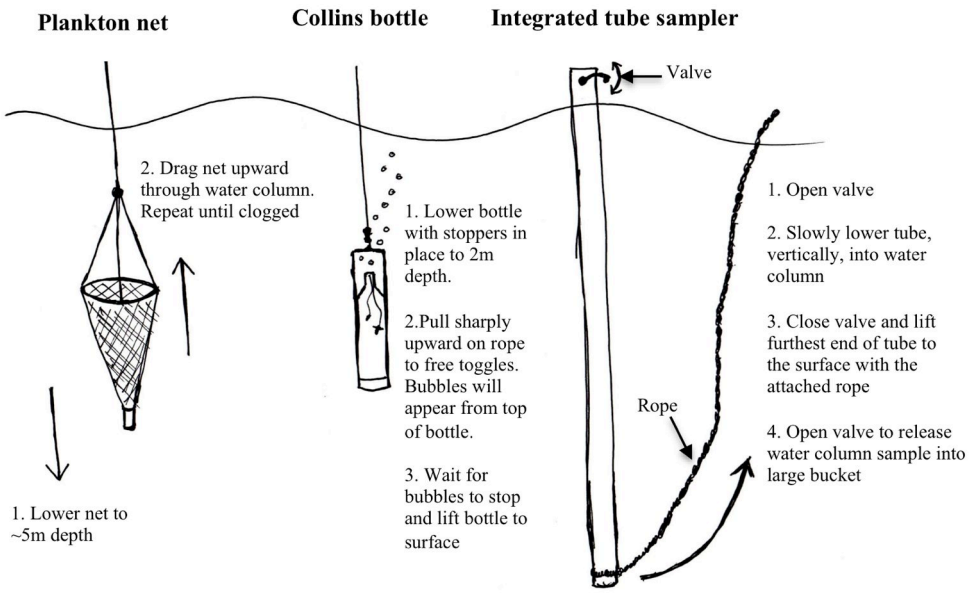


Fig 2.9 Water column sampling methods

2.2.2.4 Cell counts

Sub-samples of Lugols preserved water column samples were settled overnight in 20 mL chambers and cells identified and counted using an inverted IMT-2 microscope (Olympus). Cells were identified according to Hoppenrath et al. (2009) and Tomas (1997). Full details of the cell counting method are included in appendix A.

Table 2.2 List of Orkney Island water column samples, collection sites and dates for May 2010 and June 2011.

Year/Location	Station/site	Coordinates	Date	Sample/s
2010				
Orkney	Station 2	58°54.238'N 3°08.388'W	24.5.10	ITS, 2m depth sample
	Station 3	58°051.566'N 3°04.564'W	24.5.10	ITS, 2m depth sample,
	Station 5	58°53.293'N 3°07.527'W	24.5.10	ITS, 2m depth sample,
	Station 6	58°55.971'N 3°15.785'W	24.5.10	ITS, 2m depth sample, net sample,
	Burwick Pier	58°44.394'N 2°58.371'W	25.5.10	2m depth sample, net sample
	Stromness Pier	58°57.851N3°17.668'W	25.5.10	2m depth sample
	Stromness Pier	58°57.851N3°17.668'W	26.5.10	2m depth sample, net sample
	Station 6	58°55.971'N 3°15.785'W	26.5.10	ITS, 2m depth sample, net sample,
	Station 7	58°54.758'N 3°17.772'W	26.5.10	ITS, 2m depth sample, net sample,
	Waulkmill bay	58°59.552'N 3°04.601'W	28.5.10	Net sample
	Swanbister Bay	58°55.500'N 3°07.735'W	28.5.10	Net sample
	Kirkwall, Seafire Road	58°59.374'N 2°58.153'W	28.5.10	Net sample
Westray, Orkney	Pierowall	59°19.413'N 2°58.447'W	27.5.10	Surface water sample, net sample
	Point of Huro	59°14.133'N 2°52.518'W	27.5.10	Surface water sample
2011				
Orkney	Stromness Pier	58°57.851N3°17.668'W	19.06.11	2m depth sample, net sample
	Station 2	58°54.238'N 3°08.388'W	20.06.11	ITS, 2m sample
	Station 3	58°051.566'N 3°04.564'W	20.06.11	ITS, 2m depth sample
	Station 5	58°53.293'N 3°07.527'W	20.06.11	ITS, 2m depth sample
	Station 6	58°55.971'N 3°15.785'W	20.06.11	ITS, 2m depth sample, net sample
	Stromness Pier	58°57.851N3°17.668'W	21.06.11	2m depth sample, net sample, seaweed sample
	Station 2	58°54.238'N 3°08.388'W	22.06.11	ITS, 2m depth sample, net sample
	Station 6	58°55.971'N 3°15.785'W	22.06.11	ITS, 2m depth sample, net sample

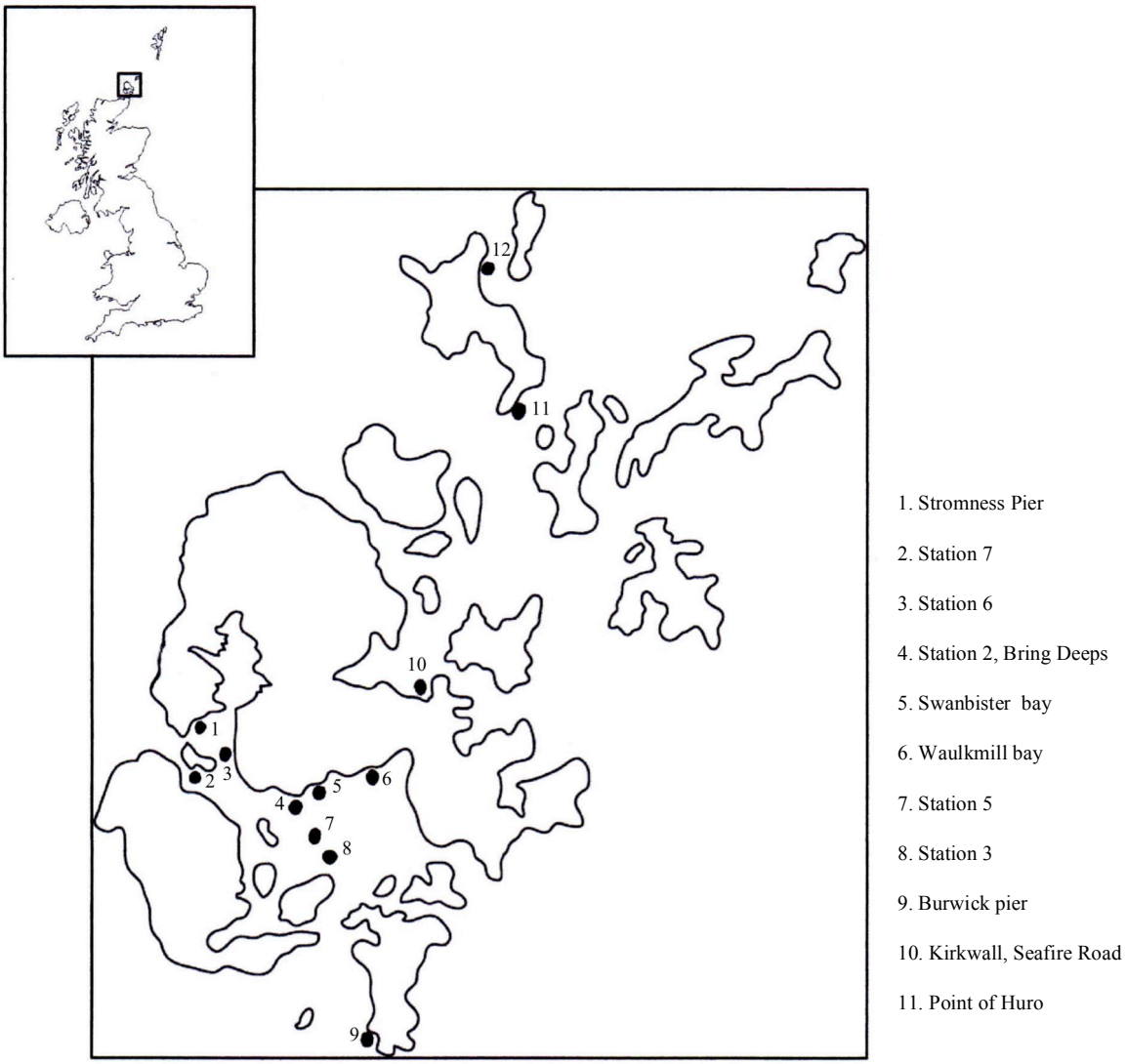


Fig 2.10 Distribution of Orkney water column sampling sites, 2010

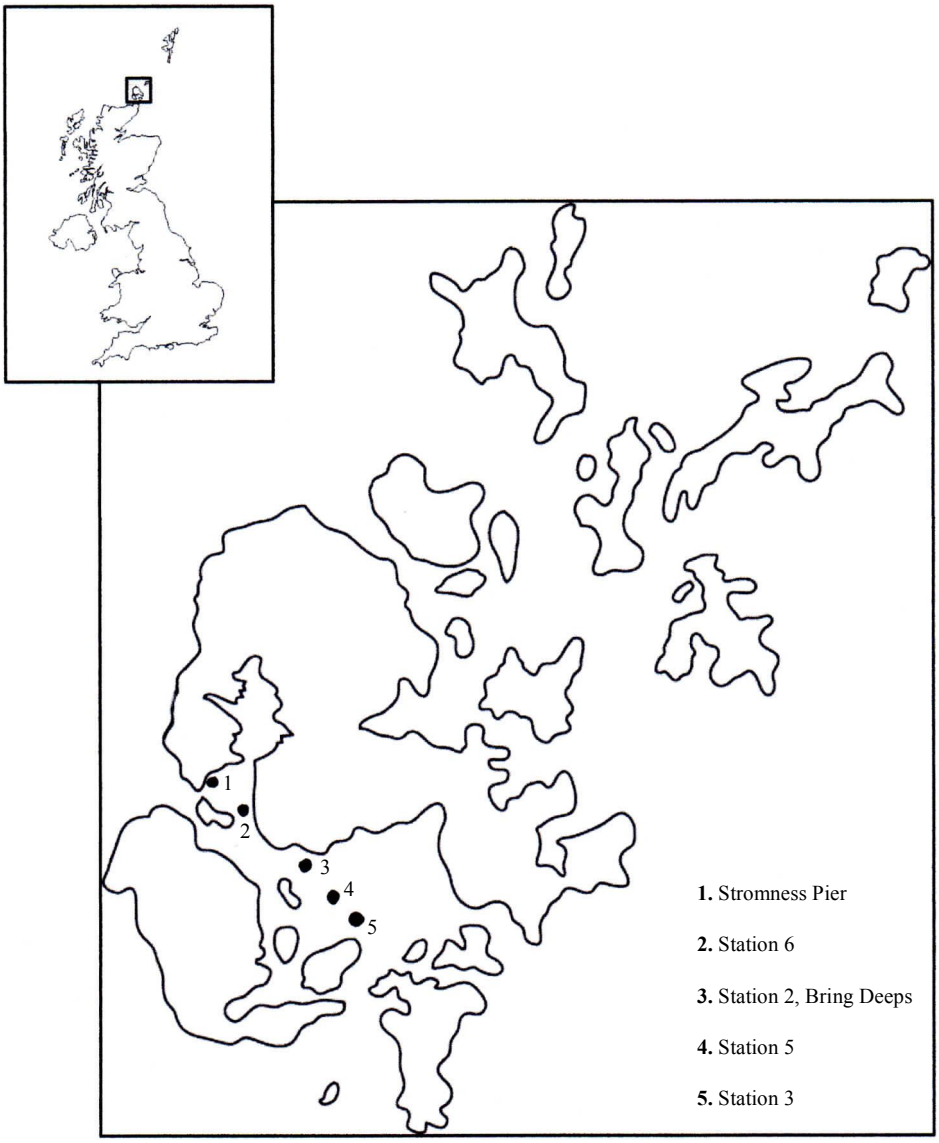


Fig 2.11 Distribution of Orkney water column sampling sites, 2011

2.3 Culture isolation

Monoclonal cultures were isolated either directly from water column samples or from motile cells germinated in sediment slurries.

2.3.1 Modified f/2 medium preparation

All isolates were germinated or isolated in f/2 medium without silicate (Guilliard, 1975), modified by the addition of sodium selenite (Na_2SeO_3, final conc. 10^{-8} M) and reducing copper sulphate ($CuSO_4.5H_2O$) from 3.93 x 10^{-8} to a final concentration of 1 x 10^{-8} M. Medium was prepared using 0.2 μm (PALL) filtered natural seawater (salinity ~34), autoclaved at 121°C for 15 minutes in 2L Teflon bottles (Nalgene) to prevent leaching of silica associated with glass vessels (Anderson et al 1984).

Stock solutions of macronutrients ($NaNO_3$, and NaH_2PO_4 H_2O), trace metals and vitamins were prepared using deionised water (dH_2O), as shown in tables 2.3 - 2.5. Prepared macronutrient, trace metal and vitamin solutions were 0.2 μm filtered and stored in sterile (autoclaved 121°C/15 mins) glass or Teflon® bottles at 4°C.

Medium was prepared by 0.2 μm filtering macronutrients, trace metals and vitamins solutions into sterile natural seawater using a syringe filter, as outlined in table 2.3. Prepared medium was stored in the dark at ~16°C.

Table 2.3 Modified f/2 components. Components added by 0.2 μm sterile filter to 1L 0.2 μm filtered, autoclaved, natural seawater.

Component	Stock solution (g/L dH_2O)	Volume/L	Final concentration in medium (M)
$NaNO_3$	75	1 mL	8.82 x 10^{-4}
NaH_2PO_4 H_2O	5	1 mL	3.62 x 10^{-5}
Trace metals solution	-	1 mL	-
Vitamins solution	-	0.5 mL	-

Table 2.4 Modified f/2 trace metals stock solution. EDTA and $FeCl_2$ dissolved in ~950 mL dH_2O, other components added and volume made up to 1L with dH_2O.

Component	Stock solution (g/L dH_2O)	Quantity/L	Final concentration in medium (M)
$FeCl_3$ $6H_2O$	-	3.15 g	1.17×10^{-5}
Na_2EDTA $2H_2O$	-	4.36 g	1.17×10^{-5}
$MnCl_2$ $4H_2O$	180.0	1 mL	9.10×10^{-7}
$ZnSO_4$ $7H_2O$	22.0	1 mL	7.65×10^{-8}
$CoCl_2$ $6H_2O$	10.0	1 mL	4.20×10^{-8}
$CuSO_4$ $5H_2O$	9.8	250 µL	1.0×10^{-8}
Na_2MoO_4 $2H_2O$	6.3	1 mL	2.60×10^{-8}
Na_2SeO_3	6.9	250 µL	1.0×10^{-8}

Table 2.5 f/2 vitamins stock solution. Thiamine HCl dissolved in ~950 mL dH_2O, biotin and cyanocobalamin added, volume made up to 1L with dH_2O.

Component	Stock solution (g/L dH_2O)	Quantity/L	Final concentration in medium (M)
Thiamine HCl (vitamin B_1)	-	200 mg	2.96×10^{-7}
Biotin (vitamin H)	1.0	1 mL	2.05×10^{-9}
Cyanocobalamin (vitamin B_{12})	1.0	1 mL	3.69×10^{-10}

2.3.2 Sediment slurries

For the preparation of sediment slurries approximately 1- 4 mL of wet, thoroughly mixed, sediment was drawn into the barrel of a 10 mL syringe with its distal end cut off. The sediment was then placed into a sterile 50 mL glass beaker. Beakers were sterilized by covering with aluminium foil and autoclaving at 121°C for 15 minutes. To maintain sterility as much as possible the foil beaker cover was replaced during

sonication and sieving. Approximately 50 mL of artificial seawater (salinity ~34) was added to the wet sediment in the beaker and the beaker placed in a sonication bath for 2 minutes. Artificial seawater was prepared by dissolving 34g/L of artificial sea salt (Tropic Marin) in dH_2O. Prepared artificial seawater was then 0.2 µm filtered, transferred to 2 L Teflon bottles (Nalgene), autoclaved at 121°C for 15 minutes and stored at ~16°C in the dark.

Sieving apparatus was set-up by placing an 80 µm mesh sieve above a 20 µm mesh sieve and both were then placed on a large metal sieve in a large bowl (see figure 2.12). A little of the sonicated sediment/artificial seawater mixture was poured onto the 80 µm mesh and washed through with artificial seawater using a wash bottle. This was continued until all the mixture had been sieved and the washings from the 20 µm mesh ran clear. It was often necessary to rub the underside of the 20 µm mesh to facilitate the flow of liquid through the mesh.

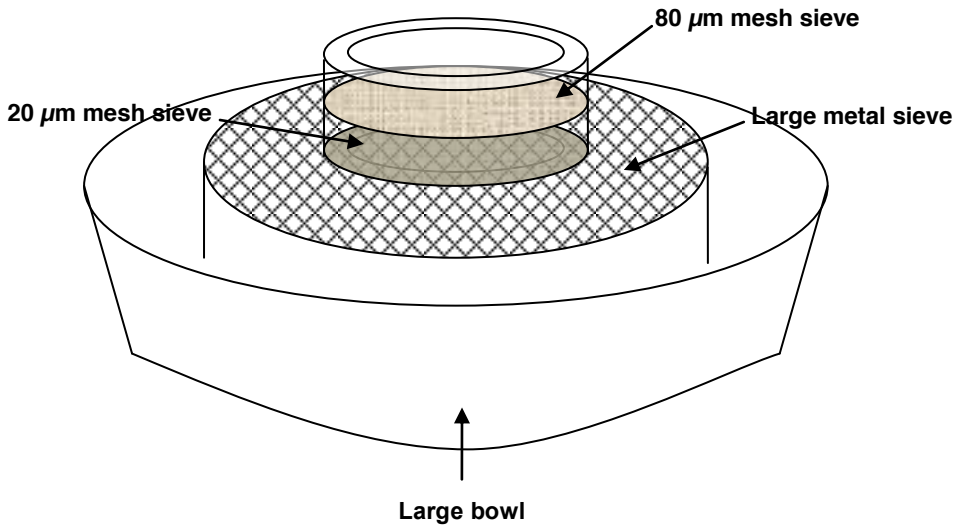


Fig. 2.12 Sieve set-up for sediment slurries

The fine sediment remaining on the 20 μm mesh was collected using a sterile Pasteur pipette and artificial seawater and placed into a clean sterile 50 mL beaker. This was then distributed into 50 mm Petri dishes (Sterilin) containing f/2 medium without silicate (Guilliard, 1975), modified by the addition of sodium selenite (Na_2SeO_3, final conc. 10^{-8} M) and reducing copper sulphate ($CuSO_4.5H_2O$) from 3.93 x 10^{-8} to a final concentration of 1 x 10^{-8} M. Medium was prepared with 0.2 μm (PALL) filtered natural seawater (salinity ~32-34) in 2L Teflon bottles (Nalgene) to prevent leaching of silica associated with glass vessels (Anderson et al. 1984). The amount of sediment added to each Petri dish was just enough to barely cover the bottom of the dish with a very fine single layer, so as not to obscure the visibility of motile cells under microscopic examination. The Petri dishes were labelled, sealed with parafilm and incubated at ~15-16°C, under cool white fluorescent light on a 14:10 light/dark cycle. Slurries were monitored every few days for motile cells and *Alexandrium spp*. type cells were isolated as outlined below.

2.3.3 Isolation of single motile cells from sediment slurries and water column samples

Single cells from both water column samples and slurry cultures were isolated by micropipette into 96 or 24 well tissue culture plates, containing modified f/2 medium. Two 96/24 well plates were used, one for washing cells and one for incubation. Micropipettes were made by drawing the tip of a glass Pasteur pipette over a blue Bunsen burner flame using forceps. The tip of each micropipette was observed at 100x magnification, using an inverted microscope, and any that had tips that were jagged or very irregular were discarded or redrawn. A micropipette was

attached to one end of a long piece of silicon tubing with a bulb on the opposite end (see figure 2.13). Cells were isolated by observing samples/slurries using an inverted microscope (Olympus) whilst manoeuvring the micropipette with one hand and operating the bulb to capture cells, with the other. To allow this the tubing was passed over the shoulders. A single cell was transferred to a clean well of the 96/24 well 'washing' plate and then transferred again to a new, clean well to wash the cell. This was repeated 3 times and the cell then transferred to a clean well of the 96/24 well 'incubation' plate (see figure 2.14 for an illustration of the process).

Isolated cells were incubated at ~15-16°C, under cool white fluorescent light on a 14:10 light/dark cycle. After several generations (~10 -14 days), cells were transferred to a 50 mm Petri dish containing fresh modified f/2 medium and grown under the same conditions until finally being transferred to 25 cm^3 Falcon® tissue culture flasks. Established cultures were maintained in f/2 modified medium, in 25 cm^3 Falcon® tissue culture flasks under the above conditions and sub-cultured every 2-4 weeks. .

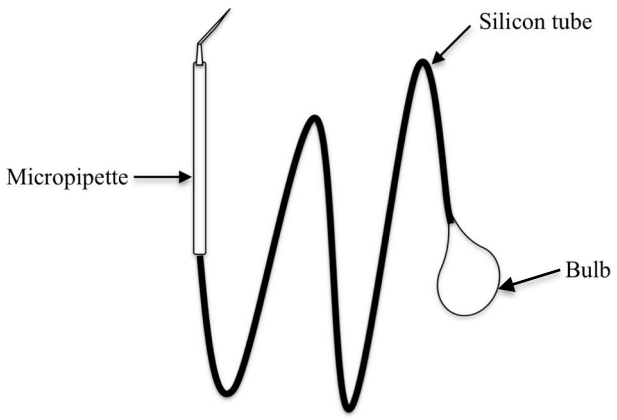


Fig. 2.13 Micropipette set-up for the isolation of single, motile dinoflagellate cells

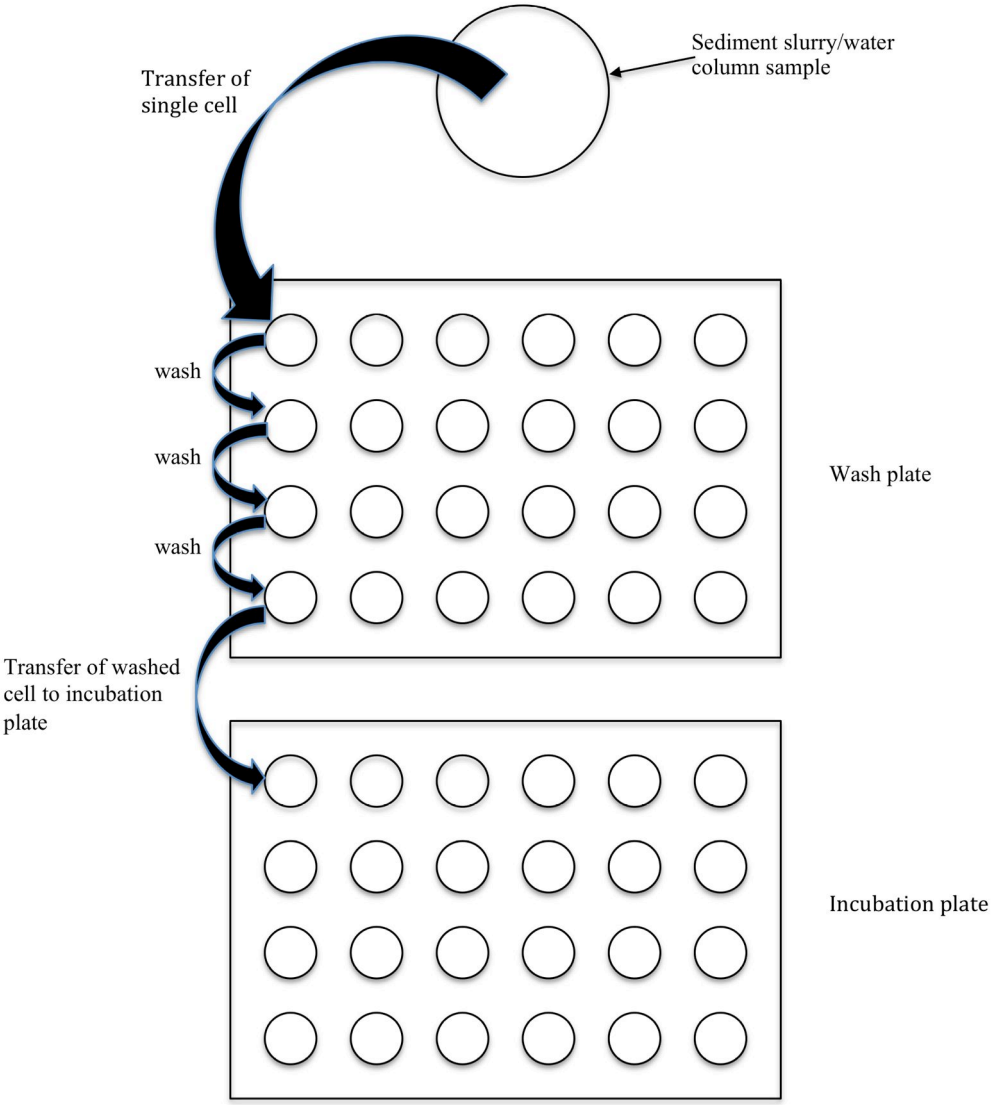


Fig. 2.14 Isolation method for single *A. tamarense* cells, showing sequential washing steps and transfer to incubation plate.

