SEXUAL REPRODUCTION AND BLOOM DYNAMICS OF TOXIC DINOFLAGELLATES FROM AUSTRALIAN ESTUARINE WATERS

by

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DECLARATION AND AUTHORITY OF ACCESS

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Naomi S. Parker

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ABSTRACT

Of the dinoflagellates known to form toxic blooms in Australian estuaries three, *Alexandrium minutum*, *Alexandrium catenella* and *Gymnodinium catenatum* produce paralytic shellfish toxins that can lead to the potentially fatal paralytic shellfish poisoning in humans. These three species and a fourth dinoflagellate *Protoceratium reticulatum* all have the capacity to reproduce both by vegetative cell division and by sexual reproduction. The product of sexual reproduction in all cases is a resting cyst, which can sink to the sediments and remain dormant for a time. Resting cyst production results in a coupling between the benthic and pelagic components of estuarine systems and forms an important part of the ecological strategy of dinoflagellates.

A variety of aspects of sexual reproduction of these four species were investigated. These included: life cycle description (*A. minutum*); mating system studies (all four species); assessing the use of sexually compatible strains in high biomass culture systems (*A. minutum*); experimental studies of encystment and excystment cues (*A. minutum* and *G. catenatum*); and a three-year field study of vegetative and sexual reproduction of *G. catenatum* in an estuary in south-east Tasmania.

The complete life cycle of *A. minutum* was described and was found to be similar to other *Alexandrium* species. *Alexandrium minutum* was also found to produce temporary resting cysts in response to cold shock and to have a short (approximately 4 weeks) minimum requisite dormancy period. The mating systems of all four species were investigated. *Alexandrium catenella* strains from three Australian populations were found to have simple heterothallic mating systems with essentially two mating types. The other three species all had mating systems of greater complexity with gradients of affinity between strains rather than discrete mating types. These mating type affinities were also found to vary over years in *A. minutum* and *G. catenatum*. Cluster analysis was demonstrated to be a useful method for describing these gradients.

Encystment and excystment of *A. minutum* and *G. catenatum* involves both biological and environmental factors. Both these dinoflagellates had defined temperature ranges for encystment and excystment and evidence was found for the action of pheromone-like substances in encystment. Low irradiance and low nutrients influenced encystment, and nutrient depletion had a greater effect on encystment of *A. minutum* than of *G. catenatum*. The influence of algicidal bacteria on both encystment and excystment was tested for the first time and found to have no effect while low oxygen and low light greatly reduced

excystment. The capacity for sexual reproduction by *A. minutum* did not significantly effect biomass production in high biomass culture systems while growth rate increased with aeration.

In the three year field study, four *G. catenatum* blooms were observed, two in summer and two in autumn. Sexual reproduction occurred throughout summer blooms but was below detection levels in autumn blooms and probably controlled by temperature. The importance of river flow for dinoflagellate blooms in this system was identified. Dinoflagellate blooms, including *G. catenatum*, were absent in the first year of the study which may be attributed to increased flushing of the estuary due to high and persistent flow of the Huon River in that year which may also have reduced diatom biomass and subsequent nutrient remineralisation. A dual strategy for the contribution of resting cysts in this system is suggested whereby frequent germination of resting cysts from shallow sediments contributes to bloom populations and overwintering biomass while deeply buried and rarely resuspended resting cysts contribute to long-term survival of the population in the estuary. The results of all investigations for *A. minutum* and *G. catenatum* were synthesised into conceptual frameworks of sexual reproduction and bloom dynamics within natural systems.

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DEDICATION

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CHAPTER 1

INTRODUCTION

GENERAL INTRODUCTION

Humans are increasingly reliant on the oceans for food, the aquaculture industry is expanding and increased human population densities impose ever greater stresses on coastal zones world wide (State of the Environment, Australia, 1996). This combination of factors poses a challenge to managers and scientists alike to find ways to use our marine environment in a sustainable way. One major concern for estuaries and coastal zones is the apparent increase of harmful algal blooms (HABs)(Hallegraeff, 1993). Although harmful and toxic algal blooms have posed a threat to ecosystems and human health for millennia, over the last thirty years, the frequency of occurrence of HABs has increased, and the distributions of many toxic species have expanded (Smayda, 1990; Hallegraeff, 1993). Factors such as increased eutrophication and the transport of organisms in ballast water or through shellfish translocation have been implicated in the increased HAB occurrence (Hallegraeff, 1993).

Many of the known harmful algal bloom species are dinoflagellates i.e. species belonging to the phylum Dinophyta, Class Dinophyceae. Fossil evidence indicates that the Dinophyta are a very ancient group, with a minimum age of 400 million years (van den Hoek et al., 1995). The majority of dinoflagellates are unicellular flagellates with two dissimilar flagella: the transverse flagellum, which usually lies in a transverse furrow and the longitudinal flagellum, which runs along a longitudinal groove. Both photosynthetic and heterotrophic species are known (van den Hoek et al., 1995).

Of the diverse range of microalgae, dinoflagellates are particularly fascinating organisms, exhibiting biological phenomena such as bioluminescence, symbiosis with invertebrates, parasitism, complex life cycles and various nutritional strategies (autotrophy, heterotrophy and mixotrophy) (Taylor, 1987). Of the more than 2000 extant species of dinoflagellate, approximately 60 are known to produce toxins (Steidinger and Tangen, 1997). Okadaic acid, a diarrhoeic shellfish toxin and tumour promoter is found in many dinoflagellates of the genera *Dinophysis* and *Prorocentrum*, and brevetoxins (neurotoxic shellfish toxins) are produced by *Gymnodinium breve* and other dinoflagellates (Ragelis, 1984). A potent mammalian neurotoxin, saxitoxin, and its analogues, the gonyautoxins, neosaxitoxin and C toxins (the paralytic shellfish toxins - PSTs) are produced by *Gymnodinium catenatum* and many species of the genus *Alexandrium* (Ragelis, 1984). Dinoflagellate toxins bioaccumulate in fish and shellfish, which can then become toxic to other parts of the food chain including humans (Ragelis, 1984).

Dinoflagellates are an important component of marine, freshwater and estuarine systems and can reach high cell densities which can colour the water (van den Hoek et al., 1997), and

contribute significantly to primary production and biomass. Blooms of toxic dinoflagellates can have major environmental and economic impacts. At the environmental level toxic dinoflagellate blooms have been found to cause fish kills (e.g. *Cochlodinium polykrikoides*, Park et al., 2001 and *Gymnodinium breve,* Steidinger et al., 1998), and to result in mass mortalities of zooplankton, pelagic and demersal fish and benthic invertebrates (e.g. *Karenia brevisulcata* in Wellington Harbour, New Zealand, Wear and Gardner, 2001). There is also evidence that toxic dinoflagellates may cause death in marine mammals (Anderson and White, 1992; Trainer and Baden, 1999). Economically, toxic dinoflagellate blooms can have huge impacts on both aquaculture and wild fisheries, resulting in closure to harvesting for shellfisheries (up to nine months of the year for a mussel farm in one Tasmanian estuary - G. Schroeter, personal communication), significant mortalities of caged finfish (Whyte et al., 2001) or reductions in fish stocks (Wear and Gardner, 2001; Chang et al., 2001).

Toxic dinoflagellate blooms have been known from Australian waters since the mid 1980s (Hallegraeff and Sumner, 1986) and have had a major impact on the shellfish industry in southeast Tasmania. As such toxic dinoflagellate blooms pose a significant threat to Australia's coastal and estuarine environment and to the growing aquaculture industry. *Gymnodinium catenatum* blooms in south eastern Tasmania have resulted in closure of shellfish farms for periods of up to nine months in some years due to unacceptable toxicity levels in shellfish (> 80μg saxitoxin / 100 g shellfish meat, USFDA). Two other PST producing dinoflagellates, *Alexandrium minutum* and *Alexandrium catenella* are also known to form irregular blooms in Australian waters (Cannon, 1990; Hallegraeff et al., 1991). All three of these dinoflagellates are able to reproduce both asexually (also known as vegetative reproduction) and sexually, the latter resulting in the production of a resting cyst (Blackburn et al., 1989; Bolch et al., 1991; Yoshimatsu, 1981).

1.1 REPRODUCTION OF DINOFLAGELLATES

Many dinoflagellates, including many toxic species, reproduce through both asexual and sexual reproduction. Detailed studies since the 1970s, by von Stosch (1964, 1965, 1972, 1973) and others (e.g. Beam and Himes, 1974; Pfiester, 1975, 1976, 1977; Walker & Steidinger, 1979) have documented the complex life history stages of various dinoflagellate species.

Asexual or vegetative reproduction is the more commonly observed form of reproduction in dinoflagellates and is the result of a haploid (1N, i.e. a single set of chromosomes) cell undergoing binary fission to produce two to four cells with the same chromosome number. Sexual reproduction is more rarely observed and involves the production of gametes that fuse to produce a diploid (2N i.e. two sets of chromosomes) zygote. Sexual reproduction in

dinoflagellates is not reproduction in the strict sense since it generally involves a reduction in cell number rather than an increase but this term is commonly used in the dinoflagellate literature and will be used throughout this thesis. Sexual reproduction facilitates gene recombination and often results in the formation of a resting cyst or hypnozygote, which is generally considered to be a survival mechanism for environmental extremes (Pfiester and Anderson, 1987).

1.2 SEXUAL REPRODUCTION OF DINOFLAGELLATES

By 1987 the sexual life cycles of 22 dinoflagellate species had been detailed (Pfiester and Anderson, 1987). There are now more than 50 dinoflagellate species for which sexual life cycles have been described (summarised in Table 1.1). Some 'typical' dinoflagellate life cycles are now recognised (Pfiester, 1989). The life cycle of *Gymnodinium catenatum* is one example of a relatively simple sexual life cycle that has been observed in several different species (Blackburn et al., 1989, Figure 1.1) with some variations. The sexual life cycle of *G. catenatum* commences with gametogenesis. Gametes are isogamous (morphologically identical to one another but different from the vegetative cell) in this case but this varies from species to species. Fusion of gametes to form a planozygote follows. The planozygote then forms a non motile resting cyst or hypnozygote, which can sink to the sediments and later germinate to produce a germling or planomeiocyte. The planomeiocyte is then able to divide, and vegetative or asexual reproduction resumes.

There are three key elements in the sexual reproduction of resting cyst forming dinoflagellates. These are: mating systems, which determine which individuals are sexually compatible; encystment, the process by which resting cysts are formed; and excystment, the germination of resting cysts. Knowledge of each of these elements is crucial to understanding the autecology of a given species.

1.2.1 MATING SYSTEMS

Although some dinoflagellates are self-fertile, i.e. sexual reproduction occurs within clonal culture (homothallic), many species are outcrossing i.e. reproductively compatible clones of different 'mating types' must be combined in order for sexual reproduction to occur (heterothallic). Mating type is the genetically determined physiological or biochemical difference between individuals that determines which other individuals they are sexually compatible with (Sonneborn, 1957). Mating systems vary in complexity from homothallic systems to heterothallic binary systems (where all individuals are either mating type (+) or mating type (-) with only (+)(-) combinations resulting in sexual reproduction), to multiple mating type systems with varying levels of compatibility between groups (Destombe and Cembella, 1990; Blackburn et al., 2001).