2.4 Genotyping of established cultures

DNA was extracted from established monoclonal cultures, the hypervariable D1-D2 region of the LSU rDNA amplified by PCR and the PCR products sequenced and analysed in order to confirm the genus/species of each culture.

2.4.1 Growth of Cultures

Cultures were grown in 100 mL f/2 modified medium at ~15-16°C, under cool white fluorescent light on a 14:10 light/dark cycle, as previously, with a starting inoculum was ~1000 cells/mL. Cells were counted every two days using a 1 mL Sedgewick Rafter cell and an Olympus compound microscope. Five mL of culture was removed and preserved with a few drops of Lugol's iodine prior to counting and all cell counts were performed in triplicate. Care was taken to sample at the same time of day for each sub-sampling. Cells were harvested in late exponential/early stationary phase (~day 10-14) to maximise DNA yield.

2.4.2 DNA extraction

Approximately 5 mL of cultured cells in late exponential/early stationary phase (8000-14,000 cells/mL) were collected by vacuum filtration onto 25 mm nitrocellulose membrane filters (Whatman). The filters were placed in 2 mL bead beater tubes containing 0.2 g glass beads with 400 µL lysis buffer (Invisorb©, Invitek) and beaten for 2 minutes at maximum speed in order to lyse the cells. The lysate solution was recovered by centrifuging for 2 minutes at 3200 rpm in a Centaur 2 centrifuge (MSE). The DNA extraction was then carried out using an Invisorb© mini plant kit (Invitek) according to the manufacturer's instructions. Extracted DNA quality and quantity was assessed using a NanoDrop™ND 1000 spectrophotometer (Thermo Scientific).

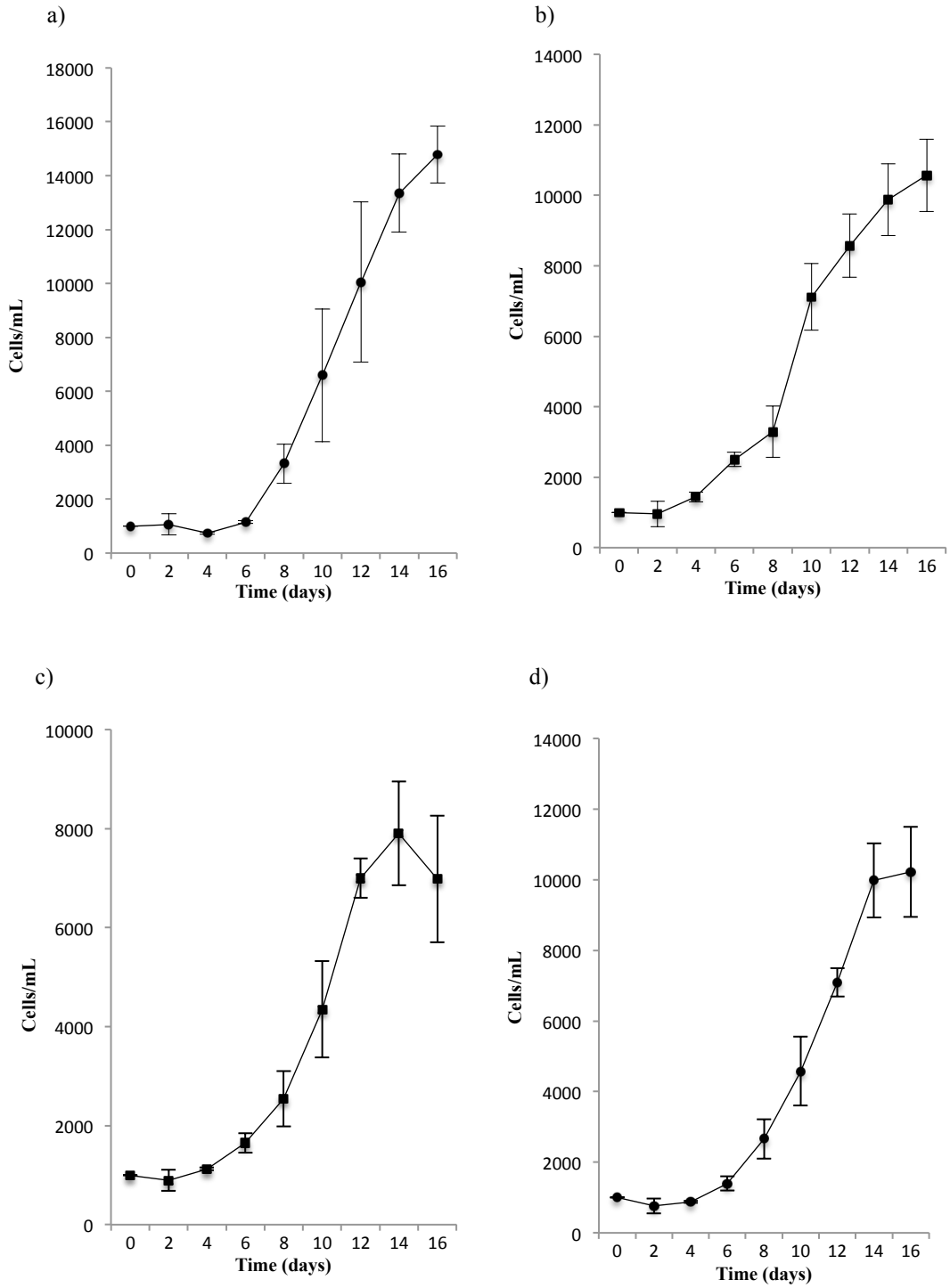


Fig. 2.15 Growth curves for a) SPC6 (Stromness Pier), b) WHC2 (Weymouth Harbour), c) BLA6 C4 (Belfast Lough) and d) BBD4 (Bedford Basin). All cultures grown at ~15 - 16°C. Error bars ± standard deviation.

2.4.3 PCR amplification of D1-D2 LSU rDNA and sequencing

PCR amplification of the D1-D2 hypervariable region of the LSU rDNA was performed in 25 µl reaction volumes (2x Accusure™ master mix (Bioline), primers D1R & D2C (Scholin *et al*, 1994; see table 2.6) at a final concentration of 0.5 µM, ultra pure PCR water (Bioline), 25-50 ng template DNA or 5 µl PCR water for no template control (NTC) reactions). PCR cycling was carried out using an Eppendorf® personal cycler with the following cycling conditions; Initial denature 96°C for 5min followed by 35 cycles of denature 95°C: 45sec, annealing 54 °C: 45 sec, extension 72°C: 1min followed by a final extension of 72°C: 10min.

Table 2.6 D1-D2 LSU rDNA PCR primers. Direction of primer is indicated by F (forward) & R (reverse).

Primer	Sequence	Origin
D1R (F)	5'ACCCGCTGAATTTAAGCATA3'	Scholin *et al*, 1994
D2C (R)	5'CCTTGGTCCGTGTTTCAAGA3'	Scholin *et al*, 1994

Following amplification, 8µL of each PCR reaction was run for 1 hour at 100V on a 1% ultra pure agarose gel (Invitrogen) made with 1x TBE buffer and precast with 5 µl 10,000x GelRed™ nucleic acid stain (Biotium). A 100 bp Easyladder I (Bioline) was run alongside the reaction wells to confirm product size. Gels were visualised and photographed using a UV visualiser to confirm product size (~700bp). The remaining 17 µL of each PCR product was purified using a QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions and eluted with

30 µL PCR water (Bioline). Twenty µL of purified PCR product, at a concentration of 10-50ng/µL, was directly sequenced in forward and reverse directions at GATC Biotech. The returned sequences were examined and aligned using ClustalW and Bioedit v7.0.9. Edited sequences were then blast searched via NCBI for similarity to existing deposited nucleotide sequences.

2.5 Results/Discussion

A total of 47 *A. tamarense* isolates were brought into culture from Belfast Lough, Orkney, Weymouth Harbour and Bedford Basin, although four were lost from culture at various stages of the project (see table 2.7). The majority of the cultures were isolated from sediment slurries, with only those from the Orkney Islands being isolated directly from water column samples collected in May 2010 and June 2011

Table 2.7 List of isolated cultures, D1-D2 LSU rDNA results and material culture was isolated from. Cultures that were identified by whole-cell FISH are marked *.

Origin	Strain	UoW ID	Species/Group	Material
Weymouth Harbour	WHA1	UoW 700	*A. tamarense* III	Sediment
	WHA2	UoW 701	*A. tamarense* III	Sediment
	WHA5	UoW 702	*A. tamarense* III	Sediment
	WHB2	UoW 703	*A. tamarense* III	Sediment
	WHB4*	UoW 704	*A. tamarense* III	Sediment
	WHC1	UoW 705	*A. tamarense* III	Sediment
	WHC2	UoW 706	*A. tamarense* III	Sediment
	WHD1*	UoW 724	*A. tamarense* III	Sediment
	WHC3*	UoW 725	*A. tamarense* III	Sediment

Continued from page 52

Origin	Strain	UoW ID	Species/Group	Material
Weymouth Harbour	WHD2	UoW 707	*A. tamarense* III	Sediment
Belfast Lough -site A6	BLA6 A6	UoW 708	*A. tamarense* III	Sediment
54º39.800'N 005º48.80'W	BLA6 B2	UoW 709	*A. tamarense* III	Sediment
	BLA6 B4	UoW 710	*A. tamarense* III	Sediment
	BLA6 B5*	UoW 711	*A. tamarense* III	Sediment
	BLA6 C4	UoW 712	*A. tamarense* III	Sediment
	BLA6 C5	UoW 713	*A. tamarense* III	Sediment
	BLA6 B1	UoW 726	*A. tamarense* III	Sediment
	BLA6 A5	UoW 727	*A. tamarense* III	Sediment
	BLA6 C1*	UoW 728	*A. tamarense* III	Sediment
	BLA6 D5	UoW 714	*A. tamarense* III	Sediment
	BLA6 A4	UoW 729	*A. tamarense* III	Sediment
	BLA6 D2	UoW 730	*A. tamarense* I	Sediment
Stromness Pier, Orkney	SPB5	UoW 715	*A. tamarense* I	Water column
58°57.851N3°17.668W	SPC6	UoW 716	*A. tamarense* I	Water column
	SPD4	UoW 717	*A. tamarense* I	Water column
	SPD6	UoW 718	*A. tamarense* I	Water column
	SPD2	UoW 731	*A. tamarense* I	Seaweed/water column
Station 7, Orkney	ST7 A3	UoW 719	*A. tamarense* I	Water column
58°54.758N 3°17.772W	ST7 B3	UoW 720	*A. tamarense* I	Water column
	ST7 B5*	UoW 721	*A. tamarense* I	Water column
	ST7 D5*	UoW 722	*A. tamarense* I	Water column
	ST7 D2	UoW 723	*A. tamarense* I	Water column
Bedford Basin, Canada	BBD4	UoW 732	*A. tamarense* I	Sediment
	BBD1	UoW 733	*A. tamarense* I	Sediment
	BBA4	UoW 734	*A. tamarense* I	Sediment
	BBB5	UoW 735	*A. tamarense* I	Sediment
	BBB3	UoW 736	*A. tamarense* I	Sediment
	BBC2	UoW 737	*A. tamarense* I	Sediment
	BBA6	UoW 738	*A. tamarense* I	Sediment
	BBD6	UoW 739	*A. tamarense* I	Sediment
	BBA2	UoW 740	*A. tamarense* I	Sediment
	BBC5	UoW 741	*A. tamarense* I	Sediment
	BBC4	UoW 742	*A. tamarense* I	Sediment
	BBB4	UoW 743	*A. tamarense* I	Sediment

2.5.1 Water column samples

Isolation of cells from water column samples collected in Orkney in 2010 proved difficult despite *Alexandrium spp*. type cells being identified in cell counts for all stations sampled in May 2010 (See table 2.8. Full cell count data for May 2010 sampling is included in appendix B). Live cells were isolated from these samples but either failed to divide and died, or the wells became contaminated with other phytoplankton (small diatoms were a particular problem) and the cells had to be re-isolated several times to ensure monoclonal cultures, free from contaminant species, were obtained.

Water column samples collected in June 2011 coincided with a large diatom bloom, with very few dinoflagellates observed (data not shown). These samples yielded only four *Alexandrium* spp. type cells and of these four cells isolated only one survived into culture. This was a small cell, confirmed to be Group I *A.tamarense,* found associated with a seaweed sample taken from Stromness Pier and isolated by Linda Percy.

Table 2.8 Water column samples collected in the Orkney Islands in May 2010, including *Alexandrium* spp. cell count data, isolation positive samples and numbers of cultures generated. N/A = not applicable.

Station/site	Coordinates	Date	*Alexandrium* cells/L	Cells isolated?	Number of cultures generated
Station 2	58°54.238'N 3°08.388'W	24.5.10	300	No	N/A
Station 3	58°051.566'N 3°04.564'W	24.5.10	250	No	N/A
Station 5	58°53.293'N 3°07.527'W	24.5.10	400	Yes	0
Station 6	58°55.971'N 3°15.785'W	26.5.10	100	Yes	4
Station 7	58°54.758'N 3°17.772'W	26.5.10	100	Yes	5

2.5.2 Sediment slurries

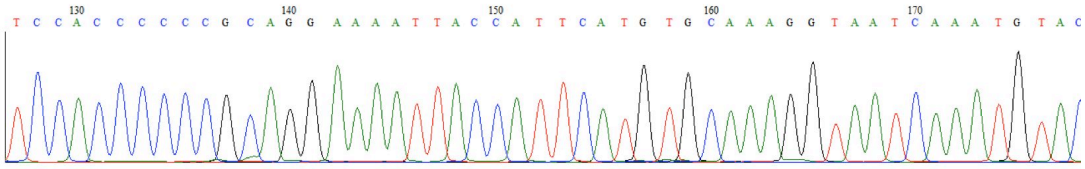
Isolation of cells from sediment slurries proved equally difficult with only sediment slurries from Belfast Lough site A6, Weymouth Harbour and Bedford Basin yielding results. All other sediment samples proved negative for the excystment of *Alexandrium spp*. type cells despite multiple attempts for each sediment sample (n= 2 - 4 slurries per location) and the conditions being the same as those resulting in successful excystment seen in the aforementioned samples. Visual screening of sediment slurries only resulted in one *A. tamarense* type hypnozygote being observed. This was in the Bay of Tuquoy sample collected from Westray in 2010. This hypnozygote did not excyst and in those samples that did yield motile *A. tamarense* cells, hypnozygotes were never observed. The reasons for this are unclear, however one explanation may be the failure of sonification to dislodge sediment particles that may have adhered to hypnozygote mucilage.

Another factor that may have influenced these results in relation to the Orkney Island samples taken in 2009, 2010 and 2011 was the predominance of shoreline sampling. According to Anderson et al. (2003) sediment sampling is preferable offshore, where exposure of sediment to air is not an issue. However, it was only possible to sample offshore, using the Ekman grab, at limited stations due to restricted time and the inability to locate areas of soft sediment in Scapa Flow. Furthermore, those offshore samples that were collected did not yield any *Alexandrium* cells. Even that from Stromness Pier, where several cultures were isolated from the water column, yielded no *Alexandrium* cells and no hypnozygotes were observed. This may suggest that the cells isolated from this site were seeded from another location.

2.5.3 Genotype of cultures

PCR amplification and sequencing of the D1-D2 region of the LSU rDNA produced useful data for 36 of the 43 extant cultures. Although, chromatograms for all Group I ribotypes displayed the presence of at least two sequences (see fig. 2.16). The remaining 7 cultures, where direct sequencing of the D1-D2 rDNA had failed, were identified using group specific probes and whole-cell fluorescent in situ hybridisation (Whole-cell FISH) (full method is given in chapter 5).

a)



b)

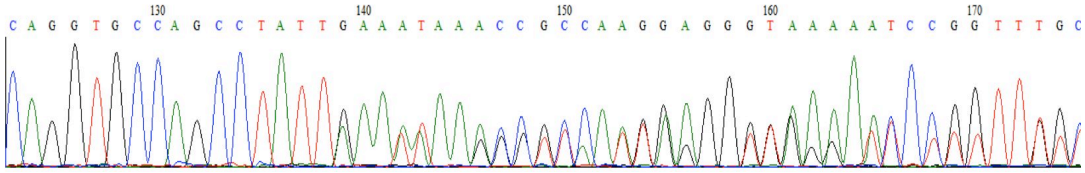


Fig. 2.16 Chromatograms showing a) single peaks of Group III culture BLD5 isolated from Belfast Lough and b) Dual peaks of Group I culture SPB5 isolated from Stromness Pier, Orkney.

Sequence data and whole-cell FISH revealed all Orkney isolates to be *A. tamarense* Group I. However this does not exclude the possibility that Group III were also present, as it could be that vegetative Group I cells were simply more amenable to the culture conditions or that the water column temperature at the time of sampling (10-11°C) were more supportive to the growth of Group I. Conversely, all but one of the 12 cultures isolated from Belfast Lough, site A6, were Group III. Again this could indicate a bias toward the culture of this group, particularly in relation to excystment conditions. These results could also indicate that Group I hypnozygotes constitute a minor component of the *A. tamarense* population in this area of Belfast Lough. These results could also suggest sampling bias as was reported by Genovesi et al. (2010) where one operator isolated only Group III *A.tamarense*, while another isolated only Group IV *A.catenella* from mixed population in Thau lagoon, France.

Interestingly, Collins et al. (2009) described similar bias when attempting to isolate *A. tamarense* cells from water column and sediment samples from a site off the north east Scottish coast. In this study the authors reported that all *A.tamarense* cells isolated from the water column were Group I, while all those isolated from sediment were Group III. Furthermore, there are remarkable similarities between the Collins et al. study and the study presented here. For example hypnozygotes were germinated at ~15°C in both studies, suggesting that this temperature may favour the germination of Group III *A. tamarense*. However, the effect of temperature on cyst germination remains unclear and other researchers have reported that temperature has no effect on the germination *A. tamarense* hypnozygotes in the range of 2 - 16°C (Perez et al. 1998).

In relation to Group I *A. tamarense* cells isolated from water column samples, the surface water temperature at the time of sample collection was reported by Collins et al. (2009) as ~10°C. This is near identical to the water column temperatures recorded during field-sampling in Orkney (10-11°C), which were also in agreement with Joyce (2005) who found that water temperatures in Scapa Flow, Orkney never exceeded 13°C. However, as with the germination of Group III *A. tamarense*, the role of temperature and the preferential growth of Group I is contentious. Particularly as Touzet et al. (2010) reported the co-occurrence of Group I and III at a site in Shetland at the only slightly higher temperature range of 12.2 -12.8°C.

As was expected from sediment samples from regions where no co-occurrence has previously been reported, all isolates from Weymouth Harbour sediment were *A.*

tamarense Group III and all Bedford Basin sediment isolates were *A. tamarense* Group I. It is noteworthy that Group I *A. tamarense* hypnozygotes were easily germinated from the Bedford Basin sediment at 15°C. A finding which contradicts both this study, in relation to Belfast Lough, and the study by Collins et al. (2009) and further supports Perez et al's (1998) assertion that temperature does not affect the germination of *A. tamarense* hypnozygotes. These results are further complicated by the fact that the water temperature profile of Bedford Basin is very similar to that of Scapa Flow, with means of 4-13°C (Bedford Institution of Oceanography, Bedford Basin monitoring programme). Furthermore the age of the Bedford Basin sediment is also worthy of note. Hypnozygotes from this sediment were germinated after >13 years in dark storage under anoxic conditions, at 4°C. Suggesting that sediment storage conditions were not a factor in the failure of hypnozygotes to germinate.

Given these facts, the idea of some form of unintended sampling bias must be seriously considered as a factor in the failure to isolate both Group I and III *A. tamarense* from both Belfast Lough and Orkney. What factors might cause such bias remain unclear and require further investigation. However, whatever the cause, this failure had significant implications for the project and resulted in the necessity to revise, in particular, the mating experiments presented in chapter 3.

CHAPTER 3

MATING COMPATIBILITY AND HYPNOZYGOTE VIABILITY: A STUDY OF THE MATING INTERACTIONS BETWEEN *A. TAMARENSE* GROUPS I AND III IN CULTURE

3.1 Introduction

Sexual reproduction in the *A. tamarense* species complex has important implications for the initiation and termination of harmful algal blooms (HAB's) associated with these species. Resistant, long-lived hypnozygotes deposited in sediments during previous blooms are the source of inoculum of motile vegetative *A. tamarense* cells, in temperate zones during spring/summer blooms (i.e. Anderson and Wall 1978;

Anderson et al. 2005; Anderson et al. 2013; Joyce et al. 2005). In addition the induction of sexual reproduction is also crucial in the decline and, sometimes abrupt, termination of blooms (McGillicuddy et al. 2013).

The induction of sexual reproduction, or gametogenesis, in *A. tamarense* in the laboratory is possible through nutrient limitation of nitrogen (Anderson et al. 1984), phosphorus (Anderson et al. 1984; Anderson and Lindquist 1985) and iron (Doucette et al. 1989). However, sexuality in the field has been shown to occur at nutrient levels above those required for vegetative growth in culture (Anderson et al. 1983) suggesting that other factors may play a role in sexuality in the natural environment. One theory is the regulation of bloom initiation and termination by an endogenous clock (Anderson and Keafer 1987; Perez et al. 1998).

Analysis of the sexual reproduction of *A. tamarense* in the laboratory is often carried out at 20°C (Anderson et al. 1984; Anderson and Lindquist 1985; Brosnahan et al. 2010; Fritz et al. 1989), this being the optimal temperature for maximum *A. tamerense* hypnozygote yield according to Anderson et al. (1984). However, the Group I isolates used in this study are from regions where the water temperature never reaches this level (see chapter 2), and it has been noted through personal observation that Group III isolates tend to have a higher growth rate than Group I isolates at temperatures above 15°C.

Past observations of successful mating compatibility between the *A. tamarense* complex morphospecies, *A. tamarense*, *A. fundyense* and *A. catenella (*Anderson et al. 1994; Mackenzie et al. 2004) suggest that these morphotypes do not represent

valid species. Conversely, mating compatibility between Groups I and III (Brosnahan et al. 2010) did uphold the separation of these groups as biological species. The separation of five ribotype groups in *A. tamarense* is based on LSU rDNA sequence analysis (Scholin et al. 1994; Lilly et al. 2007).

3.1.1 Aims and objectives

The original aim of the study was to assess the mating interactions of Group I and III *A. tamarense* isolates from the same geographic locations, where co-occurrence has been previously identified (i.e. Belfast Lough and Orkney). However, the limitations imposed by the cultures isolated for this study from these locations (see chapter 2), meant it was necessary to revise this aim considerably.

One of the major adaptations was to include Group I and III *A. tamarense* isolates from broader geographic regions where no co-occurrence of groups has been reported. This was the rationale behind utilising previously collected sediments from Weymouth Harbour, an area exclusively Group III, and Bedford Basin, Canada, an area exclusively Group I (as in chapter 2).

The design of the study is similar to that of Brosnahan et al. (2010), which showed that mating between compatible isolates of Group I and III *A.tamarense* resulted in non-viable progeny, all of which failed to survive post meiosis. Using a subset of the cultures isolated from Orkney, Belfast Lough, Weymouth Harbour and Bedford Basin, as described in chapter 2, mating and excystment studies were carried out in order to assess the following:

- The frequency of mating between inbred (same group) and out-bred (different groups), as determined by hypnozygote yield.
- The effect of temperature on hypnozygote yield.
- The germination of inbred and out-bred hypnozygotes.

3.2 Preliminary mating experiments - method selection

Since Anderson et al. (1984) asserted that optimal laboratory conditions for sexual reproduction in *A. tamarense* required the use of acid washed, borosilicate glass culture tubes, the method has been used for experiments assessing sexual reproduction and mating compatibility within the *A. tamarense* species complex (e.g. Anderson & Lindquist, 1985; Fritz et al. 1989; Brosnahan et al. 2010). However the process of washing glassware is labour and time intensive and hundreds of tubes are often necessary, particularly when large numbers of cultures are to be investigated.

Given the potential constraints that this method would pose, particularly in terms of time and resources, to the proposed study and the fact that sexual reproduction in other *Alexandrium species* (e.g. Figueroa and Garces. 2006*;* Figueroa et al. 2008 and 2011*)* and species of other dinofagellate genera (e.g. Blackburn et al. 2001; Figueroa and Bravo 2005) have been assessed using small sterile disposable plastic Petri dishes with no reported difficulty, it was decided to compare the two methods prior to commencing large scale encystment trials.