Table 1.1: Dinoflagellates for which sexual life cycles are known, their thallism and conditions for induction of sexuality species. Species names in brackets indicate synonyms/basionyms under which life cycle literature has been published for these species.

Species	Thallism	Induction of sexuality	References
Alexandrium catenella (Protogonyaulax catenella)	Heterothallic	Nutrient depletion	Yoshimatsu 1981, Yoshimatsu 1984
Alexandrium hiranoi (Goniodoma pseudogonyaulax)	Homothallic	No cue required	Kita et al. 1985, Kita et al. 1993
Alexandrium lusitanicum	Homothallic	N and Fe limitation	Silva & Faust , Blanco 1995
Alexandrium minutum	Heterothallic		Bolch et al. 1991, Parker and Blackburn in prep.
Alexandrium monilatum (monilata)	---	N limitation	Walker & Steidinger 1979
Alexandrium ostenfeldii	Heterothallic		Jensen & Moestrup 1997
Alexandrium pseudogonyaulax	---	Nutrient depletion	Montresor 1995, Montresor & Marino 1996
Alexandrium tamarense (Alexandrium excavatum / Protogonyaulax tamarensis / Gonyaulax excavata)	Heterothallic / Homothallic	Increased temperature, nutrient depletion	Dale 1977, Anderson & Wall 1978, Dale et al. 1978, Turpin et al. 1978, Anderson 1980, Destombe & Cembella 1990
Alexandrium taylori	Homothallic	N limitation	Giacobbe &Yang 1999, Garcés et al. 1998
Amphidinium carterae	Homothallic	Aging cultures	Cao Vien 1967, 1968
Amphidinium klebsii	---	Inc. salinity	Barlow & Triemer 1988
Ceratium comutum	Heterothallic	Dec. temp., day-length and light intensity	von Stosch 1965
Ceratium horridum	---		von Stosch 1964
Coolia monotis	Homothallic	Addition of mangrove extract	Faust 1992
Crypthecodinium (Gyrodinium) cohnii	Homothallic	N and P limitation	Beam & Himes 1974, Tuttle & Loeblich 1974
Cystodinium bataviense	---		Pfiester & Lynch 1980
Dinophysis fortii	---		Uchida et al. 1999
Dinophysis norvegica	---		Subba Rao 1995
Dinophysis pavillardi	Homothallic	Transfer into nutrient replete media	Giaccobe & Gangemi 1997
Gambierdiscus toxicus	---		Taylor 1979
Glenodinium lubiniensiforme	Heterothallic	Nutrient depletion	Diwald 1937
Gloeodinium montanum	Homothallic		Kelley 1989, Kelley & Pfiester 1990
Gonyaulax apiculata	---		Hickel & Pollingher 1986
Gymnodinium catenatum	Heterothallic		Blackburn et al. 1989, Blackburn et al. 2001
Gymnodinium fungiforme	---	Decreased food	Spero and Morée 1981
Gymnodinium mikimotoi	---		Ouchi et al. 1994

Table 1.1 continued: Dinoflagellates for which sexual life cycles are known, their thallism and conditions for induction of sexuality. Species names in brackets indicate synonyms/basionyms under which life cycle literature has been published for these species.

Species	Thallism	Induction of sexuality	References
Gymnodinium nolleri	Heterothallic	Temperature window, P limitation	Ellegaard et al. 1998
Gymnodinium paradoxum	Heterothallic		Von Stosch 1972
Gymnodinium pseudopalustre	---	Low temp., short day, N, P limitation	von Stosch 1973
Gyrodinium uncatenum	---		Tyler et al. 1982, Coats et al. 1984
Helgolandidinium subglobosum	Homothallic	Aging cultures	von Stosch 1972
Karenia brevis (Gymnodinium breve, Ptychodiscus brevis)	Heterothallic and homothallic strains	N limitation, cold temp., blue light	Walker 1982, Steidinger et al. 1998
Noctiluca scintillans (miliaris)	Homothallic, Heterothallic		Zingmark 1970, Hofker, 1930, Schnepf & Drebes 1993
Oxyrrhis marina	Homothallic	Change in food source	von Stosch 1972
Peridinium balticum	---	N deficiency	Chesnick 1987, Chesnick & Cox 1989
Peridinium bipes	---		Zhang & Li 1990
Peridinium cinctum f. ovoplanum	Homothallic	N deficiency	Pfiester 1975
Peridinium cunningtonii	Homothallic	N and P deficient medium	Sako et al. 1984
Peridinium gatunense	Homothallic	N deficient medium	Pfiester 1977
Peridinium inconspicuum	Homothallic	N deficiency	Pfiester et al. 1984
Peridinium limbatum	Homothallic	N deficiency	Pfiester & Skvarla 1980
Peridinium penardii	Homothallic	N and P deficient medium	Sako et al. 1987
Peridinium volzii	Heterothallic	N deficiency	Pfiester and Skvarla 1979
Peridinium willei	Homothallic	N deficiency	Pfiester 1976
Pfiesteria piscicida	---		Steidinger et al. 1996
Polykrikos kofoidii	---		Morey-Gaines & Ruse 1980
Prorocentrum lima	---		Faust 1993
Pyrodinium bahamense	Heterothallic		Corrales et al. 1995
Pyrophacus steinii	Heterothallic		Pholpunthin et al. 1999
Scrippsiella hangoei	---		Kremp & Heiskanen 1999
Scrippsiella minima	---		Gao & Dodge 1991
Scrippsiella trochoidea	---	N, P limitation	Watanabe et al. 1982, Uchida 1991
Symbiodinium microadriaticum	---		Taylor 1973
Woloszynskia apiculata	Heterothallic	N, P limitation, lower light	von Stosch 1973