3.2.1 Cultures

Eight xenic *A. tamarense* cultures were chosen (4 Group I, 4 Group III) from those previously isolated and identified as described in Chapter 2. Two cultures each from

Stromness Pier and Station 7 in Orkney, two from Belfast Lough and two from Weymouth Harbour. Cultures were maintained at 15°C in 25 cm^3 Falcon® tissue culture flasks with 50 mL f/2 modified medium, under cool white fluorescent light on an 14/10h light dark cycle. Stock cultures maintained at 15°C were acclimated to 20°C for several cell generations (~10-14 days) in f/2 modified medium. All cultures were acclimated in 25 cm^3 Falcon® tissue culture flasks with 50 mL medium as for culture maintenance and in 50 mL borosilicate glass culture tubes with 25 mL medium prior to the start of encystment trials.

Table 3.1 *A. tamarense* cultures used for preliminary encystment trials

Origin	Strain	UoW ID	Species/group based on direct D1-D2 LSU rDNA sequences
Stromness Pier, Orkney	SPC6	UoW 716	*A. tamarense* I
58°57.851N3°17.668W	SPD6	UoW 718	*A. tamarense* I
Station 7, Orkney	ST7B5	UoW 721	*A. tamarense* I
58°54.758N 3°17.772W	ST7D2	UoW 723	*A. tamarense* I
Belfast Lough -site A6	BLA6-A6	UoW 708	*A. tamarense* III
54º39.800'N 005º48.80'W	BLA6 -C5	UoW 713	*A. tamarense* III
Weymouth Harbour	WHA1	UoW 700	*A. tamarense* III
	WHC2	UoW 706	*A. tamarense* III

3.2.2 Encystment conditions

The medium, temperature, lighting and culture inoculum were consistent for both methods, with only culture vessel and medium volume being varied. Nitrogen limited f/2 modified medium (f/2-N) was used as the encystment medium. Nitrogen limited f/2 modified medium was prepared using 0.2 μm filtered natural seawater

(salinity ~32), with $NaP0_4$, trace metals and vitamins at modified f/2 concentrations (see Chapter 2.). Nitrogen was limited by replacing $NaNO_3$ with ammonium chloride (NH_4Cl) at 2.5 x10-5 M (see table 3.2). The temperature for encystment was 20°C and is consistent with other studies (e.g. Anderson & Lindquist, 1985; Fritz et al, 1989; Brosnahan et al 2010). Lighting conditions were as for culture maintenance described above.

Co-cultures were crossed in a pairwise fashion at 700 cells/mL (350 cells/mL from each isolate). Self-crosses were set up for all cultures to assess for homothallism (700 cells/mL). All co-culture and self-crosses were performed in duplicate.

Table 3.2 Modified f/2-N medium composition

Component	Stock solution (g/L dH2O)	Quantity used/L	Final Concentration in medium (M)
NH4CL	26.75	50 µL	2.5×10^{-5}
NaH2PO4 H2O	5	1 mL	3.62×10^{-5}
Trace metals solution	-	1 mL	-
Vitamins solution	-	0.5 mL	-

3.2.3 Encystment in borosilicate glass tubes

Borosilicate glass tubes (50 mL, dimensions 25 mm x 125 mm) with caps, were soaked in 5% Decon®90 for 24 hours, rinsed in tap water for 30 minutes and rinsed in deionised H_20 for 30 minutes. The tubes were then soaked in 10% HCl overnight, rinsed three times in deionised H_20 and air-dried. Tubes were then filled with 25 mL deionised H_20, capped and autoclaved at 121°C for 15 min.

The deionised H_20 was aseptically discarded into a beaker and the tubes filled with 25 mL sterile f/2-N medium using a 50 mL Zipette™ (Jencons). Tubes were inoculated with exponentially growing cells (4000-8000 cells/mL) at 700 cells/mL (350 cells/mL from each isolate, 700 cells/mL for self crosses) and then capped. Tubes containing co-cultures and self crosses were incubated at 20°C for 28 days and observed weekly by the removal of ~100 μL of the cell debris at the bottom of tube with a glass Pasteur pipette and placing in a Palmer Maloney counting chamber to check for hypnozygotes.

3.2.4 Encystment in sterile Petri dishes

Sterile 30 mm plastic Petri dishes (Sterilin) were filled with 5 mL sterile f/2-N using a 50 mL Zipette™ (Jencons). Petri dishes were inoculated with exponentially growing cells (4000 – 8000 cells/mL) at 700 cells/mL (350 cells/mL from each isolate, 700 cells/mL for self crosses) and sealed with Parafilm® to reduce evaporation. Petri dishes containing co-cultures and self crosses where incubated at 20°C for 28 days and observed weekly for hypnozygotes using an inverted microscope (Olympus).

3.2.5 Enumerating hypnozygotes

Borosilicate glass culture tube contents were poured into a large sterile Petri dish and examined for hypnozygotes using a stereomicroscope.

Petri dish co-cultures were examined directly using an inverted microscope. Hypnozygotes in positive crosses were loosened from the base of the dish

(hypnozygotes tended to adhere to surface) with a cell scraper and then transferred to sterile 15 mL centrifuge tubes. The tubes were briefly sonicated (~2 minutes) in a sonication bath to dissociated large hypnozygote aggregates and the contents then returned to the original Petri dish. All hypnozygotes in each positive cross (in duplicate) were counted and recorded.

A scoring system for hypnozygote yield similar to that of Blackburn et al. (2001) was devised. Yield is scored from 0-3 based on hypnozygotes/mL as determined by calculating the average number of hypnozygotes and standard deviation values for each co-culture or self cross (see table 3.3).

Table 3.3 Hypnozygote yield scoring system for *A.tamarense* encystment cultures

Score	Hypnozygotes/mL
0	0
1	>0 - 10
2	>10 - 100
3	>100 - 1000

3.2.6 Results/Discussion

No hypnozygotes were observed in any of the borosilicate glass tubes. Due to the inability to directly observe the cultures during incubation it is unknown whether sexual reproduction occurred to any extent in any of the tubes, i.e. if there was gamete fusion or planozygote formation and there was a failure of these to encyst.

However no such cells were observed via the removal of sub-samples or upon transfer of the tube contents to large sterile Petri dishes. Further to the issue of observation, the method suffers from the need to interfere directly with the cultures during the incubation period by removing a sample from the bottom of each tube weekly. This has the dual disadvantage of possibly introducing sources of contamination, which may adversely affect encystment, and of maybe removing fusing gametes/planozygotes and reducing hypnozygote yield. Although the latter point was not true in this instance, as no such cells were observed.

In contrast the sterile Petri dish method resulted in hypnozygotes being produced in 7 of the 36 possible crosses (see table 3.4). These comprised both Group III/III and Group I/III crosses and one Group III self-cross. Hypnozygote yields were generally >100 hypnozygotes/mL, with the exception of Group I/III cross ST7D2 x WHC2. These yields would be sufficient for germination studies. Furthermore, the method allowed for the observation of cell interactions directly without adversely affecting results and did not require large volumes of medium or laborious preparation of culture vessels.

Table 3.4 *A. tamarense* hynozygote yield scores for sterile Petri dish co-cultures.

		Group I				**Group III**			
		SP C6	SP D6	ST7 B5	ST7 D2	BLA6 A6	BLA6 C5	WH A1	WH C2
Group I	SP C6	**0**							
	SP D6	**0**	**0**						
	ST7 B5	**0**	**0**	**0**					
	ST7 D2	**0**	**0**	**0**	**0**				
Group III	BLA6 A6	**0**	**0**	**0**	**0**	**0**			
	BLA6 C5	**0**	**0**	**0**	**0**	**0**	**0**		
	WH A1	/	/	/	/	/	/	**3**	
	WH C2	**2**	**2-3**	**0-1**	**0**	**3**	**3**	/	**0**

3.3 Encystment of *A. tamarense* groups I and III at 20°C and 15°C

Pairwise crosses of all cultures detailed in this section were first performed at 20°C, in line with the above preliminary encystment trials and with previously stated research findings that indicated this was the optimal temperature for maximising *A. tamarense* hypnozygote yield. All pairwise crosses were then repeated at 15°C to assess what effect, if any, decreased temperature had on mating compatibility and/or hypnozygote yield.

3.3.1 Methods

3.3.1.1 Cultures

A total of 20 xenic *A. tamarense* complex cultures (see table 3.5) were selected for pairwise mating experiments from those listed in Chapter 2. Cultures were chosen to include, as far as possible, a broad geographical distribution and even number of Group I and III strains. Cultures were maintained at 15°C in 25 cm^3 Falcon® tissue culture flasks with 50 mL f/2 modified medium, under cool white fluorescent light on an 14/10h light dark cycle.

Table 3.5 *A tamarense* complex cultures used in pairwise mating cross experiments at 20°C and 15°C

Origin	Strain	UoW ID	Species/group based on direct D1-D2 LSU rDNA sequences
Weymouth Harbour	WHA1	UoW 700	*A. tamarense* III
	WHA5	UoW 702	*A. tamarense* III
	WHC2	UoW 706	*A. tamarense* III
	WHD1	UoW 724	*A. tamarense* III
Belfast Lough -site A6	BLA6 A6	UoW 708	*A. tamarense* III
54°39.800'N 005°48.80'W	BLA6 C4	UoW 712	*A. tamarense* III
	BLA6 C5	UoW 713	*A. tamarense* III
	BLA6 A5	UoW 727	*A. tamarense* III
	BLA6 D5	UoW 714	*A. tamarense* III
	BLA6 A4	UoW 729	*A. tamarense* III
Stromness Pier, Orkney	SPB5	UoW 715	*A. tamarense* I
58°57.851N3°17.668W	SPC6	UoW 716	*A. tamarense* I
	SPD4	UoW 717	*A. tamarense* I
	SPD6	UoW 718	*A. tamarense* I
	SPD2	UoW 731	*A. tamarense* I
Station 7, Orkney	ST7A3	UoW 719	*A. tamarense* I
58°54.758N 3°17.772W	ST7B5	UoW 721	*A. tamarense* I
	ST7D2	UoW 723	*A. tamarense* I
Bedford Basin, Canada	BBD4	UoW 732	*A. tamarense* I
	BBB1	UoW 733	*A. tamarense* I

3.3.1.2 Morphology

The selected cultures were morphologically identified according to criteria in Balech (1995) and as described and illustrated in chapter 1. The ability to form chains (indicative of *A. catenella*) was determined as the observation of chains of more than four cells in any given culture. This criterion was used as through personal

observation of cultures it has been noted that couplets of *A.tamarense* are frequent in exponentially growing cultures and, very occasionally, chains of four cells have also been observed

Isolates were analysed using traditional light microscopy and Trypan blue stain (0.4% w/v) (Sigma). Approximately 1.5 mL of exponentially growing culture (4000 - 8000 cells/mL) was transferred to 2 mL microcentrifuge tube and centrifuged for 2 min at 10,000 RPM. Approximately 1 mL of supernatant was aspirated away using a Pasteur pipette and the cell pellet re-suspended. One or two drops of Trypan blue stain was added and then one drop of the stained cells placed on a clean microscope slide and covered with a glass coverslip. Slides were examined using a compound microscope (Olympus). Cells/thecae were manipulated/rotated using a mounted needle. As slides dried out a little, cells could be gently squashed to aid shedding of theca. Images were recorded, where possible, using a 1.3mp digital camera (Brunel Microscopes). Cell size (width and length) of all isolates (n=10) was measured using an eyepiece reticule calibrated with a 1 mm stage micrometer (each division = 0.01 mm/10 μm). Samples for cell sizing were preserved with acidic Lugol's iodine, as previously (see chapter 2).

3.3.1.3 Encystment conditions

All possible co-culture pairwise mating crosses of the *A. tamarense* strains listed in table 3.4 were carried out in duplicate at 20°C and 15°C in sterile 30 mm Petri dishes (Sterilin). Duplicate self-crosses for each strain were also performed at 20°C and

15°C. The encystment method, medium and conditions were as described previously for Petri dishes.

3.3.1.4 Enumerating hynozygotes

Co-cultures and self-crosses were examined directly using an inverted microscope (Olympus). Hypnozygotes in positive crosses were loosened from the base of the dish (hypnozygotes tended to adhere to the surface) with a cell scraper. Unlike previously the hypnozygotes were not sonicated, due the uncertain affects of sonication on cyst physiology (Anderson 2003). Instead aggregates of hypnozygotes were separated as much as possible with the aid of a sterile pipette tip.

All hypnozygotes in each positive cross (in duplicate) were counted and recorded. Hypnozygote yield scores were determined for all positive crosses as described previously (see table 3.2).

Compatibility index (CI), average vigour (AV) and reproductive compatibility (RC) were calculated for all isolates at 20°C and 15°C, with the exclusion of those that were homothallic, and for inter-population and between population crosses (i.e. Belfast x Belfast, Orkney x Belfast etc.), as described in Blackburn et al. (2001). Calculations were as follows:

- CI was determined as the number of positive crosses resulting in a hynozygote yield score ≥1, divided by the total number of possible crosses (excluding self-crosses) for each strain. Where scores fell between categories,

e.g 1-2, 2-3, the lower figure was used, in this instance scores of 0-1 were excluded from CI calculations.

- AV was determined as the average of all the hypnozygote yield scores (0-3) for each strain. As for CI the lower value was used where scores fell between categories.

- RC was determined as the product of the CI and AV values for each strain.

Two tailed, paired, Student t-tests were run separately in Excel® 2011 (Microsoft Corporation, USA) for CI, AV and RC values for all Group I and III isolates at 20°C and 15°C, to assess the affect of temperature variation on mating compatibility and hypnozygote yield for each group. Tests were also run using average hypnozygote yields for combined groups, Group I and Group III at 20°C and 15°C. Significant difference was determined as a p-value of ≤ 0.05. Any p-value >0.05 was determined as showing no significant difference.

3.3.1.5 Hypnozygote storage

Hypnozygotes were transferred by pipette to sterile 1.2 mL cryovials (Nalgene). Cryovials were submerged in natural anoxic marine sediment (from Weymouth harbour) in small glass jars. The jars were sealed with Parafilm® and stored in the dark at 4°C for between 8-12 months.

3.3.1.6 Hypnozygote germination and evaluation of progeny viability

Germination of hypnozygotes for all positive crosses at 20°C and 15°C were attempted in f/2 modified medium (prepared as previously described in chapter 2). In addition germination of hypnozygotes for all positive crosses at 20°C were also attempted in f/20 modified medium prepared as previously for modified f/2 but with $1/10^{th}$ the final concentration of $NaNO_3$, $NaP0_4$, trace metals and vitamins.

Cryovials containing hypnozygotes were removed from anoxic sediment and washed thoroughly with dH_20 prior to opening. Hypnozygotes were transferred to a watch glass and observed using an inverted ITS microscope (Olympus). Individual hypnozygotes were isolated by micropipette (see chapter 2 for micropipette set-up) to wells of a 96 well plates containing 200 μL of f/2 or f/20 modified medium. Occasionally two or more hypnozygotes were transferred to a single well due to adhesion amongst them. Between 3-30 hypnozygotes were isolated from each cross, for each medium. Plates were sealed with Parafilm® to reduce evaporation and incubated at 15°C under cool white fluorescent light on a 14/10h light dark cycle for 28 days.

All hypnozygotes were observed weekly and the number of cell divisions carefully monitored. Viable progeny were those determined to have completed at least three divisions post excystment. This observation was particularly important in Group I/III hybrid hypnozygotes, as these were expected not to survive past two divisions.

Single cells germinated from single hypnozygotes that produced viable progeny were isolated, as described in chapter 2, to generate progeny clonal cultures.

3.4 Results

3.4.1 Morphology

All but two isolates were identified as *A. tamarense* morphotype. The main determining factor was the presence of a ventral pore on the 1' plate in all these cultures. The two remaining isolates, both from Orkney, were identified as *A. fundyense* morphotype due to the absence of a ventral pore on the 1', or between the 1' and 4' plates, and their inability to form chains (see fig. 3.1 for example images of theca).

Cell size was a variable characteristic for all isolates, with all displaying a wide range of cell sizes in culture. Very small (i.e. 25x25 μm) and very large cells (i.e. 40x50 μm) were observed in a number of isolates, particularly Group I. In some isolates the cells were nearly always spherical, for example WHA1. However, most displayed a range of spherical, and slightly longer than wide cells. No cell chains longer than four cells were ever observed in any of the cultures. (See table 3.6 for full morphological detail).

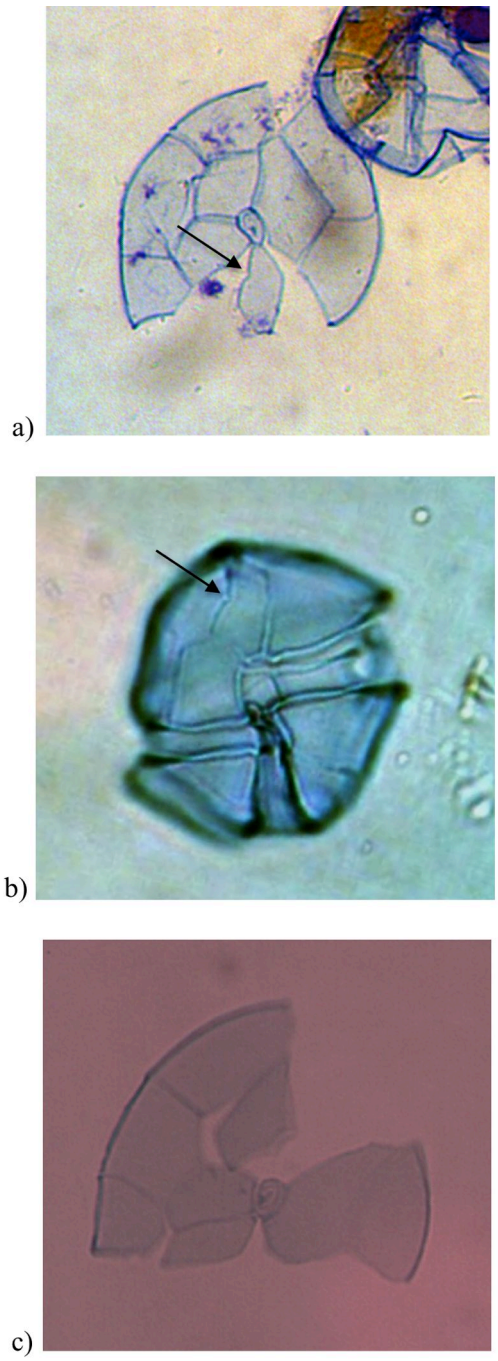


Fig. 3.1 Trypan blue stained thecae; a) epitheca of Group I *A.tamarense* BBD4 at x1000 magnification b) complete theca of Group III *A.tamarense* WHD1 at 400x magnification, and c) epitheca of Group I *A. fundyense* SPC6. Arrows indicate apical pore on 1st apical plate (1').

Table 3.6 Cell size and morphospecies designation of isolates. w= width, l=length

Location	Strain	Cell size (µm)	Ventral pore	Chain former	Morphospecies
Weymouth Harbour	WHA1	30-40w, 30-40l	Yes	No	*A. tamarense*
	WHA5	30-40w, 35-50l	Yes	No	*A. tamarense*
	WHC2	25-40w, 30-50l	Yes	No	*A. tamarense*
	WHD1	30-40w, 30-40l	Yes	No	*A. tamarense*
Belfast Lough -site A6	BLA6 A6	30-35w, 35-40l	Yes	No	*A. tamarense*
54º39.800'N 005º48.80'W	BLA6 C4	25-40w, 30-40l	Yes	No	*A. tamarense*
	BLA6 C5	30-40w, 30-40l	Yes	No	*A. tamarense*
	BLA6 A5	25-40w, 30-50l	Yes	No	*A. tamarense*
	BLA6 D5	30-35w, 30-40l	Yes	No	*A. tamarense*
	BLA6 A4	30-35w, 30-40l	Yes	No	*A. tamarense*
Stromness Pier, Orkney	SPB5	25-40w, 25-50l	Yes	No	*A. tamarense*
58°57.851N3°17.668W	SPC6	35-40w, 40-50l	No	No	*A. fundyense*
	SPD4	30-35w, 30-40l	Yes	No	*A. tamarense*
	SPD6	25-40w, 30-50l	Yes	No	*A. tamarense*
	SPD2	20-40w, 30-50l	Yes	No	*A. tamarense*
Station 7, Orkney	ST7A3	25-40w, 30-50l	Yes	No	*A. tamarense*
58°54.758N 3°17.772W	ST7B5	30-35w, 30-40l	No	No	*A. fundyense*
	ST7D2	35-40w, 40-50l	Yes	No	*A. tamarense*
Bedford Basin, Canada	BBD4	25-50w, 30-50l	Yes	No	*A. tamarense*
	BBB1	23-40w, 30-50l	Yes	No	*A. tamarense*

3.4.2 Encystment of *A.tamarense* complex cultures

3.4.2.1 General observations

Morphological differences between gametes and vegetative cells were not obvious, and as a consequence it was virtually impossible to differentiate between non-fusing gametes and vegetative cells. Gametes that were observed fusing were isogamous and generally ~30 x 30 µm. Many very small (~ 20 x20 µm), less pigmented, cells were often observed in crosses, however these were never observed fusing and large aggregations of dead small cells were often observed alongside hypnozygote

aggregations. The behaviour of gametes was very different in comparison to vegetative cells. Gametes swam in an erratic motion, frequently changing direction, frantically bumping and circling one another in what has been termed a 'mating dance'. In addition, gametes would swarm at the edge of the Petri dish. Early fusing gametes could be easily distinguished from dividing cells due to their fusing epitheca to epitheca, resulting in their flagella facing opposite directions. In contrast vegetative cells divide by diagonal cleavage of the original cell, resulting in their flagella being parallel (see fig. 3.2).

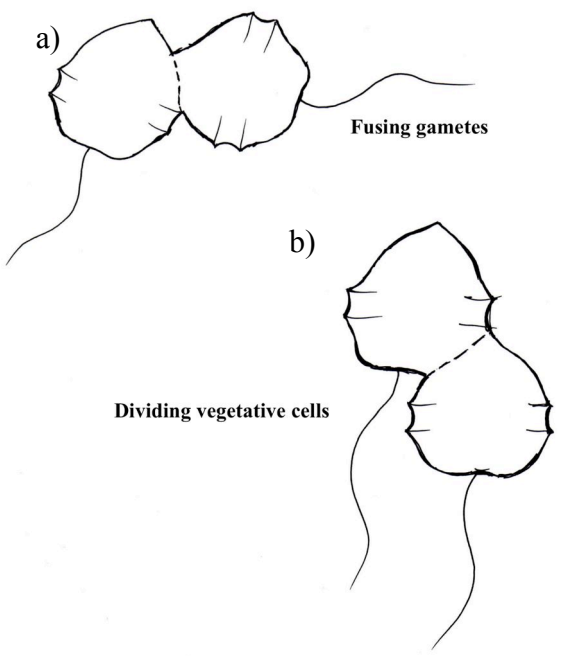


Fig. 3.2 Difference between fusing gametes and dividing vegetative cells: a) gametes fusing epitheca to epitheca, longitudinal flagella in opposing directions, b) dividing cells with parallel longitudinal flagella (adapted and redrawn from Persson et al. 2013).

Planozygotes were distinguished from vegetative cells and gametes due to their larger size, particularly in relation to width-length ratio (~50 μm wide, 60-65 μm long), slightly elongated epitheca, much darker pigment and biflagellate longitudinal flagella. As for gametes, planozygotes accumulated at the edge of the Petri dish. They often swam slower than other cells, until becoming stationary and shedding their theca. Planozygotes in all crosses persisted for more than a week before encysting, with the exception of homathallic cross WHA1 where planozygotes encysted in less than 7 days.

Hypnozygotes were found in large aggregations at the edge of the Petri dish (see fig. 3.3), except in crosses where yield was very low (<100). Sizes ranged from ~30-40 μm wide and 50-60 μm long. A halo of mucilage could often be observed around individual hynozygotes, as well as large numbers of empty planozygote thecae (see fig. 3.4).

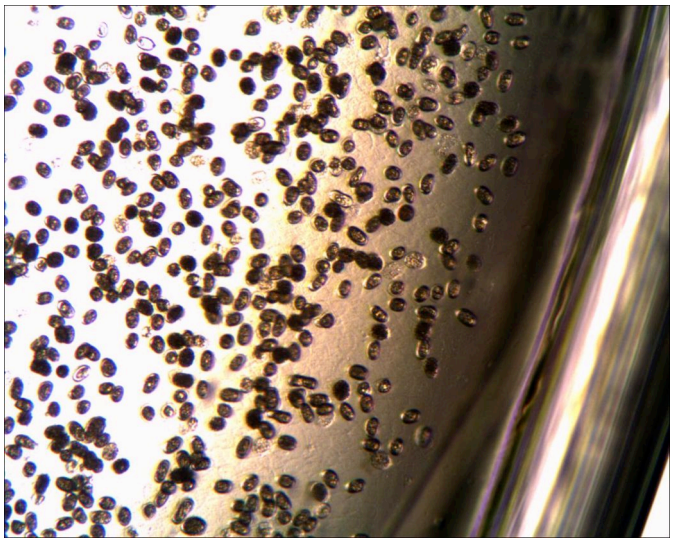
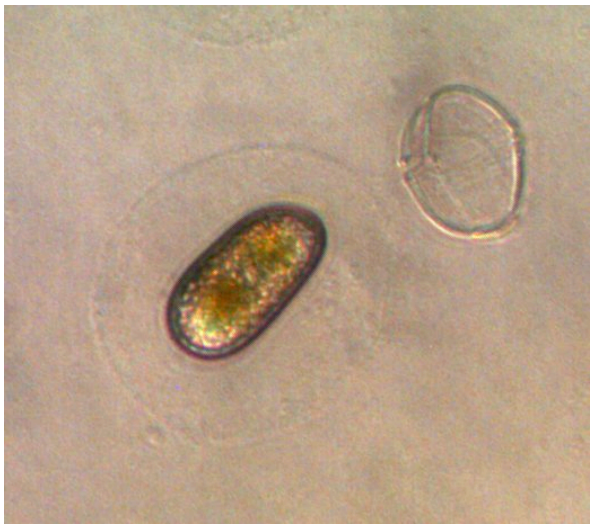


Fig. 3.3 Aggregation of hypnozygotes at the edge of Petri dish

a)



b)

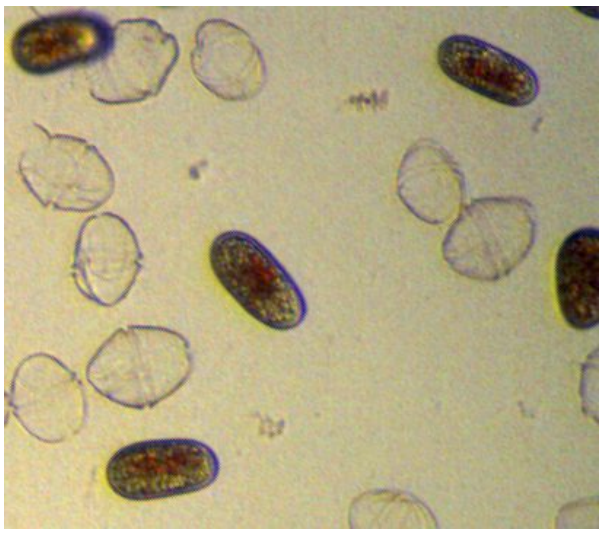


Fig. 3.4 Hypnozygotes: a) a single hypnozygote with halo of mucilage and empty planozygote theca, b) hypnozygotes and multiple empty thecae

3.4.2.2 Hypnozygote yields at 20°C and 15°C

A total of 153 co-culture crosses (excluding self-crosses) were set up in duplicate for encystment at 20°C and 15°C. Resulting in a total of 29 crosses that produced hypnozygotes at 20°C, 4 Group I/I, 12 Group III/III, 12 Group I/III and 1 Group III self-cross (strain WH A1). At 15°C there were 31 crosses that produced hypnozygotes, 7 group I/I, the same 4 as at 20°C plus 3 additional crosses, the same 12 Group I/III and the same 12 Group I/III crosses as at 20°C. Group III self-cross

WHA1 did not produce hypnozygotes at 15°C. Total hypnozygote yield varied considerably between duplicates as is evident from large standard deviation values for most crosses (see fig. 3.5). Hypnozygote yield scores (0-3) were significantly lower for all combined Group crosses at 15°C compared to 20°C ($p<0.05$). However, individually only Group III/III crosses were significantly affected by a reduction in temperature ($p<0.05$), with Group I/I and Group I/III crosses showing no significant difference in hypnozygote yield ($p>0.05$) (see table 3.7).

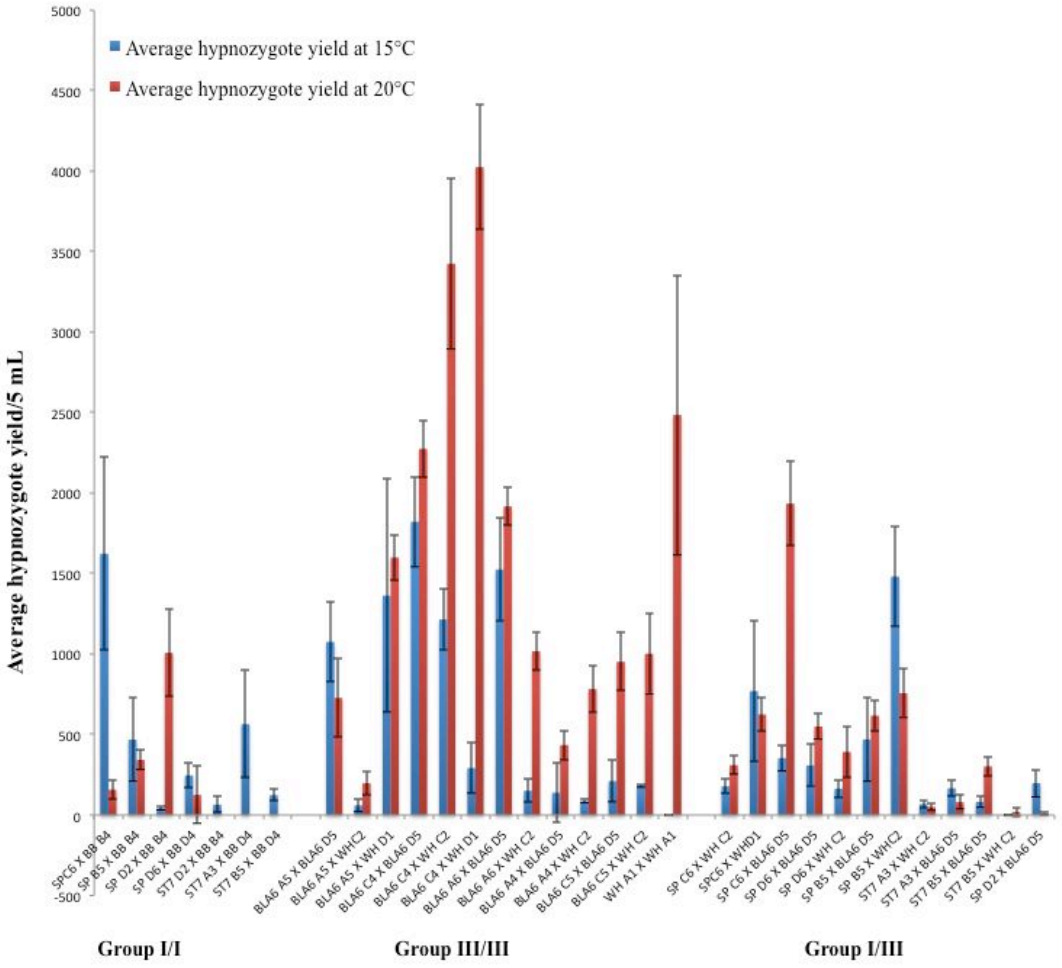


Fig. 3.5 Average hypnozygote yield for positive *A. tamarense* co-culture and self-crosses at 15°C and 20°C. Group labels indicate positive crosses for in-group (Group I x Group I = I/I, Group III x Group III = III/III) or out-group (Group I x Group III = I/III). Error bars represent ± standard deviation.

Table 3.7 Student t-test results for hypnozygote yields at 15°C and 20°C. Significant results are shown in bold.

	Difference in average hypnozygote yield at 15°C and 20°C			
	All groups	**Group I/I**	**Group III/III**	**Group I/III**
p-value	**0.02827**	0.46172	**0.00937**	0.4163

Mating grids of all crosses and hypnozygote yield scores at 20°C and 15°C could not be explained with a simple heterothallic pattern of mating compatibility. Two isolates, Group I BBB1 and Group III WHA5, failed to produce hypnozygotes in any of the possible co-culture crosses. Furthermore, there were no crosses between Group I BBD4, from Bedford Basin, and any of the Group III isolates from Belfast Lough or Weymouth Harbour. There were however hypnozygotes produced in crosses between isolates from Bedford basin/Orkney, Orkney/Weymouth Harbour, Orkney/Belfast Lough, Belfast Lough/Weymouth Harbour and Belfast Lough/Belfast Lough (see tables 3.8 and 3.9).

Table 3.8 Hypnozygote yield scores (0-3) for all possible co-culture and self-crosses at 20°C. Homothallic strain WHA1 not crossed with other strains is indicated by /.

		Group I									Group III									
		SP C6	SP D4	SP D6	SP B5	ST7 A3	ST7 B5	ST7 D2	SP D2	BB B1	BB D4	BLA6 A6	BLA6 A4	BLA6 C5	BLA6 D5	BLA6 A5	BLA6 C4	WHA1	WH A5	WH D1	WH C2
Group I	SP C6	0																			
	SP D4	0	0																		
	SP D6	0	0	0																	
	SP B5	0	0	0	0																
	ST7 A3	0	0	0	0	0															
	ST7 B5	0	0	0	0	0	0														
	ST7 D2	0	0	0	0	0	0	0													
	SP D2	0	0	0	0	0	0	0	0												
	BB B1	0	0	0	0	0	0	0	0	0											
	BB D4	2	0	0	2	0	0	0-2	3	0	0										
Group III	BLA6 A6	0	0	0	0	0	0	0	0	0	0	0									
	BLA6 A4	0	0	0	0	0	0	0	0	0	0	0	0								
	BLA6 C5	0	0	0	0	0	0	0	0	0	0	0	0	0							
	BLA6 D5	3	0	2-3	3	1-2	2	0	0-1	0	0	3	2-3	3	0						
	BLA6 A5	0	0	0	0	0	0	0	0	0	0	0	0	0	2-3	0					
	BLA6 C4	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0				
	WHA1	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	3			
	WH A5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	/	0		
	WH D1	3	0	0	0	0	0	0	0	0	0	0	0	0	0	3	3	/	0	0	
	WH C2	2	0	2-3	3	1	0-1	0	0	0	0	3	3	3	0	2	3	/	0	0	0

Table 3.9 Hypnozygote yield scores (0-3) for all possible co-culture and self-crosses at 15°C. Homothallic strain WH A1 not crossed with other strains is indicated by /.

		Group I										Group III									
		SP C6	SP D4	SP D6	SP B5	ST7 A3	ST7 B5	ST7 D2	SP D2	BB B1	BB D4	BLA6 A6	BLA6 A4	BLA6 C5	BLA6 D5	BLA6 A5	BLA6 C4	WHA1	WH A5	WH D1	WH C2
Group I	SP C6	0																			
	SP D4	0	0																		
	SP D6	0	0	0																	
	SP B5	0	0	0	0																
	ST7 A3	0	0	0	0	0															
	ST7 B5	0	0	0	0	0	0														
	ST7 D2	0	0	0	0	0	0	0													
	SP D2	0	0	0	0	0	0	0	0												
	BB B1	0	0	0	0	0	0	0	0	0											
	BB D4	3	0	2	2-3	2-3	2	1-2	1-2	0	0										
Group III	BLA6 A6	0	0	0	0	0	0	0	0	0	0	0									
	BLA6 A4	0	0	0	0	0	0	0	0	0	0	0	0								
	BLA6 C5	0	0	0	0	0	0	0	0	0	0	0	0	0							
	BLA6 D5	2	0	2	2-3	2	2	0	2	0	0	3	0-2	2	0						
	BLA6 A5	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0					
	BLA6 C4	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0	0				
	WHA1	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	/	0			
	WH A5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0		
	WH D1	2-3	0	0	0	0	0	0	0	0	0	0	0	0	0	3	2	/	0	0	
	WH C2	2	0	2	3	1-2	1	0	0	0	0	2	2	2	0	1-2	3	/	0	0	0

3.4.2.3 Compatibility index, average vigour and reproductive compatibility at 20°C and 15°C

Compatibility index (CI), average vigour (AV) and reproductive compatibility (RC) values showed some significant differences between Group I and III isolates at 20°C and 15°C. The CI value for Group I increased significantly at 15°C ($p<0.05$). However, AV and RC values for Group I isolates were not significantly affected by variation in temperature ($p>0.05$). In contrast CI values for Group III isolates were not significantly affected by variation in temperature ($p>0.05$), yet AV and RC values for Group III were significantly lower at 15°C ($p<0.05$) (see tables 3.10 and 3.11).

The results of inter-population and intra-population CI, AV and RC analysis (table 3.12) showed that, with the exception of Bedford Basin x Orkney which increased (0.25 - 0.40), CI values were not adversely affected by a reduction in temperature. Bedford Basin x Orkney AV was unchanged at 20°C and 15°C (1.6) and was the lowest of all population crosses. Bedford Basin x Orkney showed the greatest increase in RC in response to decreased in temperature (0.40 – 0.70). Belfast Lough x Weymouth Harbour had the highest AV (2.86 at 20°C) but also the greatest reduction in AV at 15°C (2.14). Belfast Lough x Belfast Lough showed a modest decrease in AV and RC. Similarly only modest decrease or increase in AV and RC were seen in Group I/III intra-population crosses Orkney x Weymouth Harbour and Orkney x Belfast Lough.

Table 3.10 Compatibility index (CI), average vigour (AV) and reproductive compatibility (RC) for Group III strains at 15°C and 20°C. Two tailed paired t-test results, significant p-values (p=≤0.05) are shown in bold.

	Strain	CI	AV	RC
15°C	BLA6 A6	0.11	2.50	0.27
	BLA6 A4	0.11	1.50	0.16
	BLA6 C5	0.11	2.00	0.22
	BLA6 D5	0.50	2.00	1.00
	BLA6 A5	0.11	3.00	0.33
	BLA6 C4	0.11	2.50	0.27
	WH A5	0	0	0
	WH D1	0.16	2.33	0.26
	WH C2	0.55	1.90	1.05
20°C	BLA6 A6	0.11	3.00	0.33
	BLA6 A4	0.11	2.50	0.27
	BLA6 C5	0.11	2.5	0.27
	BLA6 D5	0.44	2.37	1.04
	BLA6 A5	0.11	2.50	0.27
	BLA6 C4	0.11	3.00	0.33
	WH A5	0	0	0
	WH D1	0.16	3.00	0.48
	WH C2	0.50	2.44	1.22
p-values		0.17102	**0.02321**	**0.03371**

Table 3.11 Compatibility index (CI), average vigour (AV) and reproductive compatibility (RC) for Group I strains at 15°C and 20°C. Two tailed paired t-test results, significant p-values (p=≤0.05) are shown in bold.

	Strain	CI	AV	RC
15°C	SP C6	0.22	2.25	0.49
	SP D4	0	0	0
	SP D6	0.16	2.00	0.32
	SP B5	0.16	2.30	0.37
	ST7 A3	0.16	1.66	0.26
	ST7 B5	0.16	1.66	0.26
	ST7 D2	0.05	1.00	0.05
	SP D2	0.11	1.50	0.16
	BB B1	0	0	0
	BB D4	0.38	1.85	0.70
20°C	SP C6	0.22	2.00	0.44
	SP D4	0	0	0
	SP D6	0.11	2.00	0.22
	SP B5	0.16	2.66	0.42
	ST7 A3	0.11	1.00	0.11
	ST7 B5	0.05	2.00	0.10
	ST7 D2	0.05	1.00	0.05
	SPD2	0.05	3.00	0.15
	BB B1	0	0	0
	BB D4	0.22	2.00	0.44
p-values		**0.03699**	0.43601	0.05370

Table 3.12 Compatibility index (CI), average vigour (AV) and reproductive compatibility (RC) for inter-population and intra-population crosses at 15°C and 20°C.

	Temperature	CI	AV	RC
Orkney x Bedford Basin	15°C	0.44	1.6	0.70
	20°C	0.25	1.6	0.40
Orkney x Weymouth Harbour	15°C	0.20	2.0	0.40
	20°C	0.20	2.2	0.44
Orkney x Belfast Lough	15°C	0.10	2.5	0.25
	20°C	0.12	2	0.24
Belfast Lough x Weymouth Harbour	15°C	0.38	2.14	0.81
	20°C	0.38	2.86	1.09
Belfast lough x Belfast Lough	15°C	0.23	2.4	0.53
	20°C	0.23	2.6	0.60

3.4.3 Germination of hypnozygotes

3.4.3.1 Germination of hypnozygotes produced at 20°C

Upon removal from storage there was evidence of degradation of Group I/I, and some Group I/III, hynozygotes produced at 20°C. As a consequence it was only possible to isolate low numbers of potentially viable hypnozygotes for some of these crosses. Group III/III hypnozygotes were not affected to the same extent of degradation, despite being stored under the same conditions.

Excystment of hypnozygotes in f/20 modified proved negative, with only three Group III/III hypnozygotes excysting, none of which survived beyond the planomeiocyte stage. Excystment in f/2 modified medium was more successful with

hypnozygotes from all groups excysting, including hybrid Group I/III. Germinated Group I/I and III/III hypnozygotes proved viable with most excysted planomeiocytes completing at least three divisions. Germinated hypnozygotes from Group I/III hybrids did not produce viable progeny, with all failing to complete more than two divisions post excystment. The formation of the planomeiocyte inside the walls of the hypnozygote was often observed prior to excystment. Post excystment it was often possible to obverse the empty hypnozygote shells (see fig. 3.6).

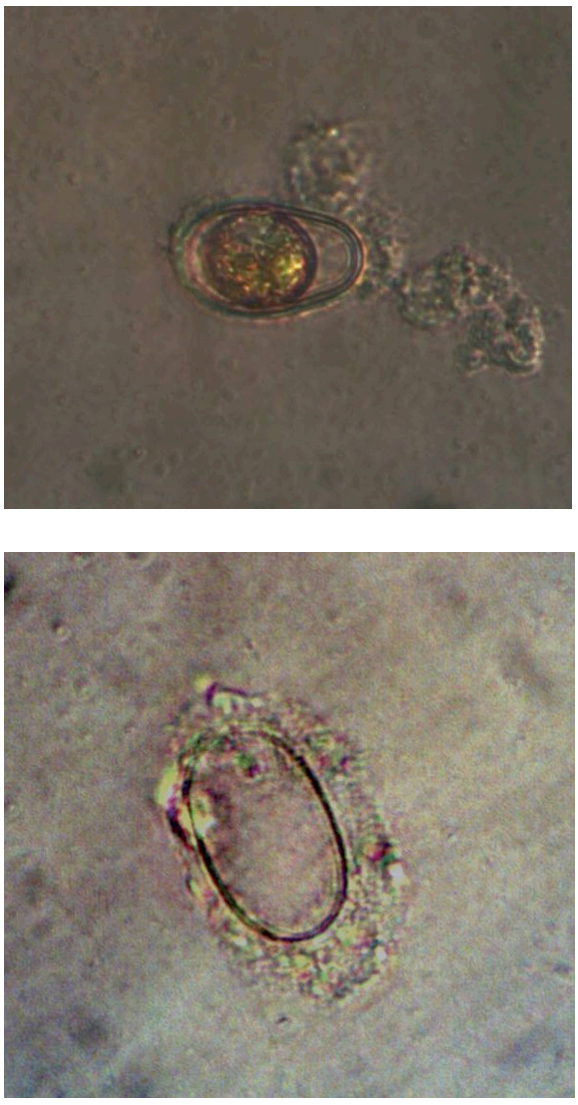


Fig. 3.6 a) Transformation of protoplast inside hynozygote prior to excystment. b) Empty hypnozygote shell.

Hypnozygotes produced in Group III/III crosses had the highest excystment success rates, with 11 out of 13 crosses having at least one successful excystment. Hypnozygotes from Group III self-cross WHA1 failed to excyst despite their normal appearance (see fig. 3.7). Hypnozygotes of only one of four Group I/I crosses successfully excysted, and only three out of ten for Group I/III crosess. Excystment rates for all groups ranged from 5-55% (table 3.13).

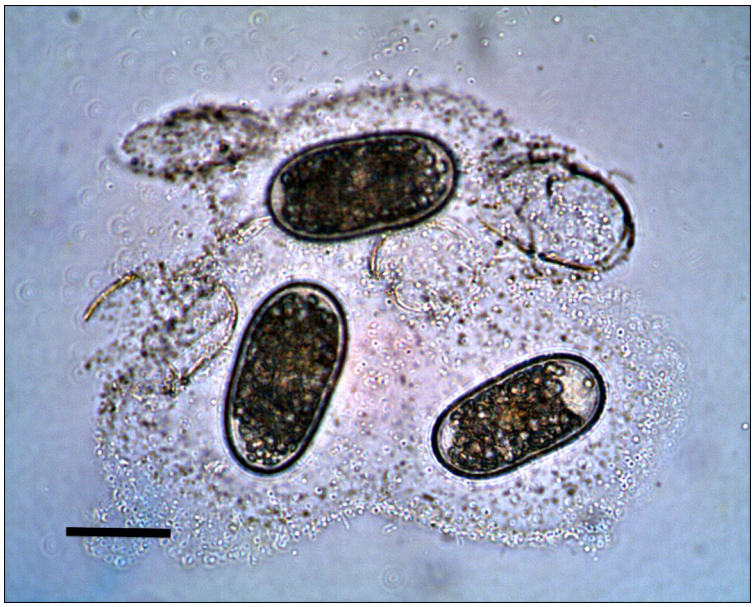


Fig 3.7 Hypnozygotes produced in WHA1 self-cross. Scale bare = 30 μm

Table 3.13 Germination of hypnozygotes produced at 20°C in f/2 modified medium.

	Co-culture	No. of hypnozygotes isolated	No. of excystments	%
Group I	SPC6 X BB D4	10	0	0
	SP B5 X BB D4	3	1	33
	SP D2 X BB D4	3	0	0
	ST7 D2 X BD D4	3	0	0
Group III	BLA6 A5 X BLA6 D5	20	3	15
	BLA6 A5 X WHC2	10	0	0
	BLA6 A5 X WH D1	20	1	5
	BLA6 C4 X BLA6 D5	30	10	33
	BLA6 C4 X WH C2	20	1	5
	BLA6 C4 X WH D1	30	14	46
	BLA6 A6 X BLA6 D5	12	1	8
	BLA6 A6 X WH C2	14	3	21
	BLA6 A4 X BLA6 D5	8	3	37.5
	BLA6 A4 X WH C2	20	11	55
	BLA6 C5 X BLA6 D5	20	1	5
	BLA6 C5 X WH C2	20	4	20
	WH A1 X WH A1	15	0	0
Group I/III	SP C6 X WH C2*	13	5	25
	SPC6 X WHD1*	20	2	10
	SP C6 X BLA6 D5	20	0	0
	SP D6 X BLA6 D5*	20	1	5
	SP D6 X WH C2	10	0	0
	SP B5 X BLA6 D5	4	0	0
	SP B5 X WHC2	10	0	0
	ST7 A3 X WH C2	3	0	0
	ST7 A3 X BLA6 D5	2	0	0
	ST7 B5 X BLA6 D5	3	0	0

Non-viable offspring (failed to complete 3 cell divisions post excystment) are denoted by*

3.4.3.2 Germination of hypnozygotes produced at 15°C

The results of the germination of hypnozygotes produced at 15°C were in complete contrast to those for hypnozygotes produced at 20°C. Firstly, there was little evidence of the degradation of hypnozygotes in storage. Secondly, there was a marked decrease in the number of hypnozygotes isolated from Group III/III crosses

that successfully excysted. In contrast, the number of Group I/I and Group I/III crosses that had at least one successful excystment increased to four out of seven for Group I/I and seven out of ten for Group I/III. All Group I/III were, however, non-viable, in agreement with the results for hypnozygotes produced at 20°C. In addition exycstment rates were higher ranging from 12.5-62.5% (table 3.14).

Table 3.14 Germination of hypnozygotes produced at 15°C in f/2 modified medium

	Co-culture	No. of hypnozygotes isolated	No. of excystments	%
Group I	SPC6 X BB D4	8	5	62.5
	SP B5 X BB D4	8	2	25
	SP D2 X BB D4	8	2	25
	SP D6 X BBD4	8	1	12.5
	ST7A3 X BBD4	8	0	0
	ST7B5 X BBD4	8	0	0
	ST7 D2 X BD D4	8	0	0
Group III	BLA6 A5 X BLA6 D5	8	2	25
	BLA6 A5 X WHC2	5	0	0
	BLA6 A5 X WH D1	8	0	0
	BLA6 C4 X BLA6 D5	8	0	0
	BLA6 C4 X WH C2	8	0	0
	BLA6 C4 X WH D1	8	2	25
	BLA6 A6 X BLA6 D5	5	0	0
	BLA6 A6 X WH C2	8	2	25
	BLA6 A4 X BLA6 D5	8	0	0
	BLA6 A4 X WH C2	8	0	0
	BLA6 C5 X BLA6 D5	8	3	37.5
	BLA6 C5 X WH C2	8	2	25
Group I/III	SP C6 X WH C2	8	0	0
	SPC6 X WHD1*	8	5	62.5
	SP C6 X BLA6 D5*	8	1	12.5
	SP D6 X BLA6 D5*	8	2	25
	SP D6 X WH C2*	8	1	12.5
	SP D2 X BLA6 D5*	8	2	25
	SP B5 X WHC2	8	0	0
	ST7 A3 X WH C2*	8	1	12.5
	ST7 A3 X BLA6 D5*	8	1	12.5
	ST7 B5 X BLA6 D5	8	0	0

Non-viable offspring (failed to complete 3 cell divisions post excystment) are denoted by*

3.4.3.3 Progeny cultures

A total of 15 clonal progeny cultures were generated from hypnozygotes from 10 different co-culture crosses. Two Group I/I progeny sibling strains and two Group III/III sibling strains (generated from the same hypnozygote) and 11 Group III/III progeny strains (table 3.15).