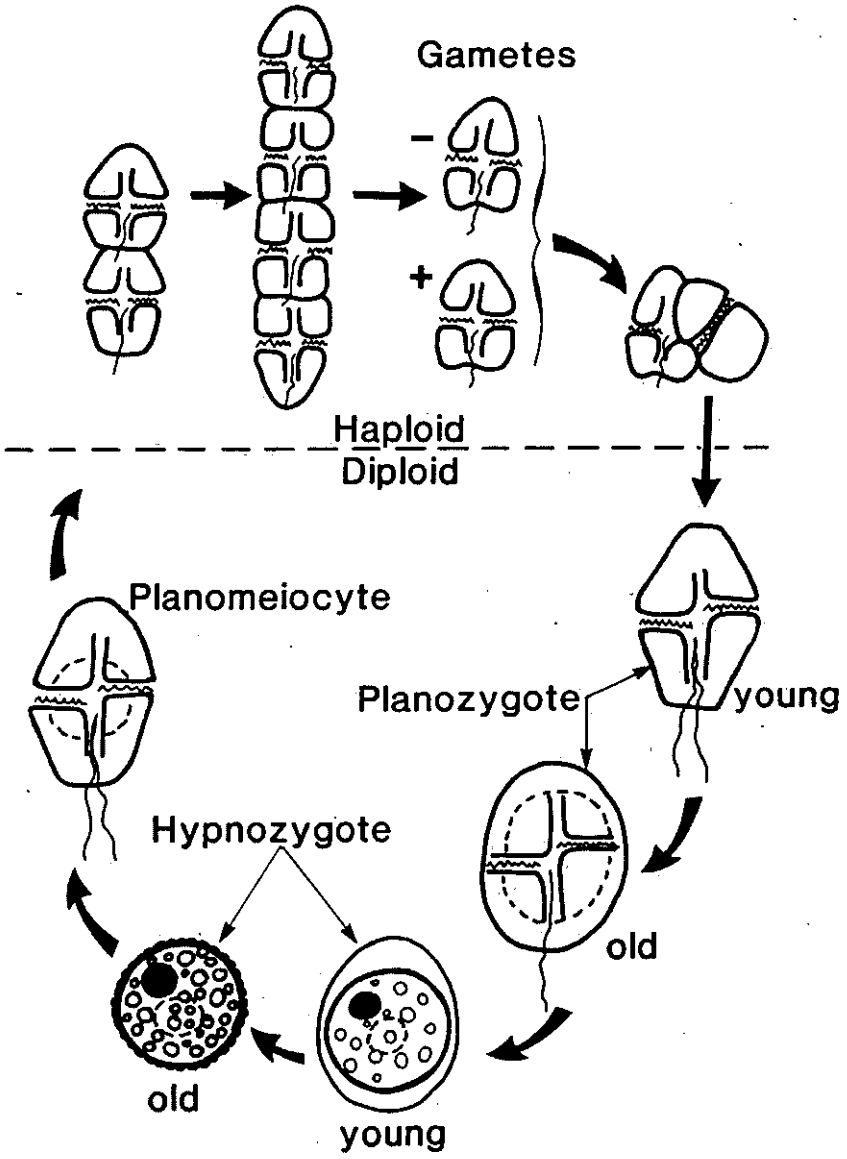

Figure 1.1 Life cycle of *Gymnodinium catenatum* (reproduced from Blackburn et al., 1989).

1.2.2 ENCYSTMENT

An understanding of the factors which trigger both encystment (resting cyst formation) and excystment (resting cyst germination) is crucial if the role of sexual reproduction in bloom dynamics is to be understood. There has long been an assumption that resting cysts simply form in response to nutrient depletion when conditions become sub-optimal for growth although there are many observations of resting cysts forming under apparently optimal nutrient conditions (e.g. Anderson et al., 1983; Pfiester et al., 1984). Over the last thirty years, many other potential encystment factors have been suggested and investigated. These include external factors such as temperature, which has been shown to have a strong influence on encystment in some species (Ellegaard et al., 1998; Sgrosso et al., 2001) and trace metals, including iron which influences encystment of *Alexandrium lusitanicum* (Blanco, 1995). Decreasing photoperiod has been determined as an encystment trigger for some species (Sgrosso et al., 2001), decreasing internal nutrient levels may also promote encystment (Probert et al., 1998) and oceanographic fronts have been identified as areas of high encystment (Tyler et al., 1982). The presence in dinoflagellates of endogenous or biological factors such as pheromone-like substances or chemical cues that facilitate sexual reproduction has also been suggested (Destombe and Cembella, 1990; Wyatt and Jenkinson, 1997), although this has not been studied directly.

1.2.3 EXCYSTMENT

Various factors have been investigated for their potential to influence excystment. Perhaps the most crucial of these factors is the length of the requisite dormancy period or internal maturation period. The requisite dormancy period is the period after resting cyst formation during which a resting cyst is unable to germinate, regardless of physical or chemical conditions. This period varies greatly from species to species. For example, the requisite dormancy period for *Gymnodinium catenatum* is a few weeks (Blackburn et al., 1989) compared with six months for *Scrippsiella hangoei* (Kremp and Anderson, 2000). Low temperatures (Bravo and Anderson, 1994; Hallegraeff et al., 1997), low light levels (Blackburn et al., 1989; Bravo and Anderson, 1994; Nuzzo and Montresor, 1999), and anoxic conditions (Bravo and Anderson, 1994; Kremp and Anderson, 2000) have all been demonstrated to either inhibit or prevent excystment. Salinity (Cannon, 1993) and growth factors (Costas et al., 1993) influence excystment in some species. Nutrient conditions on the other hand have not been demonstrated to affect excystment (e.g. Bravo and Anderson, 1994; Rengefors and Anderson, 1998).