Table 3.15 Progeny cultures generated from the germination of co-culture hypnozygotes produced at 20°C

	Co-culture	Strain	UoW ID
Group I	SP B5 X BD B4	SP B5 X BB D4B1-A2*	UoW 744
		SP B5 X BB D4B1-B2*	UoW 745
Group III	BLA6 A5 X BLA6 D5	BLA6 A5 X BLA6 D5 - D7	UoW 746
	BLA6 A5 X WH D1	BLA6 A5 X WH D1 - D2	UoW 747
	BLA6 C4 X BLA6 D5	BLA6 C4 X BLA6 D5 -B7	UoW 748
	BLA6 C4 X WH C2	BLA6 C4 X WH C2- C2	UoW 749
		BLA6 C4 X WH C2-F2	UoW 750
	BLA6 C4 X WH D1	BLA6 C4 X WH D1-A2	UoW 751
	BLA6 A4 X BLA6 D5	BLA6 A4 X BLA6 D5 -B7	UoW 752
	BLA6 A4 X WH C2	BLA6 A4 X WH C2 -C1*	UoW 753
		BLA6 A4 X WH C2- F1*	UoW 754
		BLA6 A4 X WH C2 - G1	UoW 755
	BLA6 C5 X BLA6 D5	BLA6 C5 X BLA6 D5-D8	UoW 756
	BLA6 C5 X WH C2	BLA6 C5 X WH C2-C2	UoW 757
		BLA6 C5 X WH C2-E2	UoW 758

Sibling strains germinated from the same hypnozygote are indicated by*

3.5. Discussion

3.5.1. Morphology

The morphological criteria used to distinguish *A. tamarense*, *A. fundyense* and *A. catenella* has often been criticised due to the fine-scale detail and experience required to identify isolates. Indeed the identification of isolates for this study was difficult and time consuming. In particular, locating morphologically informative thecal plates was tedious and identifying the presence or absence of, for example, a ventral pore proved difficult under normal light microscopy. However, this analysis did yield some useful information that relates to previously reported mating compatibility between morphotypes.

Two Group I isolates from Orkney identified as *A. fundyense* successfully mated with BBD4, an *A. tamarense* from Bedford Basin. Hypnozygotes from one of these crosses excysted, resulting in viable progeny. This is in agreement with previous work by Anderson et al. (1994) and the contention that these morphotypes do not represent valid species (Anderson et al. 1994; Scholin et al. 1995).

Invalidation of morphotypes as species does not, however, preclude their usefulness. Morphology provides not only a description of what these organisms look like, but may also be useful in determining potential toxicity. The latter point is primarily in relation to *A. fundyense* and *A. catenella* that are found exclusively in toxic clades according to rDNA analysis (Lilly et al. 2007; Scholin et al. 1994; Scholin et al. 1995).

3.5.2 Aggregation of gametes, planozygotes and hypnozygotes

The behaviour of cells under nutrient depleted conditions in this study is similar to that observed by other researchers. In particular, the observation of aggregations of gametes, planozygotes and hypnozygotes at the edge of the Petri dish have been described in other dinoflagellate species including *A. taylori* (Figueroa et al. 2006) and *Scrippsiella lachrymosa* and *A. fundyense* (Persson et al. 2008).

Persson et al. (2008) suggest that the phenomenon may relate to physical properties at the edge of the vessel being similar to that of the pycnocline in stratified water columns, an area where thin layers of *Alexandrium* cells are known to accumulate (Wyatt and Jenkinson 1997). The resulting density gradient, or increased viscosity, in this region could act to retain gametes and planozygotes increasing the likelihood of cell-cell contact or increase the concentration of pheromones or chemical signals (Persson et al. 2008; Wyatt and Jenkinson 1997).

Cell contact between gametes is obviously important in the formation of zygotes, but it has also been shown to be of importance in the encystment of *Gyrodinium instriatum* planozygotes. When Uchida (2001) isolated individual planozygotes of *G. instriatum* to wells where they were separated from vegetative cells by a membrane none of them encysted, instead dividing and returning to vegetative growth. This may explain the observed persistence of *A. tamarense* planozygotes at the edge of the Petri dish in this study and the deposition of hypnozygotes in large agreggations always near the wall of dish. This aggregation was virtually always observed, with the exception of crosses where yield was very low (<100) and then hypnozygotes were found scattered across the bottom of the dish.

Gametes were virtually impossible to distinguish from vegetative cells in terms of size and morphology. Fusing gametes were, however, easily identifiable and very similar to those described by Fritz et al. (1989). With the exception that the smaller size and lighter pigment noted by these authors was not obvious in the study presented here. The finding that gametes were not identifiable in terms of smaller size or pigment is similar to that of Persson et al. (2008) in *A. fundyense.* In addition these authors also noted that very small cells were not observed fusing, as in this study. These cells may represent vegetative cells that have become smaller, with less chlorophyll content in response to nutrient stress, similar to the 'starved' cells described by Persson et al. (2012).

In contrast to gametes planozygotes were easily differentiated due to their larger size, darker pigment, elongated epitheca and biflagellated longitudinal flagella, similar to those described by Fritz et al. (1989). The time between formation and encystment for planozygotes in this study is in agreement with Anderson et al. (1983) at >1 week, with the exception of homothallic isolate WHA1 whose planozygotes encysted in less than 7 days.

3.5.3 Mating type

The observed mating compatibility amongst isolates could not be explained with simple heterothallism. Mating compatibility between isolates did not follow any obvious pattern and often where mating would have been expected if isolates were either a simple + or – mating type, none occurred. For example, there were no hypnozygotes produced in any of the crosses between BBD4 (Bedford Basin) and

Belfast Lough or Weymouth Harbour. Despite several of these isolates sharing common compatible strains with Orkney isolates that did cross with BBD4. Furthermore, two isolates, WHA5 from Weymouth Harbour and BBB1 from Bedford Basin, failed to produce hypnozygotes in any crosses and WHA1 produced high numbers of hypnozygotes in self-crosses at 20°C.

Complex heterothallism in the *A. tamarense* complex has been observed (Brosnahan et al. 2010) and this type of mating system has been reported in other *Alexandrium* species including *A. minutum* and *A. tamutum* (Figueroa et al. 2007) as well as *Gynodinium catenatum* (Blackburn et al. 2001). Although *G. catenatum* has been disputed as being solely heterothallic by Figueroa et al. (2010). There have also been contradictory reports of simple heterothallism in *A. tamarense* and *A. catenella* (Anderson 1994; Parker 2002) similar to that of *Lingodinium polyedra* (Figueroa and Bravo 2005). In addition Fritz et al (1989) appeared to use a homothallic isolate of *A. tamarense* (as *A. excavatum*), however it is unclear from their description if this isolate was actually the result of a mixture of sibling strains.

Whether, the results reported here genuinely reflect incompatibility between isolates, particularly between Bedford Basin BBD4 and Group III isolates, or are the result of some unknown condition (e.g. temperature, nutrients, salinity) restricting sexuality between these isolates is uncertain. However, they do provide more evidence of the complex mating interactions of *A. tamarense* in culture.

3.5.4 Effect of temperature on hypnozygote yield

The results presented in relation to temperature differ from other studies not only in terms of maximum hypnozygote yield/temperature relationship, but they also show an effect of temperature on mating compatibility within Group I isolates. Anderson et al. (1984) reported maximum hypnozygote yield for *A. tamarense* at 20°C. The study presented here showed no significant change ($p>0.05$) in hypnozygote yield for Group I/I or Group I/III crosses at 15°C and 20°C. There was, however, a significant decrease ($p<0.05$) in Group III/III hypnozygotes at 15°C compared to 20°C.

These differences in hypnozygote yield between Groups I and III are not that surprising given that the Group I isolates used this study were isolated from areas where temperature is lower (Orkney and Bedford Basin 4-13°C) in comparison to that for the Group III isolates (Weymouth 7.8-17.3°C; Belfast Lough 2-21°C). So it is likely that these differences represent an adaptation to local environmental conditions. However, the significant decrease in Group III/III hypnozygote yield at 15 °C does suggest that the commonly used condition of 20°C for the encystment of *A. tamarense* may favour encystment of Group III isolates in culture.

The fact that there was no significant change ($p>0.05$) in hypnozygote yield for Group I/III crosses is interesting as it suggest that Group III isolates in co-culture with Group I isolates were not subjected to the same negative effect of temperature as those in Group III/III co-cultures. Why this may be needs to be further investigated, as it may have implications for mating interactions of the two groups in nature. Particularly if it is the case that Group I isolates are able to elicit a mating response in Group III isolates at lower temperatures.

The effect of decreased temperature on the mating compatibility of Group I/I crosses was unexpected. The significant increase ($p<0.05$) in CI for Group I isolates at 15°C was primarily the result of three additional crosses that did not occur at 20°C. This may indicate that sexual reproduction and encystment of these isolates is favoured at low temperatures. As was reported by Ichimi et al. (2001) in Japan, where growth and encystment of *A. tamarense* at 7.5-9°C was observed in the field. These conditions are probably similar to those in Scapa Flow, Orkney where Joyce (2005) reported that water temperature never exceeded 13°C, and where temperatures were recorded as 10-11°C in and May and June during field-sampling for this study (chapter 2).

3.5.5 Frequency of mating between inbred and out-bred groups

The frequency of mating as a function of hypnozygote yield revealed some interesting results. At 15°C and 20°C, inbred Group III/III crosses had the highest AV values, followed by Group I/III, with Group I/I having the lowest AV (see table 3.12). Given that these crosses were carried out under the same conditions, using the same medium batch, at the same time and with the same inoculum, these data suggest that these Group I isolates are more likely to produce hypnozygotes with compatible Group III isolates.

Whether this data is a true reflection of the mating interactions of Group I and III in natural populations of co-occurrence is unknown. But it may explain, at least in part, the apparent decrease in shellfish toxicity associated with PSP toxins in Scotland since the early 1990's (Bresnan et al. 2008). Particularly if hybrid Group I/III progeny conform to laboratory determined non-viable status (see section 3.4.3) under

natural environmental conditions. Furthermore, Eckford-Soper (2013) have reported that Group III isolates from Scotland appear to out compete Scottish Group I isolates in terms of growth and nutrient uptake in mixed cultures.

3.5.6 Germination of hypnozygotes

The germination success of hypnozygotes for all co-cultures was low in comparison to the reported 80-100% germination of wild mature *A. tamarense* hypnozygotes in the laboratory (Anderson et al. 1983). Brosnahan et al. (2010) had similarly poor excystment success and suggested that lower nutrient medium may improve germination rates. Figueroa et al. (2005) had reported 90% germination of cultured *A. catenella* hypnozygotes in un-enriched seawater after 20 days, compared to 40% after 60 days in L1 medium. However, our results for the excystment of *A. tamarense* hypnozygotes in f/20 modified medium suggest that lower nutrient levels do not increase their germination.

The degradation of our hypnozygotes during storage at 4°C is similar to that reported by Figueroa et al. (2008) for *A. peruvianum,* also stored for several months in cryovials, in anoxic sediment at 4°C. Brosnahan et al. (2010) also stored hypnozygoes under similar conditions, and so it is probable that the method of storage of hypnozygotes does not adequately mimic natural conditions to allow maturation of cultured hypnozygotes as in nature. Resulting in lower viability of cultured hypnozygotes compared to wild type.

Furthermore, the majority of the hypnozygotes formed in co-culture crosses resembled those described in Genovesi et al. (2009) as type-1. These hypnozygotes

are darker and more granulated than wild type hypnozygotes. Whether this difference in appearance represents differences in storage products that may affect the maturation and long-term survival of cultured hypnozygotes is unknown. However, given that several cultures for the study presented here had been isolated from natural sediments stored at 4°C, in dark anoxic conditions for >13 years, it seems clear that the storage and/or production of cultured hypnozygotes do not result in the same robust physiology as long-lived wild hypnozygotes. Moreover, it has been reported that *L. polyedra* hypnozygotes have excysted from sediment cores estimated to be ~80 years old (Lundholm et al. 2011),

The germination rates of hypnozygotes produced at 15°C and 20°C seem to suggest that Group III/III crosses produced at 15°C have lower germination success than those produced at 20°C. Conversely, these results also suggest Group I/I and I/III produced at 15°C have higher germination success than those produced at 20°C. However, these data are contradicted by germination studies for progeny in chapter 4. As a consequence it is impossible to infer anything about the possible effect of encystment temperature and the germination success of cultured hypnozygotes.

Consistent with Brosnahan et al. (2010) none of the Group I/III hybrids survived into culture. Further strengthening their assertion of these groups as biological species and adding to the probability that there is no gene flow between the groups in areas of co-occurrence. Viable progeny were isolated from Group I/I and Group I/III and brought into culture. These isolates were used in further study of the mating type of *A. tamarense* presented in chapter 4.

CHAPTER 4

ANALYSIS OF MATING TYPE: PREDICTING THE MATING COMPATIBILITY OF *A. TAMARENSE* PROGENY

4.1 Introduction

The mating interactions of *A. tamarense* in culture are complex. As a consequence it is often not possible to attribute isolates with a simple + or - mating type resulting in their mating system being defined as complex heterothally, as described in chapter 3. However it has been demonstrated that mating type in *A. catenella* follows Mendelian inheritance (Sako et al. 1992). Furthermore, it was noted by these

researchers that the toxin profile of progeny also followed Mendelian inheritance, but segregated independently of mating type.

Given the apparent stable inheritance of mating type, why have so many different models of mating system been proposed for *A. tamarense*? If progeny inherit the mating type gene/s of either parent without significant recombination, then are mating types fixed within populations? This could possibly mean that there are populations within populations of either Group I or III, that are as equally reproductively isolated as Group I and III appear to be.

4.1.1 Aims

The main aim of these experiments was to determine the mating type of progeny isolated from mating crosses described in chapter 3, by crossing them with each parental isolate. Unfortunately it was not possible to isolate tetrad sibling progeny and so the experiments were limited in how far they could assess the ratio of mating type inheritance. The second aim was to see whether mating type determination could be used to predict positive crosses for progeny with other isolates, based on the mating interactions of their parents as demonstrated in chapter 3. And lastly to assess the germination and viability of hypnozygotes produced in progeny/parent and predictive crosses.

4.2 Materials and methods

4.2.1 Cultures

Six *A. tamerense* progeny isolates, including two sets of siblings, from those generated from the germination of hypnozygotes produced at 20°C (chapter 3, table 3.10) were used for initial parent/progeny encystment experiments (table 4.1).

Table 4.1 Progeny and parental isolates used for parent/progeny mating experiments

	Co-culture/parent isolates	**Progeny isolate**
Group I	SP B5 X BB B4	SP B5 X BB B4B1-A2*
		SP B5 X BB B4B1-B2*
Group III	BLA6 A4 X WH C2	BLA6 A4 X WH C2 -C1*
		BLA6 A4 X WH C2- F1*
	BLA6 C4 X WH D1	BLA6 C4 X WH D1-A2
	BLA6 C4 X BLA6 D5	BLA6 C4 X BLA6 D5 -B7

Sibling isolates germinated from the same hypnozygote are indicated by*

4.2.2 Encystment conditions

All crosses were carried out in duplicate sterile 30 mm Petri dishes at 15°C with encystment conditions (medium, light, inoculation, incubation period) as previously described in chapter 3.

4.2.3 Determining mating type

Progeny sibling isolates were crossed with each other and crossed with both parent isolates. Single progeny isolates were crossed with both parents. Duplicate self-crosses were performed for all isolates. Mating type was determined by the result of which parent (if any), progeny isolates crossed with. Progeny were deemed to have the same mating type as the parent that they did not cross with. Mating types were designated either '+' or '-'.

4.2.4 Testing mating type

To test the mating type of progeny, sibling isolates BLA6 A4xWHC2-C1 and BLA6 A4xWHC2-F1, and isolate BLA6 C4XWH D1-A2 were crossed with *A.tamarense* isolates previously shown to be compatible with the parent of the same mating type using data in chapter 3 (tables 3.6 and 3.7). Encystment conditions were as previously described at 15°C in chapter 3. All crosses were carried out in duplicate. *A. tamarense* isolates used for predictive crosses are shown in table 4.2.

Table 4.2 *A. tamarense* isolates used in predictive mating crosses

Origin	Isolate	UoW ID
Weymouth Harbour	WHC2	UoW 706
	WHD1	UoW 724
Belfast Lough -site A6	BLA6 A6	UoW 708
	BLA6 C4	UoW 712
	BLA6 C5	UoW 713
	BLA6 A5	UoW 727
	BLA6 D5	UoW 714
	BLA6 A4	UoW 729
Stromness Pier, Orkney	SPB5	UoW 715
	SPC6	UoW 716
	SPD6	UoW 718
Station 7, Orkney	ST7A3	UoW 719

4.2.5 Hypnozygote enumeration and storage

Hypnozygotes from all positive crosses were counted and scored as described previously in chapter 3. Hypnozygotes were stored for 6 months in cryovials, in anoxic sediment, at 4°C as previously described.

4.2.6 Hypnozygote germination and evaluation of progeny viability

Five to ten hypnozygotes from each positive parent/progeny, sibling and predictive cross were isolated to 96 well plates containing 200 μL f/2 modified medium and incubated at 15°C, 14/10h light dark cycle, for 28 days as previously described in chapter 3.

4.3 Results

4.3.1 Progeny/parent crosses

Progeny isolates BLA6 C4 X WH D1-A2 and BLA6 C4 X BLA6 D5-B7, produced hypnozygotes with only one parent isolate. Sibling isolates SP B5 X BB B4B1-A2 and SP B5 X BB B4B1-B2 were not compatible with each other and both produced hypnozygotes with the same parent, BB D4. Sibling isolates BLA6 A4 X WH C2 - C1 and BLA6 A4 X WH C2–F1 were compatible with each other and each produced hypnozygotes with an alternate parent. No progeny isolates produced hypnozygotes in self-crosses. Progeny and parent isolates were designated a '+' or '-' mating type based on these data (see tables 4.3, 4.4 – 4.7).

Table 4.3 Mating type of progeny and parent isolates

	Isolate	Mating type
Group I	SP B5	+
	BB D4	–
	SP B5 X BB B4B1-A2*	+
	SP B5 X BB B4B1-B2*	+
Group III	BLA6 A4	+
	WH C2	–
	BLA6 A4 X WH C2 -C1*	+
	BLA6 A4 X WH C2- F1*	–
	BLA6 C4	+
	WH D1	–
	BLA6 C4 X WH D1-A2	–
	BLA6 D5	-
	BLA6 C4 X BLA6 D5 -B7	+

4.3.2 Predictive crosses

Predictive mating of progeny isolates with *A. tamarense* isolates previously compatible with the parent isolate of the same mating type, resulted in the production of hypnozygotes in all but one of the co-culture crosses (BLA6 A4 X WH C2–F1) x ST7 A3. Hypnozygote yields for all predictive crosses were lower than for original parent crosses (see tables 4.6 and 4.7).

Table 4.4 Sibling progeny SP B5 x BB D4-A2 and SP B5 x BB D4-B2. Hypnozygote yield for progeny and parent crosses (previous yield between parents in shown in bold).

	SP B5 x BB D4^{A2}+	**SP B5 x BB D4^{B2}+**	**SP B5+**	**BB D4−**
SP B5 x BB D4^{A2}+	0			
SP B5 x BB D4^{B2}+	0	0		
SP B5+	0	0	0	
BB D4−	0-1	0-1	**2-3**	0

Table 4.5 Progeny BLA6 C4 x BLA6 D5-B7. Hypnozygote yield for progeny/parent crosses (previous yield between parents in shown in bold).

	BLA6 C4 x BLA6 D5^{B7}+	**BLA6 C4+**	**BLA6 D5−**
BLA6 C4 x BLA6 D5^{B7}+	0		
BLA6 C4+	0	0	
BLA6 D5−	2	**3**	0

Table 4.6 Progeny BLA6 C4 x WHD1-A2. Hypnozygote yield for progeny/parent crosses and predictive crosses based on mating type + or **-** (previous yield between parents and predictive isolates are shown in bold).

		Group III				Group I
		BLA6 C4 x WHD1^{A2}–	**BLA6 C4+**	**WH D1−**	**BLA6 A5+**	**SP C6+**
Group III	**BLA6 C4 x WHD1^{A2}–**	0				
	BLA6 C4 +	3	0			
	WH D1−	0	**2**	0		
	BLA6 A5+	2	**0**	**3**	0	
Group I	**SP C6+**	3	**0**	**2-3**	/	0

Table 4.7 Sibling progeny BLA6 A4 X WH C2 -C1 and BLA6 A4 X WH C2- F1. Hypnozygote yield for progeny/parent crosses, and predictive crosses based on mating type + or - **(**previous yield between parents and predictive isolates are shown in bold). Predictive cross that failed to yield hypnozygotes is marked*

		Group III									Group I			
		BLA6 A4 x $WHC2^{Cl}$+	BLA6 A4 x $WHC2^{Fl}$–	BLA6 A4+	WHC2–	BLA6 A5+	BLA6 A6+	BLA6 C4+	BLA6 C5+	BLA6 D5–	SP B5+	SP C6+	SP D6+	ST7 A3+
Group III	BLA6 A4 x $WHC2^{Cl}$+	0												
	BLA6 A4 x $WHC2^{Fl}$–	1-2	0											
	BLA6 A4+	0	2	0										
	WHC2–	1	0	**2**	0									
	BLA6 A5+	/	1	/	**1-2**	0								
	BLA6 A6+	/	1	/	**2**	/	0							
	BLA6 C4+	/	0-2	/	**3**	/	/	0						
	BLA6 C5+	/	1	/	**2**	/	/	/	0					
	BLA6 D5–	2	0	**0-2**	**0**	/	/	/	/	0				
Group I	SP B5+	/	0-1	/	**3**	/	/	/	/	/	0			
	SP C6+	/	0-1	/	**2**	/	/	/	/	/	/	0		
	SP D6+	/	1-2	/	**2**	/	/	/	/	/	/	/	0	
	ST7 A3+	/	0*	/	**1-2**	/	/	/	/	/	/	/	/	0

4.3.3 Hypnozygote germination and viability of progeny/parent and predictive crosses

The germination of hypnozygotes produced in progeny/parent and predictive crosses varied according to group. Group III/III sibling cross BLA6 A4 X WH C2 -C1 and BLA6 A4 X WH C2- F1 failed to excyst. As did Group I/I progeny/parent crosses. For Group III/III progeny/parent and predictive crosses, excystment ranged from 0 – 70%. All hypnozygotes that excysted in Group III/III crosses were viable. Two Group I/III predictive crosses excysted, with excystment success at 10% and 60% (see table 4.8). However, these did not survive for more than two divisions post-excystment.

Table 4.8 Germination of hypnozygotes produced in progeny/parent and predictive crosses

	Co-culture	No. of hypnozygotes isolated	No. of excystments	%
Group I	(SPB5 X BBD4-A2) X BB D4	5	0	0
	(SPB5 X BBD4-B2) X BB D4	5	0	0
Group III	(BLA6 A4 X WHC2-C1) X (BLA6 A4 X WH C2-F1)	10	0	0
	(BLA6 A4 X WHC2-C1) X WHC2	10	0	0
	(BLA6 A4 X WHC2-F1) X BLA6 A4	10	4	40
	(BLA6 A4 X WHC2-F1) X BLA6 A5	10	4	40
	(BLA6 A4 X WHC2-F1) X BLA6 C4	10	2	20
	(BLA6 A4 X WHC2-F1) X BLA6 C5	10	6	60
	(BLA6 C4 X BLA6 D5-B7) X BLA6 D5	10	6	60
	(BLA6 C4 X W D1-A2) X BLA6 C4	10	5	70
	(BLA6 C4 X WHD1-A2) X BLA6 A5	10	7	70
Group I/III	(BLA6 A4 X WHC2-F1) X SP B5	10	0	0
	(BLA6 A4 X WHC2-F1) X SP C6	10	0	0
	(BLA6 A4 X WHC2-F1) X SP D6*	5	3	60
	(BLA6 C4 X WHD1-A2) X SP C6*	10	1	10

Non-viable progeny (failed to complete 3 cell divisions post excystment) are denoted by*

4.4 Discussion

4.4.1 Mating type

It was possible to determine the mating type of progeny through progeny/parent crosses and sibling crosses. The observed mating types appear to fit a Mendelian pattern of inheritance and also fit a simple heterothallic model with each isolate being attributed as a + or -. Furthermore it was possible to predict mating compatibility, with only one predictive cross failing to produce hypnozygotes. These data suggest that mating type is fixed in these isolates, at least under the experimental conditions. Furthermore, they also support the notion that the mating system in *A. tamarense* is simple heterothallism, with two mating types.

These results are in complete contrast to those presented in chapter 3. Which is not surprising given that the mating compatibility of parental isolates was known prior to the experiments being carried out. But they do highlight a possible reason for the different mating systems of *A. tamarense* reported in the literature (e.g. complex heterothallism Brosnahan et al. 2010; simple heterothallism Anderson 1994; Parker 2002). It is a probability that researchers could isolate or acquire, by chance, compatible isolates that follow a + and – model of mating. For example if only isolates in table 4.6 were used in mating crosses under the same conditions presented here the conclusion would be that the mating system in *A. tamarense* is simple heterothallism.

In contrast, the conditions used to induce sexuality and encystment could explain why complex heterothallism has been observed in *A. tamarense*. It has been noted in

the heterothallic fungus *Opiostoma quercus* that not all isolates are equally fertile under mating test conditions, resulting in poorly assigned mating types (Wilken et al. 2012). It is possible that different isolates of *A. tamarense* may be induced sexually under different environmental conditions and therefore display a range of fertility. This would explain why some isolates produced no hypnozygotes in any co-culture crosses in chapter 3.

This explanation is easier to understand when it is considered in relation to the diversity of natural populations of *A. tamarense*. For example Alpermann et al. (2010) reported the high genotypic diversity of *A. tamarense* Group I off the North East coast of Scotland. In this study 88 individual isolates were examined using microsatellites and AFLP, all were found to be unique and not of clonal origin. Such diversity is likely to be advantageous in adapting to changing environments (Barret and Schutler 2008). So it is probable, given the importance of encystment in the life cycle of *A. tamarense*, that multiple environmental cues are involved in the sexual reproduction and encystment of different lineages.

The conditions used to induce sexuality in *A. tamarense* in culture have been limited primarily to nutrient limitation (e.g. nitrogen Anderson et al. 1984; phosphorus Anderson et al. 1984; Anderson and Lindquist 1985; and iron Doucette et al. 1989). The fact that induction of sexuality under limitation of different nutrients occurs in this organism is interesting and adds weight to the idea that multifactorial initiation factors are probably involved in sexual reproduction of *A.tamarense* in the field. A consequence of the limited conditions used to assess the mating interactions of *A.*

tamarense in culture, including the sole use of nitrogen limitation and the limited temperature range in this study, is that it may result in false negatives. As was observed for some Group I isolates at 20°C in chapter 3.

4.4.2 Germination of hypnozygotes

Hypnozygote yields for progeny/parent and predictive crosses were lower than their counterpart crosses presented in chapter 3. However, there were marked differences in the success of germination of these hypnozygotes in comparison to hypnozygotes produced at 15°C in chapter 3, particularly in relation to Group III/III crosses. The data presented in chapter 3 seemed to suggest that Group III hypnozygotes produced at 15°C had lower germination success than those produced at 20°C. The data for germination of progeny/parent and predictive crosses presented here contradict this entirely. Two Group III/III crosses had excystment rates of 70%, the highest percentage of excystment during this study.

These results could be the result of several factors. Firstly, hypnozygote yield may not be a good indicator of the potential for excystment. Higher yield does not necessarily equate to higher rates of excystment. Secondly, storage time may have affected results. Hypnozygotes for mating type study were stored for only 6 months, compared to up to 12 months for crosses reported in chapter 3. This timeframe was enough for the mandatory dormancy period to have passed, but not long enough for degradation of hypnozygotes to occur. This might suggest that degradation of

cultured hypnozygotes begins after mandatory dormancy has passed and hypnozygotes enter quiescence.

CHAPTER 5

RIBOTYPE VARIATION IN *A. TAMARENSE* GROUPS I AND III ISOLATED FROM THE UK

5.1 Introduction

Ribosomal genes (rDNA) in eukaryotes occur as tandem arrays arranged at single or multiple chromosome loci (Naidoo et al. 2013). Each rDNA cistron contains genes encoding the small ribosomal sub-unit (18S), and two large sub-unit ribosomal RNA's (5.8S and 28S) organised as a single transcription unit and separated externally by non-transcribed spacers (NTS) (Eickbush and Eickbush, 2007; Naidoo et al. 2013). Internal organisation of rDNA cistron includes an external transcribed spacer region and two internal transcribed spacers (ITS1 and ITS2) (see fig 5.1).

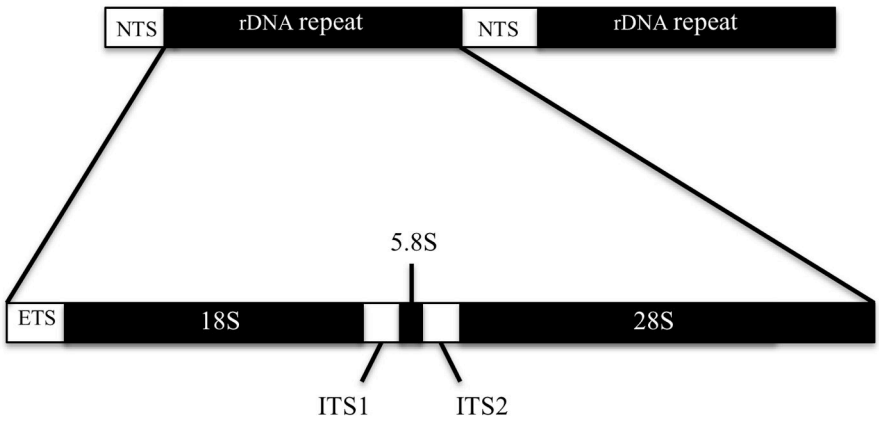


Fig. 5.1 Organisation of ribosomal RNA (rDNA) genes in eukaryotes. NTS – Non-transcribed spacer, ETS – External transcribed spacer, ITS – Internal transcribed spacer.

High cellular demand for ribosomes means that eukaryote genomes typically encode hundreds (and often more) of copies of the rDNA cistron (Eickbush and Eickbush, 2007). Arrays of rDNA cistrons have been shown to undergo evolution as a single unit, resulting in the homogenisation of the DNA sequence in each cistron of a tandem array within individuals and species (Naidoo et al. 2013). This phenomenon has been termed 'concerted evolution'. The mechanism of concerted evolution is unclear, but it is thought that processes such as unequal crossing over may result in mutations either being eliminated or spread uniformly throughout repeat cistrons (Eickbush and Eickbush; Naidoo et al. 2013). Under concerted evolution sequence variation of rDNA in species and individuals is kept minimal, whilst differences between species can accumulate (Naidoo et al. 2013). As a consequence ribosomal genes have become the marker of choice for phylogenetic studies and the discrimination of species.

Molecular research of *Alexandrium* has tended to focus on both the large (e.g. Lilly et al. 2005; Lilly et al 2007; Kim et al 2007; McCauley et al. 2009; Scholin et al 1994) and small (e.g. Montresor et al. 2004; Scholin et al. 1993) ribosomal subunit genes (LSU rDNA & SSU rDNA respectively). In 1994 Scholin et al. (1994) described the clustering of D1-D2 LSU rDNA sequences of geographically dispersed isolates of *A. tamarense*, *A. fundyense* and *A. catanella* into five clades according to geography, not morphology. This led to the proposal of division of the *A. tamarense* species complex into five groups with geographical nomenclature i.e. North American, Western European, Temperate Asian, Tropical Asian and Tasmanian. In 2007 Lilly et al. published similar research using 110 globally dispersed isolates. However, whilst the isolates were still divided into five distinct clades they did not find a link to geography as in Scholin et al. (1994). A new group numbering (I-V) was proposed and is still in routine usage. Interestingly, clades in both studies were also separated by toxicity with each clade consisting entirely of toxic or non-toxic isolates. The phylogenetic tree from Lilly et al. (2007) is shown in fig. 5.2.

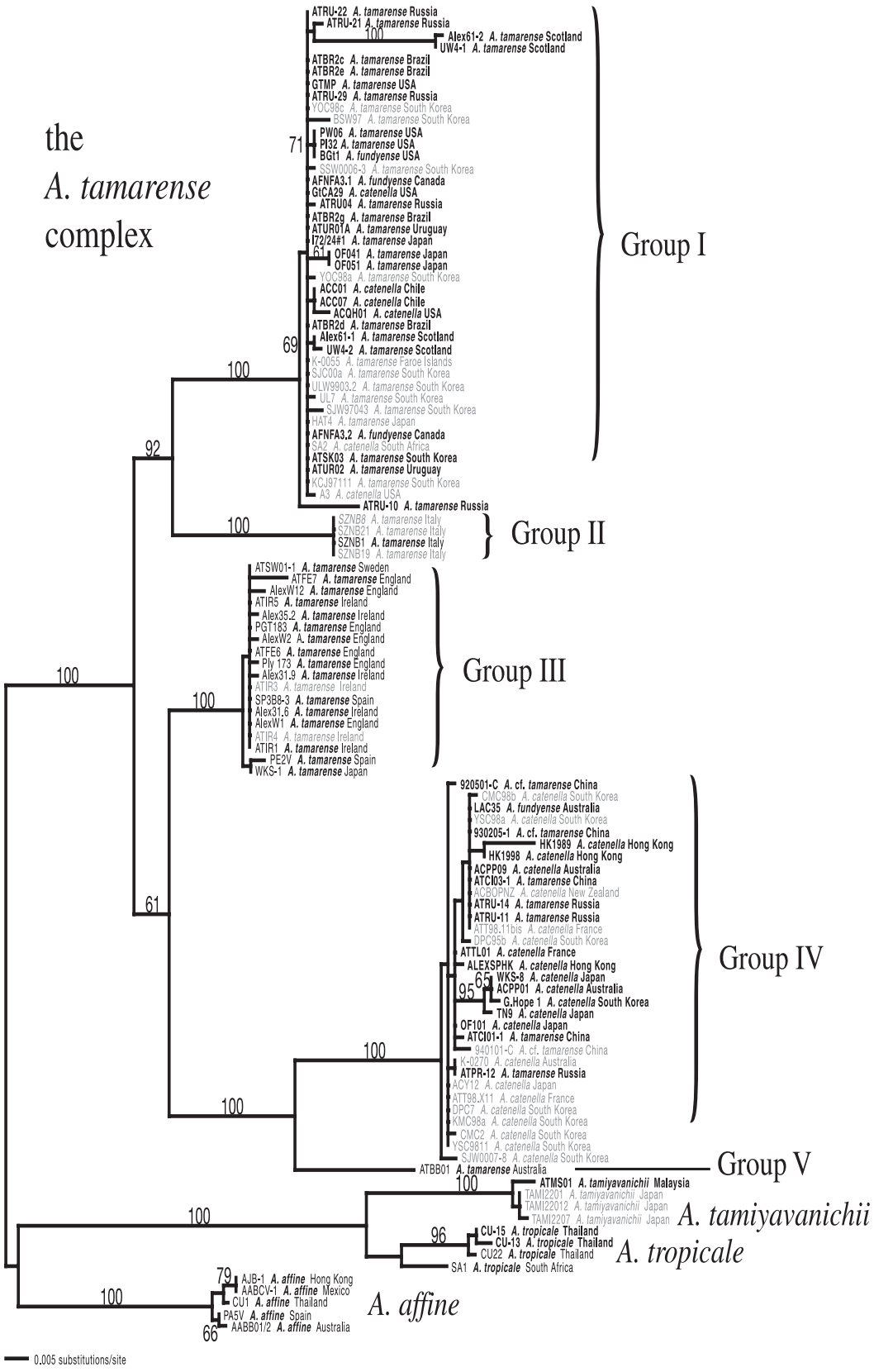


Fig. 5.2 Lilly et al's. 2007 phylogenetic tree of *A. tamrarense* complex LSU rDNA and the distinction of five clades according to ribotype (Lilly et al. 2007).

In addition to standard PCR and sequencing of rDNA hybridisation technologies, such as fluorescent in situ hybridisation and microarrays have also been developed. These have primarily been used for field samples, targeting ribosomal RNA (rRNA) (e.g. Gribble et al. 2005; John et al. 2005; Sako et al. 2004; Taylor et al. 2014; Touzet et al. 2008). These technologies have made the discrimination of *A. tamarense* groups in field samples possible without the need for sequencing and in depth morphological analysis.

4.1.1 Rationale and aims

During real-time PCR analysis of sediments (data not shown) it was noted that both Group I and III primers were amplifying product in one of the cultured DNA samples that was being use as a positive control for the Group I assay. The isolate was SPC6 a Group I *A. tamarense/fundyense* from the Orkney Islands. A single cell nested PCR/real-time PCR protocol was implemented to assess if the observed dual amplification of Group I and III DNA within SPC6 was an artefact of non-clonal culture during isolation and then to detect if it was present in other isolates.

In addition whole cell fluorescent in situ hybridisation (whole-cell FISH) using group specific fluorochrome conjugated oligonucleotide probes was used to establish if there was expression of more than one ribotype in any of the tested isolates.

5.2. Methods

5.2.1 Single cell PCR/nested real-time PCR

Single cell analysis was employed to overcome the possibility that any detected dual ribotype may be the result of non-clonal culture. Two isolates from Belfast Lough and Weymouth Harbour were analysed along with three additional isolates from Orkney (see table 5.1). As the real-time PCR primers sit within the D1-D2 region of the LSU rDNA (see fig 5.3), this region was amplified by standard PCR and the D1-D2 LSU rDNA amplicons used as a template for the group specific real-time PCR assays (see table 5.2 for primers).

Table 5.1 *A. tamarense* isolates used for single cell PCR/nested real-time PCR and whole cell fluorescent in situ hybridisation.

Origin	Strain	UoW ID	Group	Morphospecies
Stromness Pier, Orkney	SP C6	UoW 716	*A. tamarense* I	*A. fundyense*
58°57.851'N 3°17.668'W	SP D6	UoW 718	*A. tamarense* I	*A. tamarense*
Station 7, Orkney	ST7 B5	UoW 721	*A. tamarense* I	*A. tamarense*
58°54.758'N 3°17.772'W	ST7 D2	UoW 723	*A. tamarense* I	*A. tamarense*
Belfast Lough -site A6	BLA6 A6	UoW 708	*A. tamarense* III	*A. tamarense*
54º39.800'N 005º48.80'W	BLA6 C5	UoW 713	*A. tamarense* III	*A. tamarense*
Weymouth Harbour	WH A1	UoW 700	*A. tamarense* III	*A. tamarense*
	WH C2	UoW 706	*A. tamarense* III	*A. tamarense*

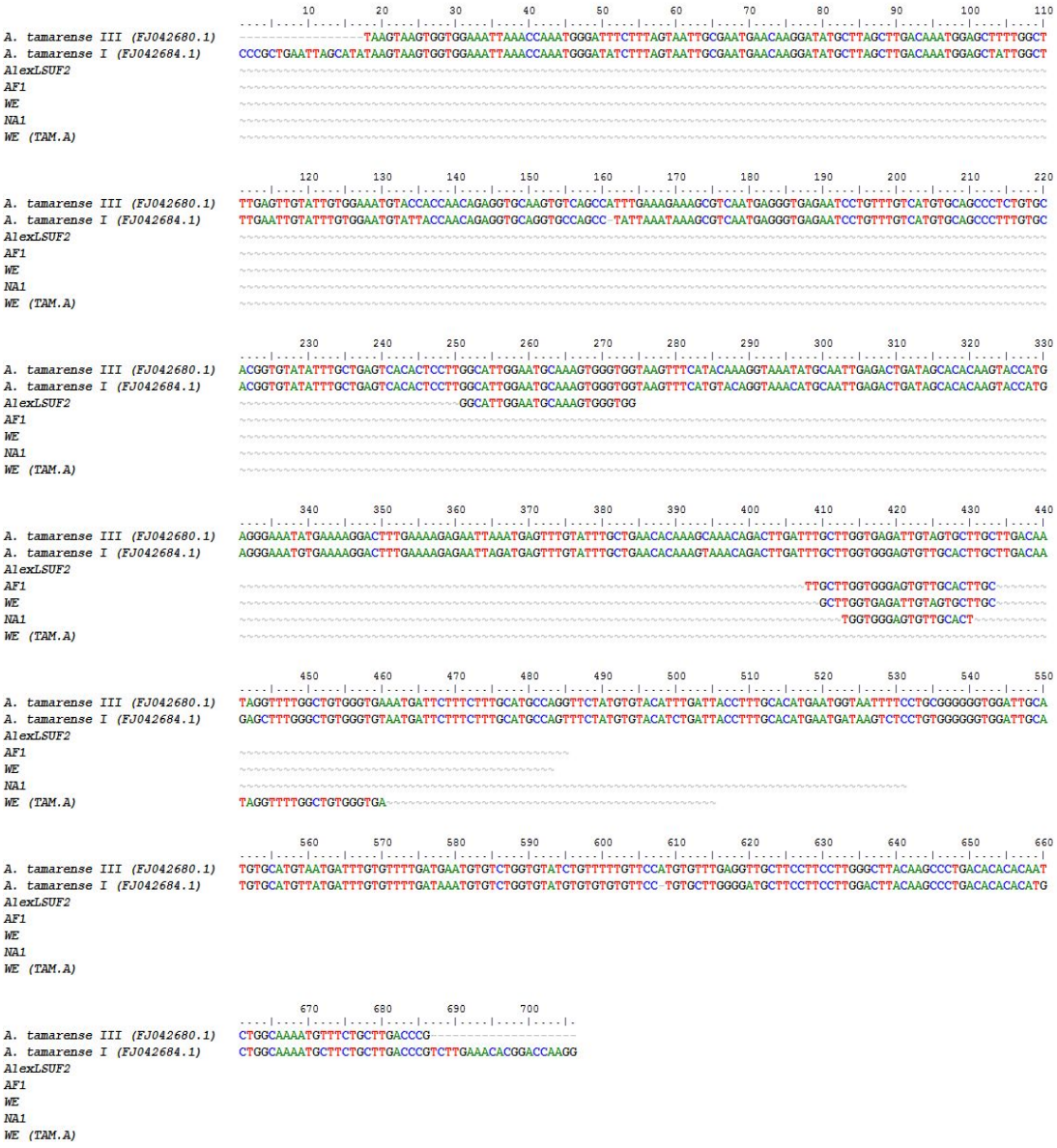


Fig 5.3 *A. tamarense* Group I and III D1-D2 LSU rDNA alignment showing position of real-time PCR primers (AlexLSUF2, AF1 & WE) and whole-cell FISH probes (NA1 & WE (TAM.A))

5.2.1.1 PCR amplification of LSU rDNA D1-D2 region

Single cells were isolated by micropipette (see chapter 2) and placed directly into 0.2 mL PCR tubes with 25 µL PCR reaction mix (2x Accusure® master mix Bioline), D1R/D2C primers (0.4 µM), ultra-pure PCR water (Bioline). PCR amplification of

the D1-D2 region of the LSU rDNA was carried out under the following cycling conditions; Initial denature 95°C for 5min followed by 30 cycles of denature at 95°C for 15sec, annealing at 55°C for 15sec, extension at 72°C for 10sec. PCR products were run on a 1% ultrapure agarose gel (Invitrogen) with a 100 bp ladder (Bioline) to confirm product size (~700 bp). The remaining PCR product was cleaned as for culture ribotyping. Cleaned PCR products were analysed using a nanodrop ND 1000 spectrophotometer (Thermo Scientific) and diluted to 1ng/µL with ultrapure PCR water (Bioline).

Table 5.2 PCR and real-time PCR primers**.** Direction of primer is indicated by F (forward) & R (reverse).

Primer	Sequence	Origin
D1R (F)	5'ACCCGCTGAATTTAAGCATA3'	Scholin et al. 1994
D2C (R)	5'CCTTGGTCCGTGTTTCAAGA3'	Scholin et al. 1994
AlexLSUF2 (F)	5'GGCATTGGAATGCAAAGTGGGTGG3'	Dyhrman et al. 2006
AF1 (Group I R)	5'GCAAGTGCAACACTCCCACCAAGCAA3'	Dyhrman et al. 2006
WE (TAM.A) (Group III R)	5'GCAAGCACTACAATCTCACCAAGC3'	Percy et al, unpublished

5.2.1.2 Nested real-time PCR of D1-D2 LSU rDNA

5.2.1.2.1 Standards

Ten-fold serial dilution standard curves, ranging from 10^6 - 10^2 copies per reaction, were constructed for Group I and III amplicons to assess PCR efficiency and provide threshold values for each assay. Standards were produced using cloned plasmids containing the D1-D2 LSU rDNA of either Group I or III (these were previously

created and donated by Linda Percy). The D1-D2 region was amplified as for single cells using primer set D1R/D2C. The PCR products were run on a 1% agarose gel and PCR products cleaned as previous for single cells. Cleaned PCR products were quantified using a nanodrop ND 1000 spectrophotometer (Thermo Scientific) and copy number calculate as follows:

Number of copies = (amount (ng) x $6.022x10^{23}$) / (length (bp) x $1x10^{9}$ x 650)

An initial 50 µL stock standard of 10^{9} copies/µL was prepared using the equation C1V1=C2V2. Example for PCR product with concentration of 50 ng/µL:

(50 x $6.022x10^{23}$)/ (700bp x $1x10^{9}$ x 650) = 6.62 $x10^{10}$ copies/µL

(10^{9} x 50)/($6.62x10^{10}$) =1.5

1.5 µL PCR product + 98.5 H_20 = 10^{9} copies/µL

Ten-fold serial dilutions from 10^{8} - 10^{2} copies/µL were prepared using ultrapure PCR water (Bioline).

5.2.1.2.2 Reactions

All real-time PCR reactions were run in triplicate on a RotorGene 6000 (Qiagen) with a total reaction volume of 25 µL (2x SYBR Green RotorGene master mix (Qiagen), forward primer AlexLSUF2 and group specific reverse primers AF1 or WE at a final concentration of 0.4µM, ultrapure PCR water (Bioline) + 1 µL LSU D1-D2 standard (10^{6} -10^{2} copies/µL) or PCR water for NTC). Cycling conditions for all reactions was as follows; Initial denature 95°C for 5 minutes, denature 95°C: 5

seconds, annealing/extension 60°C: 10 seconds, repeated for 30 cycles. Cycling was followed by melt curve analysis, from 70°C - 90°C in 0.5°C increments, to assess reaction specificity and for evidence of contamination/primer dimer. Separate assays for single cell Group I and III amplicons were run under the same cycling conditions as for standard curves with 1 ng of LSU D1-D2 PCR as a template.

Following amplification, 20 µL of each real-time PCR reaction was run for ~45 minutes at 100V on a 3% ultrapure agarose gel made with 1x TBE buffer and precast with GelRed (Biotium). A 100 bp ladder (Bioline) was used to confirm product size (~200bp). The gel was visualised and photographed quickly under UV light, as previously. Product bands were excised from the gel under low UV illumination using a sterile scalpel. The products were extracted and purified using a Qiaquick gel extraction kit (Qiagen) according to the manufacturer's instructions. Twenty µL of purified real-time PCR product were directly sequenced at GATC Biotech using the corresponding Group specific reverse primer.

5.2.1.3 Sequence analysis

A UPGMA phylogenetic tree of real-time PCR sequences aligned with *A. tamarense*, *A. fundyense*, *A. catenella* and *A. minutum* sequences retrieved from Genbank, was constructed using Mega version 6.0 (Tamura et al. 2013), Kimura-2 parameter gamma distributed substitution model and 1000 bootstrap replicates as previously. *A. minutum* sequences were used as an out-group to root the tree.

5.2.2 Whole-cell fluorescent in situ hybridisation

Whole-cell FISH was used to assess if there was dual expression of Group I and III ribosomal RNA (rRNA) in any of the isolates analysed using single cell PCR/nested real-time PCR.

5.2.2.1 Fixation/preservation

Approximately 2 mL of exponentially growing culture (4000-8000 cells/mL) was transferred to a 15 mL centrifuge tube, the volume was made up to 14 mL with the 0.2 μm filtered natural seawater and fixed with 0.75 ml formalin (5% v/v = final conc. 1.8% v/v formaldehyde). Formalin fixed samples were allowed to stand briefly (<20 mins) at room temperature (16 -18°C) before being centrifuged for 5 minutes at 5000xg. The supernatant was aspirated away and the cell pellet re-suspended in 14 mL ice-cold methanol. All samples were either processed immediately or stored at -20°C.