1.3 THE ROLE OF SEXUAL REPRODUCTION IN THE BLOOM DYNAMICS OF DINOFLAGELLATES

Despite the increased knowledge of sexual life cycles in dinoflagellates, there are still relatively few studies of the mating systems of this group of organisms. Many species with known sexual life cycles have been identified as being either homothallic or heterothallic (see Table 1.1) but of those that are heterothallic, few have been studied in further detail. Encystment and excystment also remain unstudied in many species. The logistics of carrying out these studies has been a precluding factor, with development of methods for resting cyst production in the laboratory being one problem, and the large numbers of strains required for mating system studies another. As a result, the autecological significance of sexual reproduction of dinoflagellates remains largely unknown, in particular the extent of genetic recombination in the natural environment.

Understanding the role of resting cysts in the bloom dynamics of toxic dinoflagellates is crucial to the prediction of harmful algal blooms and the management of the coastal and estuarine environment and aquaculture industry. Definition of the role of cysts requires an understanding of the environmental and endogenous cues for excystment and encystment.

Resting cysts can play a variety of ecological roles. They may: (1) function as a "seed" population to inoculate overlying waters and initiate blooms; (2) act as a survival mechanism to permit the species to withstand environmental extremes; (3) be agents for population dispersal into new regions; (4) be a means for genetic recombination (Wall, 1971); (5) act as direct sources of toxicity through their ingestion by shellfish; and (6) be a major factor in the decline of bloom populations through their formation (Anderson, 1984). In brief, resting cysts can contribute in many ways to a successful ecological strategy that enables dinoflagellates to proliferate in time and space.

The role of resting cysts in bloom dynamics has been investigated in a few dinoflagellate species including *Alexandrium tamarense* (Anderson and Keafer, 1985, 1987), *Amphidinium carterae* (Sampayo, 1985), *Gymnodinium pseudopalustre* (Tyler and Heinbokel, 1984), *Gymnodinium sanguineum* (Voltolina, 1993), and *Scrippsiella trochoidea* (Ishikawa and Taniguchi, 1996). For some species, resting cysts have been shown to play a crucial role in bloom initiation through synchronised excystment (e.g. *Alexandrium tamarense*, Anderson and Keafer, 1985) while in other species bloom decline is triggered by mass encystment (e.g. *Gymnodinium pseudopalustre*, Tyler and Heinbokel, 1985). Resting cysts have also been shown to maintain a perennial vegetative population until favourable conditions for bloom development are present (e.g. *Scrippsiella trochoidea*, Ishikawa and Taniguchi, 1996).

1.4 AUSTRALIAN TOXIC DINOFLAGELLATES

Sexual reproduction of four dinoflagellate species that are significant in Australian waters was investigated in this thesis. The study of *Gymnodinium catenatum* included extensive field observations along with laboratory studies of its mating system and cues for encystment and excystment. The study of *Alexandrium minutum* included the elucidation of its life cycle, studies of mating system and the investigation of cues for encystment and excystment. The mating systems of *Alexandrium catenella* and *Protoceratium reticulatum* were also studied.

1.5 *GYMNODINIUM CATENATUM*

Gymnodinium catenatum Graham (Fig. 1.2) produces paralytic shellfish toxins (PSTs) which bioaccumulate in shellfish under bloom conditions, and can cause paralytic shellfish poisoning in humans and other mammals (Anderson and White, 1989). There are many recorded cases of paralytic shellfish poisoning in humans as a result of *G. catenatum* blooms including deaths in some cases (Mee et al., 1986). *Gymnodinium catenatum* is found primarily in temperate waters of North America, Europe and Japan (Steidinger and Tangen, 1997) (Fig. 1.3) but also in Malaysia (Hallegraeff and Fraga, 1998). In Australia, *G. catenatum* was first observed in 1980 in south eastern Tasmania (Hallegraeff and Sumner, 1986) where it has formed blooms periodically ever since. Within Tasmania, *G. catenatum* is found along the east coast from Recherche Bay to Georges Bay and in the Derwent and Huon Estuaries (Bolch and Hallegraeff, 1990). Viable cysts and/or vegetative cells of *G. catenatum* have also been found from mainland Australian waters near Newcastle (Bolch, unpublished data), and along the south coast of Victoria from Portland to Port Welshpool (Sonneman and Hill, 1996).

Vegetative cells of *G. catenatum* are distinctive, forming long chains of up to 64 cells in length and occasionally longer (Fig. 1.2a). The resting cyst of *G. catenatum* was described by Anderson et al. (1988) as having an outer surface composed of hundreds of 1-3 μm polygons (Fig. 1.2b). Such a microreticulate resting cyst is formed by only a few related dinoflagellates (Ellegaard and Moestrup, 1999). The complete sexual and vegetative life cycle of *G. catenatum* was described by Blackburn et al. (1989).

Hallegraeff et al. (1995) determined from historical data (toxin levels, meteorological and physical data) that *G. catenatum* blooms in Tasmania are most likely to occur after a high rainfall event preceded by a dry summer with subsequent calm weather, resulting in a stratified (and stable) water column. It is not yet clear how these physical effects promote a biological response from *G. catenatum*. Research has shown that *G. catenatum* cysts have short dormancy periods and can germinate under a variety of conditions (Blackburn et al.,

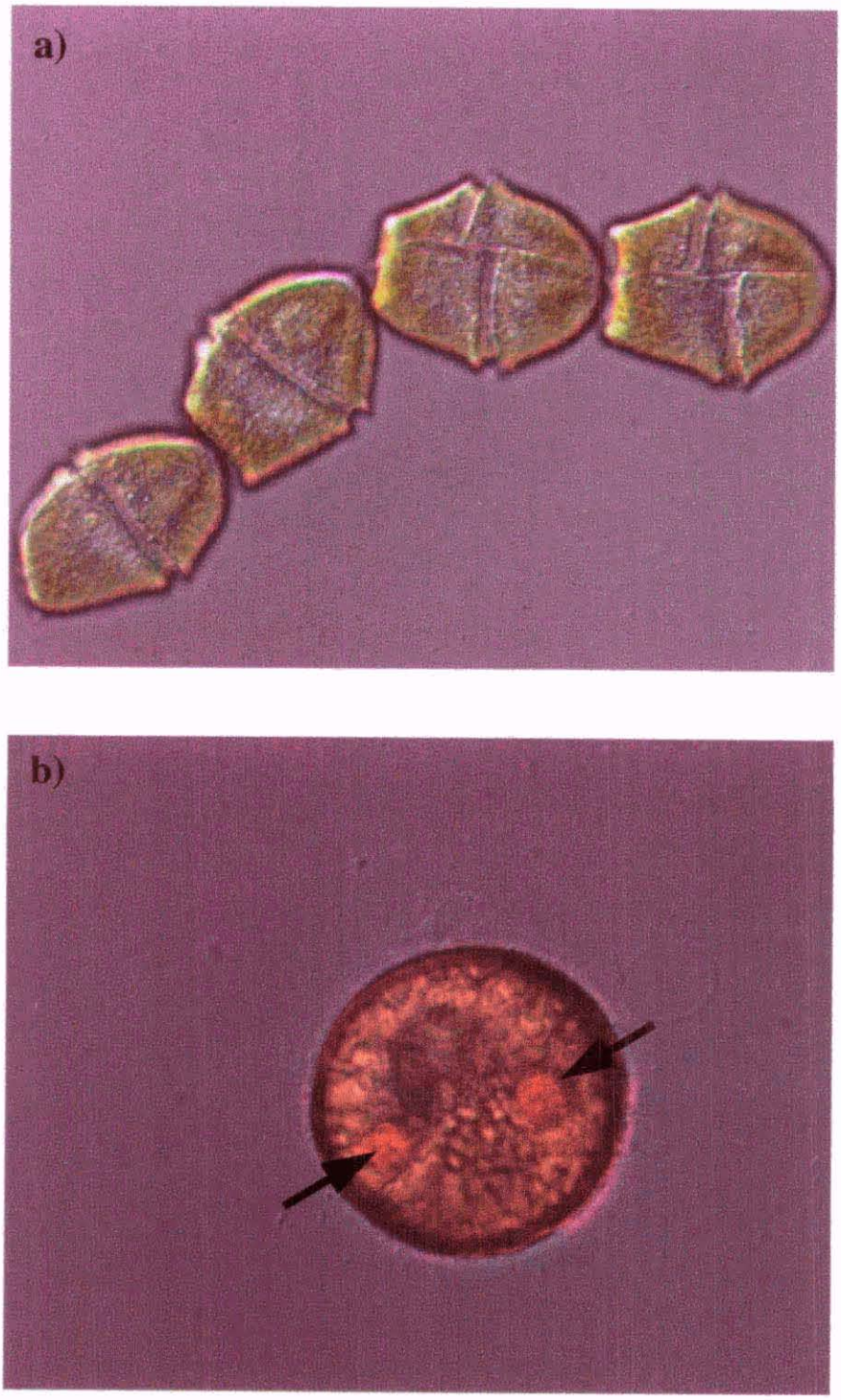

Figure 1.2: Light micrographs of *Gymnodinium catenatum* a) chain of 4 cells and b) resting cyst; arrows indicate 2 red accumulation bodies