5.2.2.2 Hybridisation

Hybridisation buffers were prepared and the hybridisation carried out according to the method set out in Touzet et al. (2007). Buffer recipes are shown in tables 5.3 – 5.5.

Table 5.3 25x SET: 3.75M NaCl, 25mM EDTA, 0.5 M Tris-HCL pH 7.8

Reagent	Volume (mL)	Final concentration
5M NaCl	75	3.75M
0.5M EDTA	5	25mM
2,5M Tris-HCL pH 7.8	20	0.5M

Table 5.4 Hybridisation buffer: 5x SET, 20% formamide, 0.1% IGEPAL, 25 µg mL Poly A

Reagent	Volume	Final concentration
25x SET	20 mL	5x
Formamide (100%)	20 mL	20%
IGEPAL	0.1 mL	0.1%
Poly A (1000 µg mL)	25 µL	25 µg/mL
H20	59.875	--

Table 5.5 Hybridsation buffer with probes

Reagent	Volume	Final concentration
Hybridisation buffer	4987.5 µL	-------
WE (TAM.A) 1 µg/µL	6.25 µL	1.25 ng/µL
NA1 1 µg/µL	6.25 µL	1.25 ng/µL

Whole cells were hybridised using previously developed group specific oligonucleotide probes (Touzet et al. 2008; Miller and Scholin 1998. See table 5.6). Probes were labelled at the 5' end with AlexFluor® 488 or 555 respectively. Two different dyes were used to facilitate the use of dual probe hybridization. Approximately 2-3 mL of ice-cold methanol preserved sample was filtered, by hand vacuum to avoid lysis of cells, onto 13 mm 1.2 µm isopore polycarbonate membrane filters (Whatman), held in custom filter holders similar to those in Miller and Scholin (1998) (see fig 5.4 for filter set-up).

Table 5.6 Oligonucleotide probes for whole-cell FISH

Probe/Group	Sequence	AlexFluor®	Reference
WE (TAM.A)/Group III	5'TCACCCACAGCCAAAACCTA3'	488	Touzet et al. 2008
NA1/Group I	5'AGTGCAACACTCCCACCA3'	555	Miller & Scholin 1998

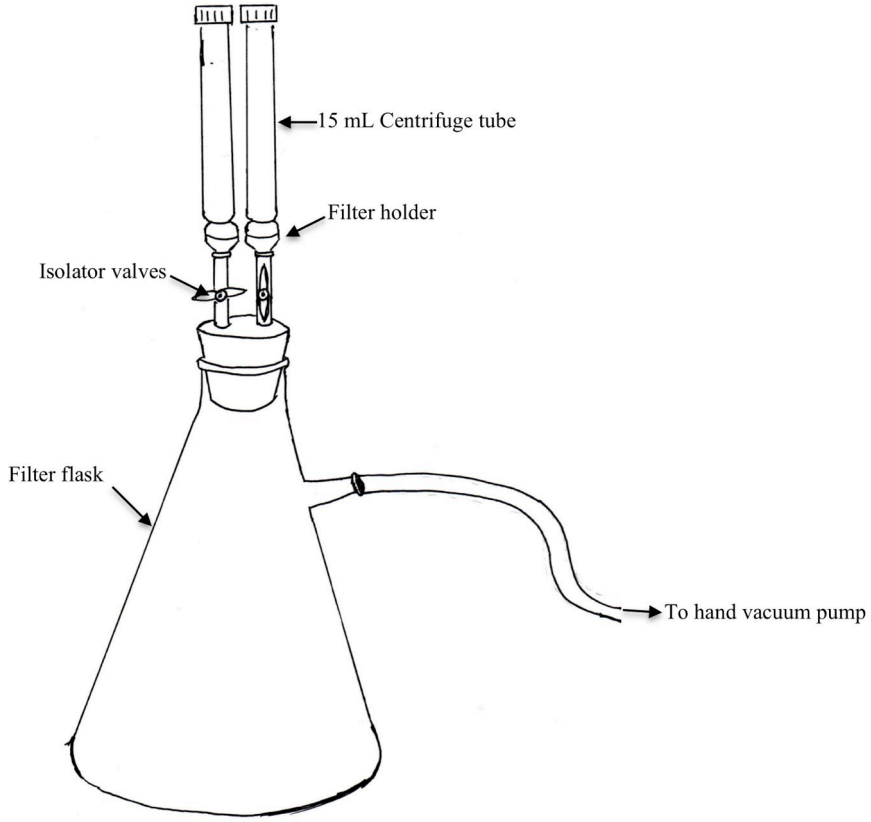


Fig 5.4 Diagram of custom filter set-up for whole-cell FISH

Filters were pre-hybridised with 400 µL hybridisation buffer for 2 mins at room temperature. Filters were then hybridised in 400 µL hybridisation buffer with NA1 & WE probes for ~60 minutes at 50°C. Hybridised filters were washed with 800 µL 0.2x SET buffer for 1 minute at 50°C and then washed with 800 µl 0.2x SET buffer for 1 min at room temperature to remove unbound probes. Filters were placed onto a labeled microscope slide, with 1 drop of Slowfade Gold (Invitrogen) and a glass cover slip added. Filters were examined using an Axiostar fluorescence microscope (Zeiss) fitted with FITC and Cy3 filters. Images were taken using a 1.3mp digital camera where possible (Brunel Microscopes). Additional control samples were

prepared as above, but without the addition of probes. These samples were instead subjected to the 'hybridisation' step with 400 μL of hybridisation buffer without probes. Controls were used to assess for auto-fluorescence of cells.

5.3 Results

5.3.1 Single cell PCR/real-time PCR

5.3.1.1 Single cell PCR

PCR amplification of the D1-D2 region of the LSU rDNA using single cells was possible for all selected isolates and all products were confirmed by agarose gel electrophoresis to be ~ 700 bp (see Fig. 5.5).

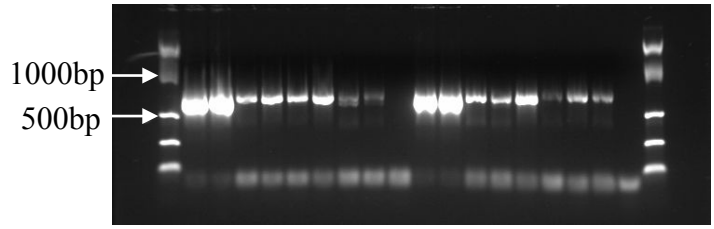
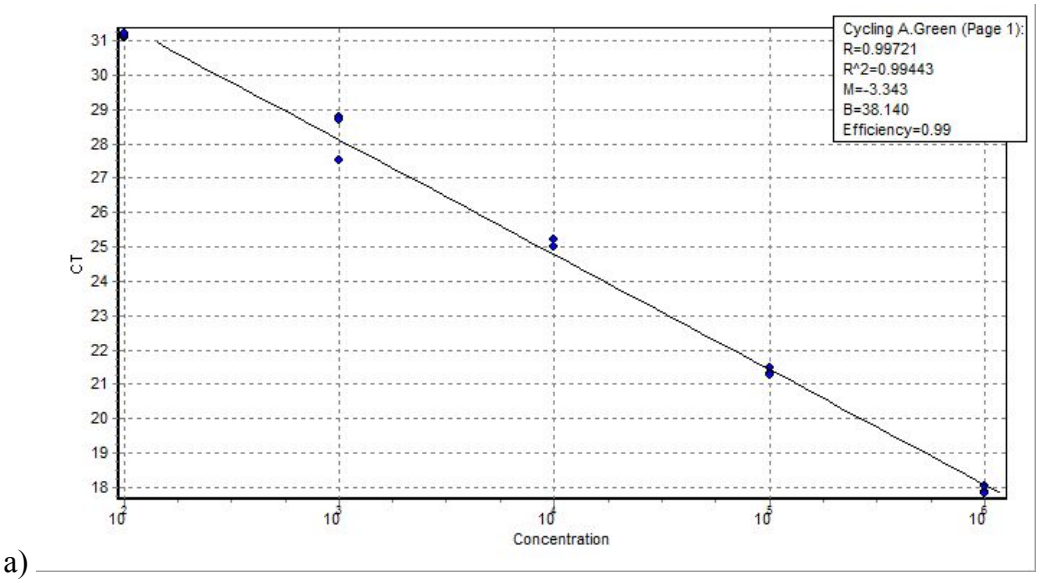


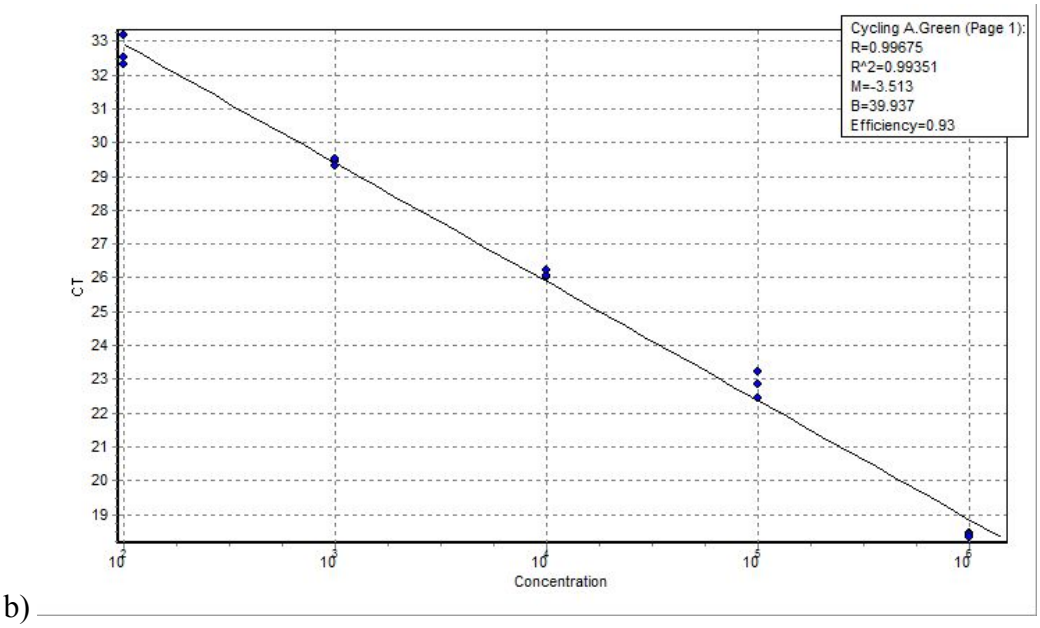
Fig. 5.5. 1% agarose gel of single cell D1-D2 LSU rDNA PCR products

5.3.1.2 Nested real-time PCR of D1-D2 LSU rDNA

Reaction efficiency for both Group I and III primers sets was good, at 99% and 93% respectively (see fig. 5.6). Melt curves displayed single peaks with no obvious sign of primer dimer or non-specific product (see fig. 5.7).



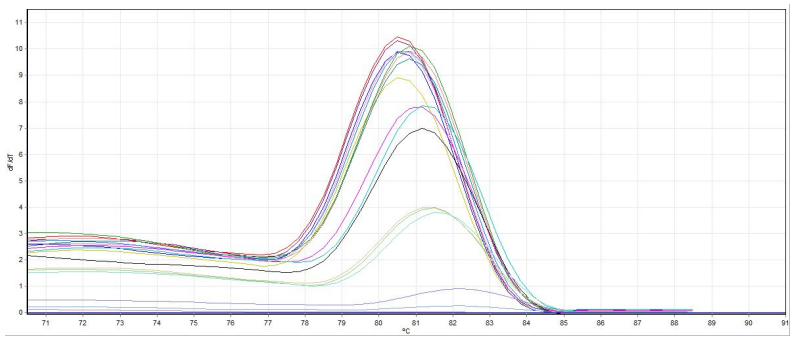
a)



b)

Fig. 5.6 Standard curves for D1-D2 LSU rDNA: a) Group I, AF1 primer. b) Group III, WE primer.

a)



b)

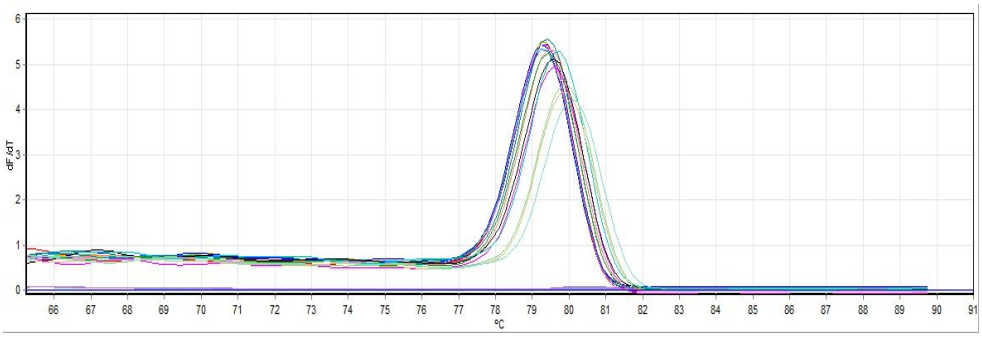
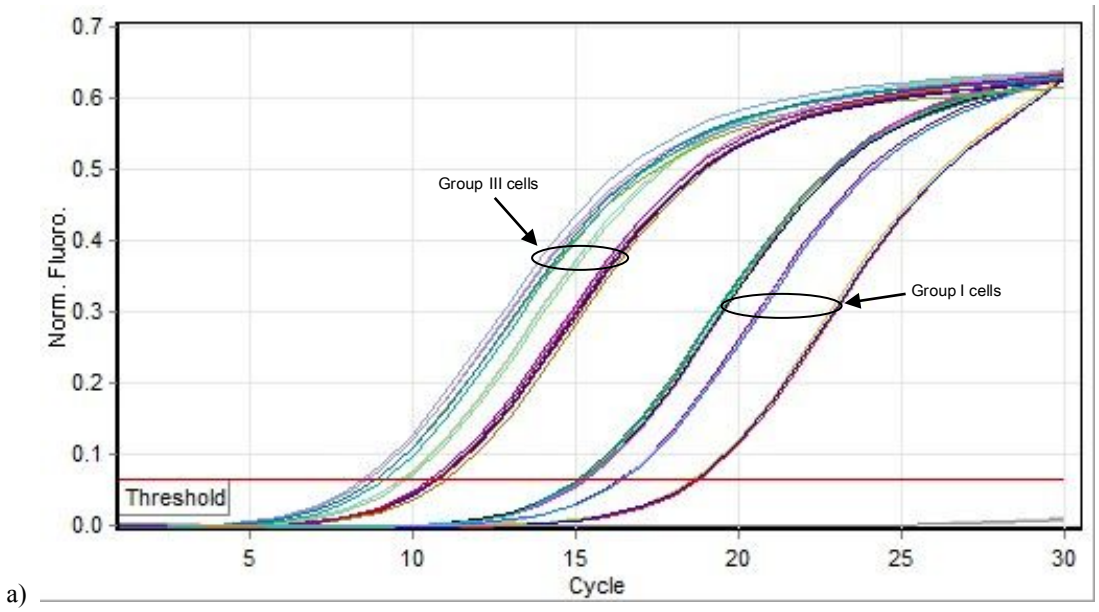
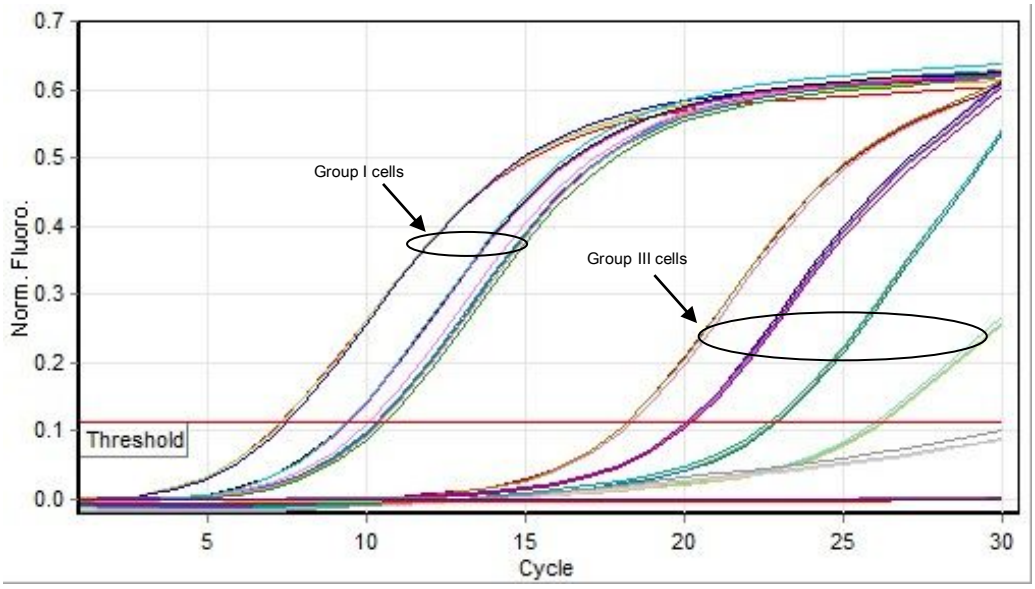


Fig. 5.7 Melt curves for D1-D2 LSU rDNA standards: a) Group I, b) Group III

Group I and Group III LSU rDNA products were apparent in real-time PCR assays for all cells/isolates tested. The ratio of each group appeared to be dependent on a dominant ribotype within each cell/isolate, with Group I cells displaying lower ct values for the Group I assay and Group III cells having lower ct values for the Group III assay (see fig. 5.8).



a)



b)

Fig. 5.8 Single cell real-time PCR cycling data. a) WE/Group III assay, b) AF1/Group I assay. Direct sequencing ribotypes of cells are indicated on each assay and clearly demonstrate the inverse relationship between ct value and starting template.

Real-time PCR products for all cells/isolates were confirmed to be ~182 bp using agarose gel electrophoresis (Figure 5.9). Direct sequences obtained from real-time PCR products appear to support the idea that all isolates, with the exception of BLA6

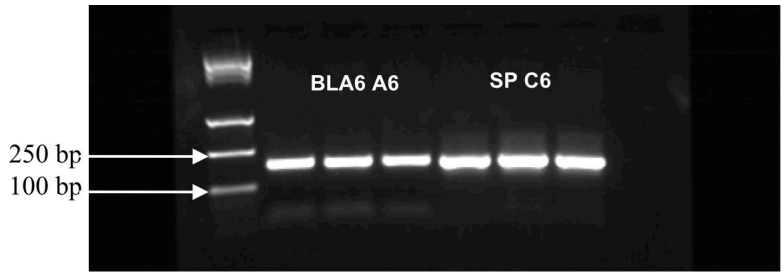


Fig. 5.9 3% agarose gel of *A. tamarense* Group I specific real-time PCR products for BLA6 A6 (Group III) and SP C6 (Group I). Difference in product quantity is evident from band width/intensity.

A6, which returned two identical Group III sequences, have at least two ribotypes. A phylogenetic tree of the aligned sequences was able to separate the two distinct groups (Figure 5.10). All Group III sequences were identical in all analysed isolates, and to Group III sequences retrieved from Genbank. In comparison there was far greater variation between the Group I sequences, even between isolates from the same location.

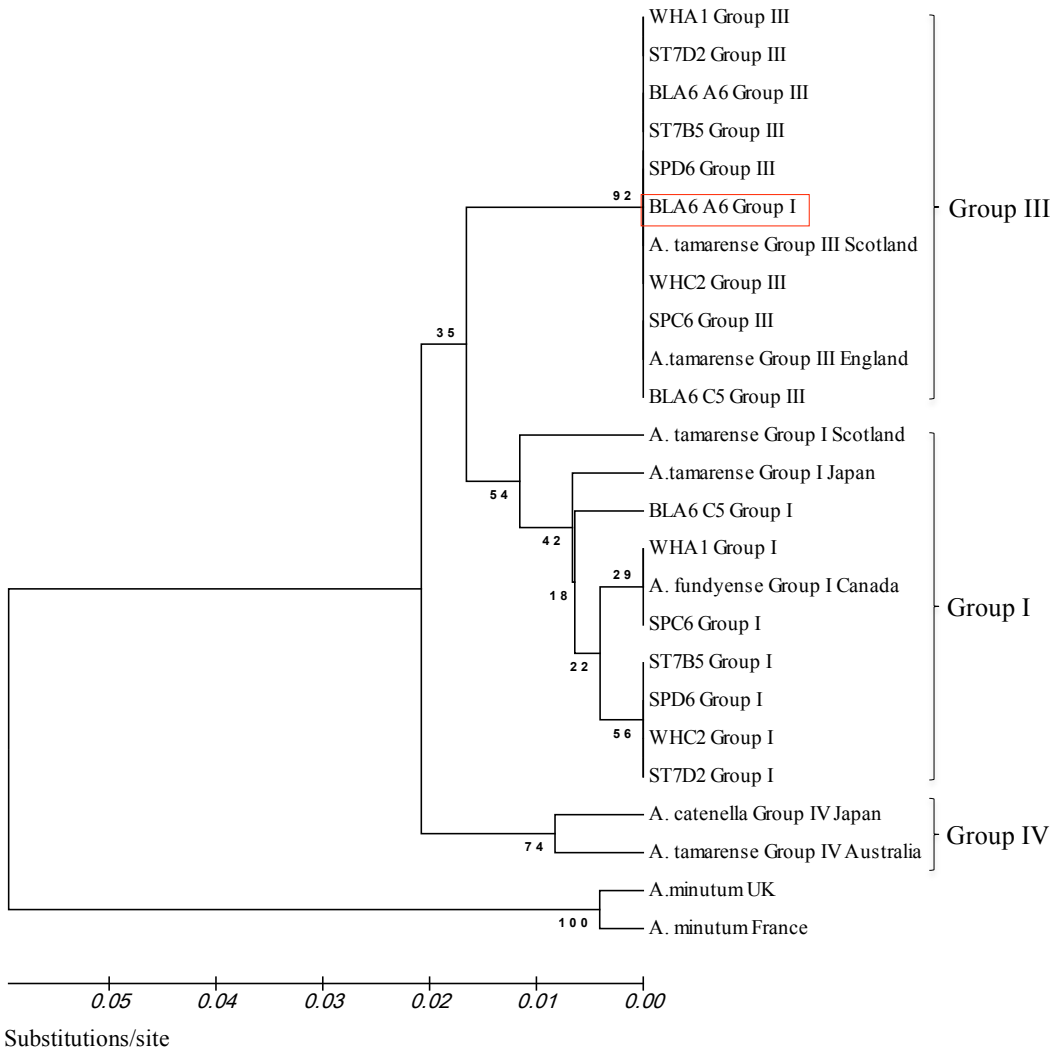


Fig 5.10 UPGMA phylogenetic tree of single cell/real-time PCR Group I and Group III sequences. Tree constructed using Mega version 6.0 (Tamura et al. 2013), Kimura-2 parameter Gamma distributed substitution model and 1000 bootstrap replicates. The anomalous BLA6 A6 Group I sequence is outlined in red.

5.3.2 *Whole-cell fluorescent in situ hybridisation*

Results of whole-cell FISH analysis for the nine isolates selected for single cell/real-time PCR were in agreement with the D1-D2 LSU rDNA sequence data used to initially ribotype isolates (see chapter 2), as there was no evidence of dual expression of rRNA in any of the isolates tested. No auto-fluorescence of controls was noted,

apart from a small round body in all cells, which can be seen in experimental cells in Fig. 5.11.