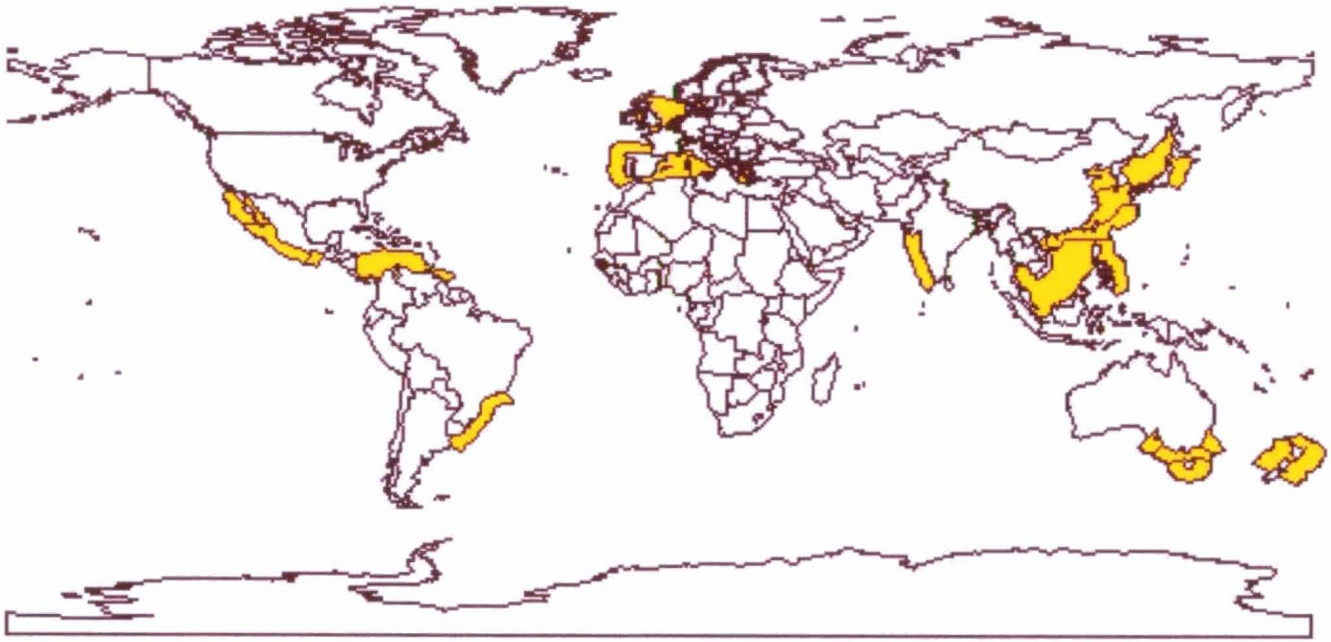

Figure 1.3: Known distribution of *Gymnodinium catenatum* by bioregions.
Map courtesy of CSIRO Marine Research, Centre for Research on Introduced marine pests.

1989). Their formation in culture can be triggered by nitrate and phosphate deficiency as in many other species (Bravo, 1986) although this is not essential for encystment (Blackburn et al., 1989). For Spanish strains darkness has been demonstrated to retard germination and either low (<11°C) or very high (>30°C) temperatures can prevent germination (Bravo and Anderson, 1994). Anoxia is believed to prevent germination in this species as in other dinoflagellates based on field observations, although this has not been directly tested (Blackburn et al., 1989).

1.6 *ALEXANDRIUM MINUTUM*

Alexandrium minutum Halim (Fig. 1.4) also produces paralytic shellfish toxins (Oshima et al., 1989), which under bloom conditions bioaccumulate in shellfish. Populations of *A. minutum* have been documented from various coastal regions (Fig. 1.5) including Egypt, Italy, Turkey, France, Spain, Portugal, France England and the USA (Steidinger and Tangen, 1997). In Australia, populations have been observed in the Shoalhaven River of New South Wales, the Swan River in Western Australia and in the Port River in South Australia (Furlani, 1996). The most consistent bloom formation occurs in the Port River, Adelaide, South Australia (Cannon, 1990).

The resting cyst of *A. minutum* has been described in Bolch et al. (1991) (Fig. 1.4b). These cysts are clear, surrounded by a mucoid sheath, roughly hemispherical in shape, circular in outline when seen from above, and reniform when viewed from the side. The life cycle of *A. minutum* had not previously been fully elucidated in culture, and dormancy requirements were also unknown prior to this study.

1.7 *ALEXANDRIUM CATENELLA*

Alexandrium catenella (Whedon and Kofoid) Balech is a chain-forming dinoflagellate that produces paralytic shellfish toxins (Figure 1.6). The distribution of *A. catenella* includes North America, Chile, Argentina, South Africa and Japan (Steidinger and Tangen, 1997). *Alexandrium catenella* was first described from Australian waters in 1986, and occurs in a variety of locations along the south and east coasts of mainland Australia, including Port Phillip Bay, Victoria, (Hallegraeff et al., 1988) Sydney Harbour, New South Wales (Hallegraeff et al., 1998), and most recently in South Australia in the Port River Estuary. The recent discovery of *A. catenella* cysts and vegetative cells in South Australian waters indicates that the range of this species may be spreading (N. Parker and T. Ault, unpublished data). Yoshimatsu (1981, 1984) have investigated the resting cyst dormancy requirements and described the mating system of Japanese *A. catenella* strains. From Australian strains, previous work has shown that the Sydney Harbour *A. catenella* strains have a much shorter