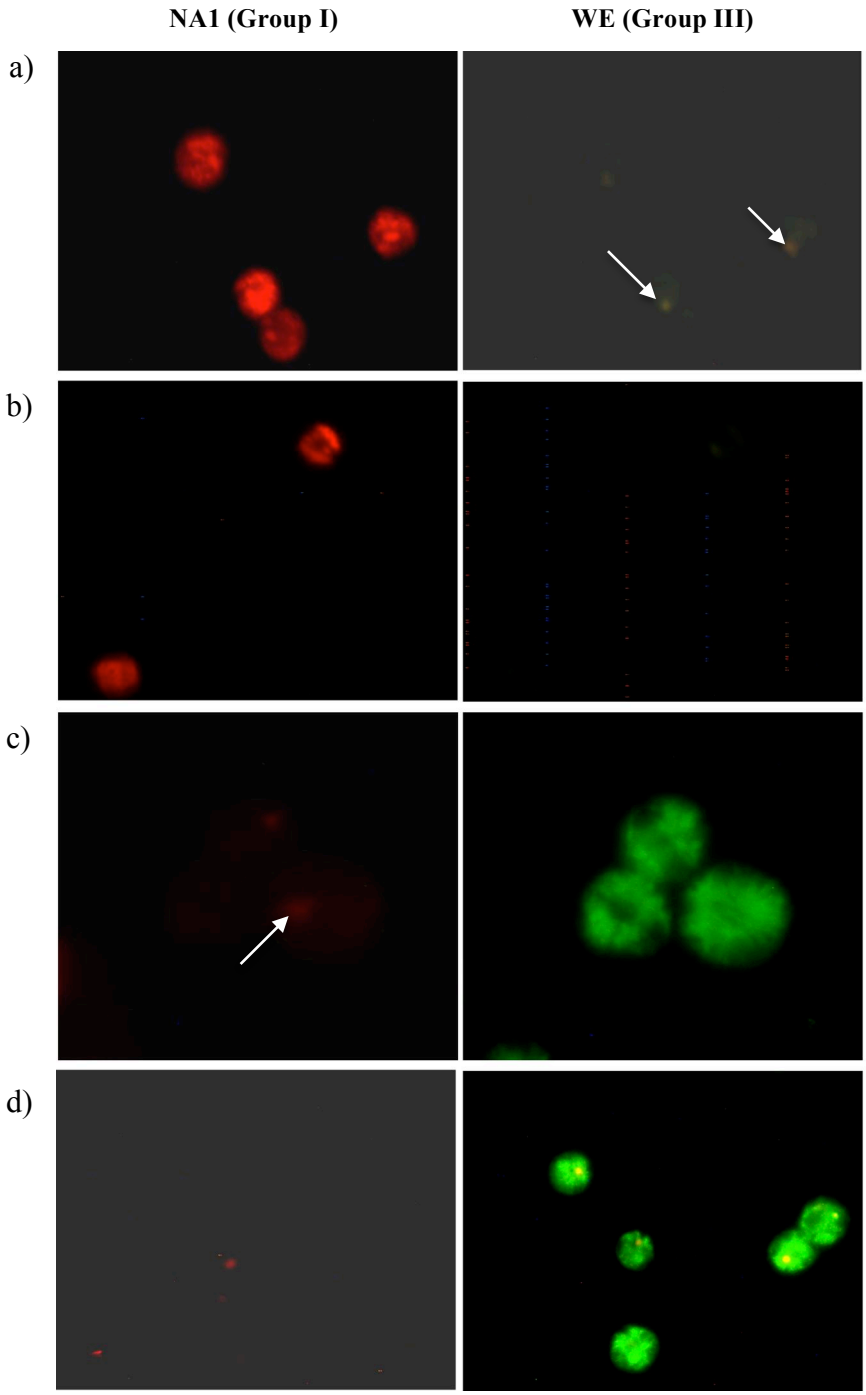


Fig. 5.11 Comparative images of whole-cell FISH dual probe assays Group I probe NA1, on the left, Group III probe WE (TAM.A) on the right. a) ST7B5 (Group I), b) SPC6 (Group I), c) WHC2 (Group III) and d) BLA6 A6 (Group III). Arrows indicate small round auto-fluorescent bodies.

5.4 Discussion

Similar findings to the data obtained from this analysis have not (to the best of my knowledge) been previously reported in *A. tamarense*. Comparable findings have been made in Scotland in *A. tamarense* cultures isolated from regions where the two groups co-occur (Marine Scotland, unpublished). But this work was not carried out on single cells and did not display the wide geographical distribution of the phenomenon indicated by the data presented here.

The amplification of Group I and III rDNA in Weymouth Harbour isolates was surprising. As it indicates that the presence of dual ribotypes in *A. tamarense* cells is not restricted to areas where groups are known to overlap and is therefore likely not to be the result of hybridisation. However, this work is in its infancy and needs to be further studied in more depth, in order to fully assess its validity and rule out experimental error. Nevertheless, there have been remarkably similar findings in other species.

For example, Gribble and Anderson (2007) reported high intra-individual variation of the D1-D6 region of LSU rDNA in species of *Protoperidinium*, *Diplopsalis* and *Preperidinium*. Reverse transcription PCR (RT-PCR) of rRNA showed less diversity suggesting that variation could be attributed to pseudogenes in these species. These results are very similar to that presented here, in that expression of rRNA and the diversity of rDNA did not correlate. However, it still remains possible that there

could be very low level expression of alternate rRNA in our isolates that was unable to be detected using whole-cell FISH.

Individuals of the Grasshopper *Podisma pedestris* have been shown to have several highly divergent ribosomal groups. However, only one group, a putatively functional group, was shown to occur at high copy number (Keller et al. 2006). Similar to *A. tamarense* the genome of *Podisma pedestris* is large, ~ 18,000 Mb, and it was suggested that there might be a causal link between genome size and the accumulation of pseudogenes in this species (Keller at al. 2006). It could therefore be possible that the extremely large size of the *A. tamarense* genome, estimated to be ~200,000 Mb (Hackett et al. 2005), could result in the accumulation of pseudogenes. Also, our study presented here focussed only on Group I and III rDNA and it would be interesting to see if rDNA sequences corresponding to other *A. tamarense* groups were also present in these isolates.

Pseudogenes associated with SSU rDNA have been reported by Scholin et al. (1993) in *A. fundyense*. It was found that North American isolates (Group I) had two distinct small subunit rDNA genes, termed A and B. In subsequent analysis B gene transcripts were not detected in total RNA extracts, whereas A gene transcripts were. Leading to the assertion that the B gene is probably a pseudogene. Additionally, Scholin et al. (1994) also reported that *A. fundyense* isolates that displayed the A and B genes also have at least two distinct classes of LSU rDNA. This may explain the higher diversity amongst Group I sequences from real-time PCR products, presented here.

A positive relationship between high rDNA copy number and polymorphisms of rDNA in individuals has been demonstrated in some species of ciliates (Gong et al. 2013). Both Group I and III *A. tamarense* have been shown to have particularly high copy numbers of rDNA. With Brosanhan et al. (2010) estimating from ~$7x10^4$ copies in Group III and up to ~10^6 copies in Group I. It is therefore possible that the diversity in rDNA sequences in our isolates is also related to high rDNA copy number.

Whilst we must treat the results of this preliminary study with caution, they also warrant further investigation. Given that so many techniques for the molecular identification and discrimination of *A. tamarense* complex species rely on the use of rDNA and rRNA, the possibility for misidentification, over estimating species abundance or distribution could be huge.

Further investigation into ribotype variation in individual isolates may also provide insights into the relationship between ribotype and mating compatibility in *A. tamarense*. A question we have been unable to address in this study. Some experiments using quantitative real-time PCR (qPCR) to assess copy number of rDNA Group I and III variants in single cells have been conducted (data not shown). However the data showed widely variable copy number between single cells of the same isolate and these were abandoned. Although, closer study of the literature has revealed that similar results have been reported by Galuzzi et al. (2009) for *A. catenella* and *A. taylori*. Galuzzi et al. (2009) found that rDNA copy number per cell in different isolates of *A. catenella* ranged from ~$1.8 x10^5$ – $5 x10^5$ and for *A. taylori*

from $7x10^3$ – $3.3x10^4$. They also noted that rDNA copy number in *A. taylori* was variable dependant on growth phase. A finding that would fit our unpublished data.

This later finding also corresponds with that of Brosnahan et al. (2010) who reported that rDNA copy number is reduced dramatically in *A. tamarense* Group I and III upon encystment. The reduction in rDNA from vegetative cells to hypnozygotes went from to ~$7x10^4$ reduced to $3x10^4$ copies in Group III and from up to 10^6 reduced to $4.5x10^3$ in copies Group I, despite hypnozygotes being diploid. In hybrid hypnoygotes the Group III rDNA was reduced to as little as ~$3x10^3$ copies. There must therefore be a mechanism for the reduction in the number of specialised rDNA chromosomes, recently described by Figueroa et al. (2014), in the *A. tamarense* complex. Investigation into the mechanism of rDNA copy number reduction and what role, if any, ribotype variation in Group I and III *A. tamarense* might play in allowing the successful formation and maturation of hypnozygotes between groups, but not the survival of progeny (as seen in chapter 3 and 4), needs to be carried out.

CHAPTER 6

CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

Previous studies of sexual reproduction in the *A.tamarense* species complex have often been contradictory. For example induction of mating is rare in nutrient replete medium and requires the limitation of nitrogen (Anderson et al. 1984), phosphorus (Anderson et al. 1984; Anderson and Lindquist 1985) or iron (Doucette et al. 1989) in the laboratory. Yet sexual reproduction has been observed in the field at nutrient levels above those that support vegetative growth in culture (Anderson et al. 1983). Hypnozygote 'seed beds' have been associated with recurrent blooms in particular areas (Anderson and Wall 1978; Anderson et al. 2005) and yet some studies have found no correlation between large blooms and increased hypnozygote deposits in sediments (Gracia et al. 2013).

Much of the data presented in this thesis are no less contradictory. However they do provide some novel insights into the mating interactions of Group I and III *A. tamarense* in culture, additional insights into the possible rDNA variation in these species, and further evidence for the potential of bias to influence isolation of a particular ribotype group.

One of the major limiting factors for the work presented in this thesis was the isolation of *A. tamarense* isolates. The fact that all isolates from Orkney were Group I, and that virtually all isolates from Belfast Lough were Group III, meant it was necessary to redesign the mating experiments. This resulted in additional isolations from other geographic regions. Similar bias, in relation to the isolation of *A. tamarense,* has been reported by other researchers (i.e. Collins et al. 2009; Genovesi et al. 2010), and so this is not a problem unique to this study.

A further limitation of the mating data presented in this thesis is the number of isolates used and the limited number of variables tested i.e. nitrogen limitation at 15°C and 20°C. This is a criticism that could also be levelled at a large number of other studies investigating mating systems in dinoflagellates. However, the isolation of cultures is labour intensive and there are finite numbers of cultures it is possible to maintain and keep healthy. Furthermore, mating experiments are extremely time consuming, even small numbers of isolates result in many pairwise crosses, and there are restrictions on the number of crosses it is possible to set up and monitor accurately at any one time.

Despite their limitations the mating experiments presented in chapters 3 and 4 did provide some novel data regarding the mating interactions of *A. tamarense* in culture. The most notable data was the variable effect of temperature on the encystment of Group I and III. The significant ($p<0.05$) reduction in hypnozygote yield for Group III/III crosses at 15°C, as compared to yield at 20°C, indicate that sexual reproduction of Group III isolates may be favoured at higher temperatures. This may become an important factor in competition between the two groups in areas of co-occurrence such as Orkney and Shetland, if seas in these areas become warmer. In addition the higher average vigour (AV) of Group I/III co-culture crosses, as compared to Group I/I, indicate that Group I isolates are more likely to reproduce with a compatible Group III isolates, that is they are more likely to out-breed, at least in culture. The implications of this in terms of natural areas of co-occurrence are huge, as it could result in the Group I population being greatly diminished. As discussed in chapter 3, this may be a factor in the apparent decrease in PSP events in Scotland in the past few decades.

Another interesting aspect regarding temperature and mating compatibility arising from this data was the apparent repressive effect of higher temperature on the mating interactions of some Group I isolates. Three additional successful Group I/I crosses occurred at 15°C as compared to 20°C. This not only suggests that lower temperatures may be preferential for sexual reproduction in Group I isolates, but it also highlights the potential perils of making assumptions about mating compatibility based on limited conditions.

The ability to predict successful crosses of *A. tamarense* progeny based on parental mating compatibility, as demonstrated in chapter 4, suggests that mating type follows a Mendelian pattern of inheritance, as previously noted (Sako et al. 1992), and that mating type may be fixed within populations. This may indicate that there are reproductively isolated strains of the same genotype in areas of the UK, perhaps exploiting different environmental niches.

Germination rates of cultured hypnozygotes in chapter 3 and 4 were low in comparison to wild type. However, they are in agreement with other studies and so this data adds weight to the idea that cultured hypnozygotes do not have the same longevity in cold storage as their wild type counterparts. Group III hypnozygotes faired a little better in chapter 4, after a shorter storage period. This data, along with that of other studies in the literature suggest that the method of hypnozygote storage is insufficient for long-term storage and does not effectively replicate the environment of natural anoxic sediment.

This study was not able to address the question of whether ribosomal genes, and hence ribotype, influence the system of mating compatibility in *A. tamarense*. It did however produce some novel data regarding the potential for dual ribotypes in Group I and III isolates from a range of locations in the UK. The data obtained using a single cell nested PCR/qPCR suggest that at least two variants of rDNA are present in these isolates and that these variants correspond to Group I and III ribotypes as defined by Lilly et al. (2007). As stated in the discussion in chapter 5, caution of the data presented is warranted. However, there are reasonable grounds to suspect that

these data may be accurate and it is also clear that the *A. tamarense* complex with their extremely large genomes, pseudogenes and high rDNA copy numbers are model organisms to escape from concerted evolution.

6.2 Future work

6.2.1 Laboratory mating experiments

Further mating experiments are required to establish if mating compatibility of isolates is fixed, or if compatibility can be induced with previously non-compatible isolates under different environmental conditions. As was the case for some Group I isolates. Further variables need to be examined, including a broader temperature range, different nutrient limitation, salinity and light. A multi-variable approach similar to that used by Figueroa et al. (2011) should be employed to assess the effect of change in more than one parameter at a time.

6.2.2 Development of long-term cultured hypnozygote storage

The issue of long-term storage of cultured hypnozygotes needs to be addressed. Degradation of hypnozygotes was a major problem in this study and has likely affected others, given that it is often reported that cultured hypnozygotes have lower germination success rates than wild type. The development of new storage methods that better replicate the natural environment may result in more robust cultured hypnozygotes and a better understanding of germination characteristics of these species in the natural environment.

6.2.3 Analysis of rDNA variation using next generation sequencing

Further analysis of rDNA variation in *A.tamarense* using next generation sequencing is planned in order to establish if variation exists and to what extent. To this end work has begun on the design of primers to amplify a ~400 bp fragment of the D1-D2 LSU rDNA, incorporating all five *A. tamarense* complex ribotype groups. In addition, RT-PCR of rRNA expression could be carried out to assess if a single, or dominant, ribotype is being expressed as suggested by the whole cell FISH analysis.

6.2.4 Study of the variability and effects of sampling bias

Unintentional sampling bias may have affected the isolation of *A. tamarense* in this study. As other researchers have also reported this, an analysis of how sampling bias may affect research and data involving species isolated from natural samples would be interesting. Much of what is known about the mating interactions of *A. tamarense* is reliant solely on laboratory mating experiments and, therefore, isolates that have been isolated from nature at some point. Some interesting questions arise such as: are there any patterns of bias? For example are culture conditions favouring certain lineages? Is there any way to reduce bias in isolation?

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APPENDIX A

ENUMERATION OF CELLS IN LUGOL'S IODINE PRESERVED FIELD SAMPLES

This method was used to count cells in field samples as described in chapter 2. It permits the enumeration (cells L^{-1}) of specific genera/species either as a whole population or as a more limited set of target cells i.e. harmful genera/species. The basic principle of the method is as follows; an aliquot of the field sample is poured into a sedimentation chamber of known volume and allowed to settle for a minimum time (dependant on the volume settled). During this period cells will sediment onto a glass plate in the base of the chamber. Cells can then be counted using an inverted microscope and the number of cells L^{-1} estimated using some simple calculations.

Equipment

- Sedimentation chambers
- Sedimentation platform
- Inverted microscope
- Counting 'clicker'
- Stage micrometer (for calibration)
- Callipers (for calibration)

Before carrying out the counting, all equipment was calibrated to ensure an accurate calculation of cell numbers.

Sedimentation chambers

The basic anatomy of a sedimentation chamber is illustrated below. Sedimentation chambers routinely used in this study were 20 mL in volume, although there are chambers with smaller volumes for samples with particularly high cell density or larger volume for low cell density samples.

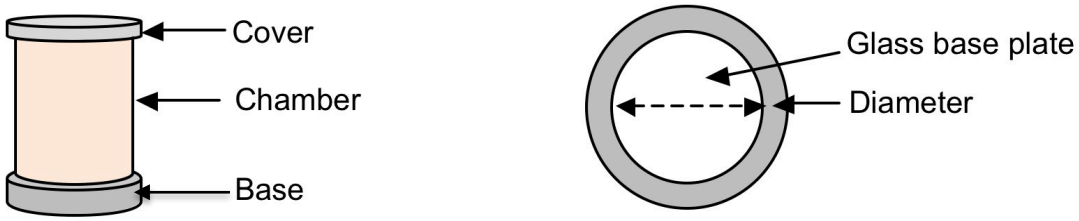

Diagram of Sedimentation Chamber

It was necessary to know the area of the glass base of each sedimentation chamber used. The diameter (in mm) of the glass base was accurately measured using callipers. The area (mm^2) was then calculated using the equation to calculate the area of a circle: πr^2.

Diameter of the bases of all nine 20 mL chambers used in this study = 25mm
Radius of base = 12.5mm
Area of base = πr^2
= π x $12.5mm^2$
= $490mm^2$

Inverted microscope

If cells were high in density it was not practical to count the number across the entire base area. In this case 10 random fields of view (FOV) were counted. The area of the FOV changes at different magnifications. The magnification utilised for the majority of FOV counts was x200. To calculate the area of FOV, the diameter (mm) of FOV was measured using a 1 mm stage micrometer . The area can then be calculated in the same way as for the chamber base.

Size of FOV of Olympus inverted microscope at x200 magnification = 0.98mm
Radius of FOV = 0.49mm
Area of FOV = πr^2

$= \pi \times 0.49mm^2$

$= 0.754mm^2$

Next it was necessary to calculate the number of FOV's at a particular magnification, this is known as the microscope field factor.

$$\text{Microscope Field Factor} = \frac{\text{Area of base of counting chamber}}{\text{Area of FOV}}$$

$$= \frac{490}{0.754} = 650 \text{ FOV's at x200 magnification}$$

Method

1. Sedimentation chambers were assembled by placing a glass plate (cover slip) in the base, adding a small amount of vacuum grease to the thread of the chamber and screwing into the base to form a tight seal.
2. Field samples were acclimated to room temperature (if refrigerated), this minimises bubble formation.
3. Samples were inverted ~50 times to thoroughly mix.
4. Samples were poured into the sedimentation chamber and a cover placed on the top of the chamber. NB: This should form a vacuum seal and there should be no bubbles visible.
5. The sedimentation chamber was placed on the sedimentation platform and allowed to settle for ~12-24h.
6. The base of the chamber was viewed at low magnification (x40 or x100) to assess the cell density.
7. The following criteria were used to decide whether to count the number of target cells across the entire base or random FOV's. It was sometimes necessary to count different cells within the same sample using different methods, depending on their relative density. It was also necessary to view smaller cells at a higher magnification (x400) to accurately identify them.

Low Density Count – less than 4 targets cells per FOV. A whole count of the number of cells within the entire chamber base was carried out at either x100 or x200 magnification depending on cell size. Cells L^{-1} were calculated for low density counts using the following equation:

$$\text{Number of cells observed} \times \left[\frac{1000}{\text{volume settled (mL)}} \right]$$

High Density Count – more than four target cells per FOV (x200 magnification). Cells within 10 random FOV's were counted and an average per FOV obtained. Cells L^{-1} were calculated for high density counts using the following equation:

$$\left(\frac{\text{Total number of cells counted}}{\text{10 (Number of FOV's counted)}} \right) \times \text{microscope field factor} \times \left(\frac{1000}{\text{volume settled (mL)}} \right)$$

Cell counts were recorded on an excel spreadsheet. A counting sheet similar to the one on the following page was used to record data during counting.

Example of Cell Count Sheet

Sample: ……..

Location: ……

Date of collection: ……..…....

Date of count: ……..

Counted by: ……

Volume of sedimentation chamber: ……....**ml**

Genus	Species	Whole count or FOV?	Cells counted	Cells/L	% total
Alexandrium					
Scrippsiella					
Dinophysis					
Pseudo-nitzschia					
Ceratium					
Prorocentrum	*lima* *minimum*				
Lingulodinium	*polyedrum*				
Protoceratium	*reticulatum*				
Protoperidinium	*crassipes/curtipes* *depressum*				
Thalassiosira					
Chaetoceros					
Penate diatoms					
Athecate dinoflagellates					

Calculations: Whole counts (low density): Number of cells observed x $\left[\frac{1000}{\text{volume settled (ml)}}\right]$

FOV counts (high density): $\left[\frac{\text{Total number of cells counted}}{\text{Number of FOV's counted}}\right]$ x microscopic field factor x $\left[\frac{1000}{\text{volume settled (ml)}}\right]$

APPENDIX B

CELL COUNT DATA FOR FIELD SAMPLES - MAY 2010

Station 2 - 24.5.10

Genus/species	**Cells/20 mL**	**Cells/L**
Scrippsiella sp.	22	1100
Guinardia delicatula	1560	78000
Alexandrium sp.	6	300
Gymnodimium sp.	21	1050
Pseudonitzchia sp. (small <80µm)	845	42250
Protoperidinium bipes	3	150
Ceratium linateum	1	50
Dinophysis acuta	4	200
Protoperidinium cf.mite	2	100
Athecate dinoflagellates (~10 - 20 µm)	6	300
Chaetoceros sp. (~20µm)	1820	91000
Thalassiosira sp. (25 µm)	1170	58500
Thalassiosira sp. (15 µm)	975	48750
L. minimus (~3µm D)	26	1300
Prorocentrum minimum	143	7150
Prorocentrum cf. balticum	1	50
Gyrodinium sp.	4	200
Karenia	8	400
cf. P. curvipes	1	50
Pseudonitzchia sp. (large >80µm)	520	26000
P. ovatum	1	50
cf.Leptocylindrus danicus	910	45500
Chaetoceros sp. (~10µm)	975	48750

Station 3 - 24.5.10

Genus/species	**Cells/20 mL**	**Cells/L**
Scrippsiella sp.	12	600
Guinardia delicatula	40	2000
Alexandrium sp.	5	250
Gymnodimium sp.	10	500
Pseudonitzchia sp. (small <80µm)	19	950
Protoperidinium bipes	5	250
Dinophysis sp.?	1	50
thecate dinoflagellates (~10 - 20 µm)	6	300
Thalassiosira sp. (30 µm)	5	250
Prorocentrum minimum	24	1200
Karenia	11	550
Pseudonitzchia spp. (large >80µm)	3	150
cf. Alexandrium spp. (small ~15x20µm)	1	50

Station 5 - 24/05/10

Genus/species	**Cells/20 mL**	**Cells/L**
Scrippsiella sp.	27	1350
Prorocentrum minimum	9	450
Guinardia sp.	715	35750
Alexandrium sp.	8	400
Guinardia deliculata	380	19000
cf. Heterocapsa sp.	2	100
Gymnodimium sp.	14	700
Thalassiosira sp. (15 µm)	325	16250
Pseudonitzchia sp. (large >80µm)	325	16250
Chaetoceros sp. (10µm)	16	800
cf. Gonyaulax sp.	1	50
Pseudonitzchia sp. (small <80µm)	650	32500
Thalassiosira sp. (50 µm)	9	450
Dinophysis acuta	3	150
Protoperidinium bipes	2	100
cf. Alexandrium sp. (small ~20 µm)	1	50
Ceratium linateum	1	50
Dionphysis acuminata	3	150
cf. Odontella sp.	8	400
Protoperidinium sp. (60 µm)	1	50
Athecate dinoflagellates (~10 - 20 µm)	12	600

Station 6 -26/05/10

Genus/species	**Cells/20 mL**	**Cells/L**
Scrippsiella sp.	8	400
Guinardia delicatula	1528	76400
Alexandrium sp.	2	100
Gymnodimium sp.	1	50
Pseudonitzchia sp. (large >80µm)	103	5150
Chaetoceros sp. (10µm)	4	200
cf. Gonyaulax sp.	1	50
Pseudonitzchia sp. (small <80µm)	44	2200
Thalassiosira sp. (50 µm)	325	16250
Protoperidinium bipes	2	100
Ceratium linateum	2	100
Dinophysis cf.acuminata	1	50
Protoperidinium cf.mite	1	50
Athecate dinoflagellates (~10 - 20 µm)	295	14750
Chaetoceros sp. (25µm)	10	500
Dictyocha sp.(25µm)	1	50
Rhizosolenia sp. (200µm L x 10 µm w)	1	50
cf.Leptocylindrus danicus	748	37400
Prorocentrum cf. Compressum	423	21150
Thalassiosira sp. (30 µm)	260	13000
Thalassiosira sp. (20 µm)	195	9750
Small thecate dino >20 µm	325	16250
L. minimus (~3µm D)	488	24400

Station 7 – 26/05/10

Genus/species	**Cells/20 mL**	**Cells/L**
Ceratium lineatum	2	100
Prorocentrum micans	2	100
Dinophysis acuminata	3	150
Scrippsiella sp.	6	300
Gymnodimium sp.	7	350
Protoperidinium mite	2	100
Alexandrium sp.	2	100
cf. P. curvipes	2	100
P. bipes	3	150
Thalassiosira sp. (45µm)	51	2550
Dactyliosoleu sp.	1	50
Pseudonitzchia sp. (>80 µm)	97	4850
cf. Gonyaulax sp.	1	50
Guinardia delicatula	2632	131600
Leptocylindrus sp.	65	3250
Chaetoceros sp.	97	4850
Thalassiosira sp. (35µm)	65	3250
Pseusonitzchia sp. (small < 80 µm)	65	3250