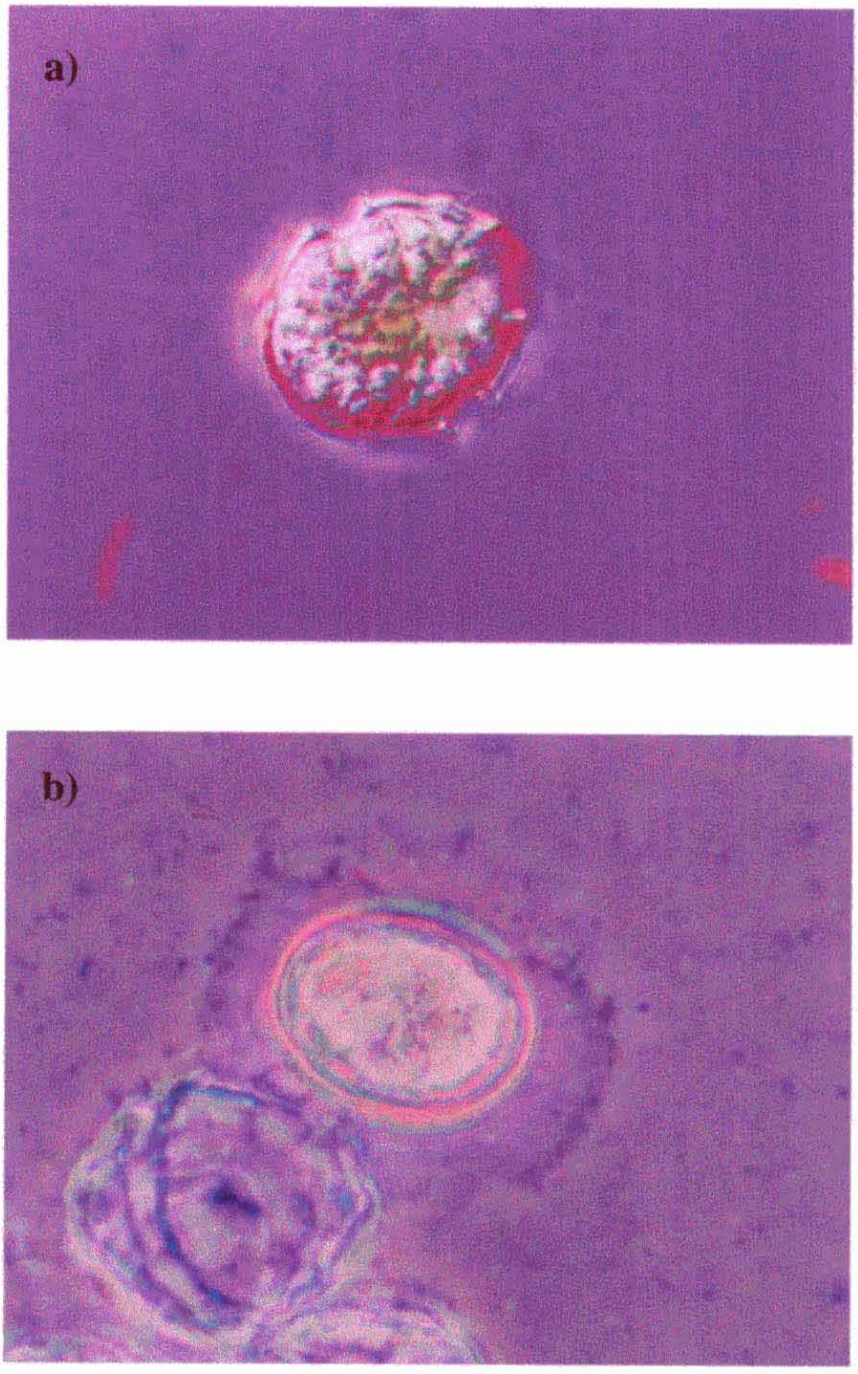

Figure 1.4: Light Micrographs of *Alexandrium minutum* a) vegetative cell and b) resting cyst.

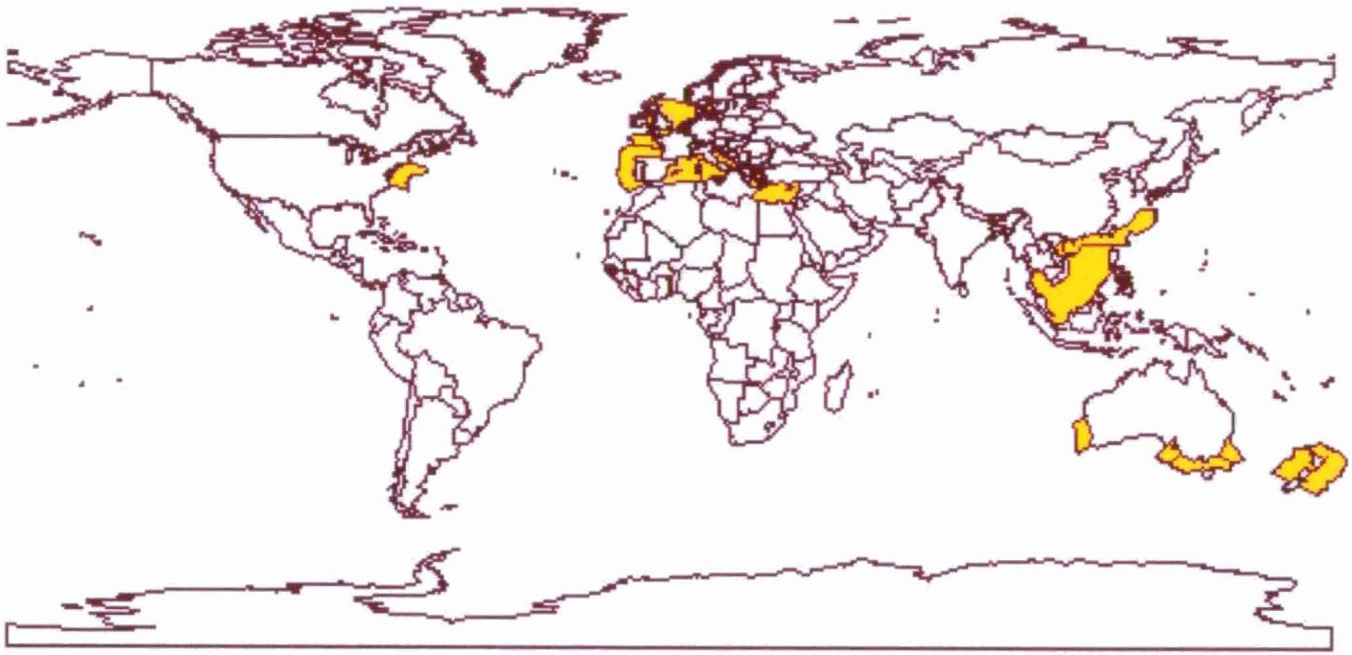

Figure 1.5: Known distribution of *Alexandrium minutum* by bioregions.
Map courtesy of the CSIRO Marine Research Centre for Research on Introduced marine pests.

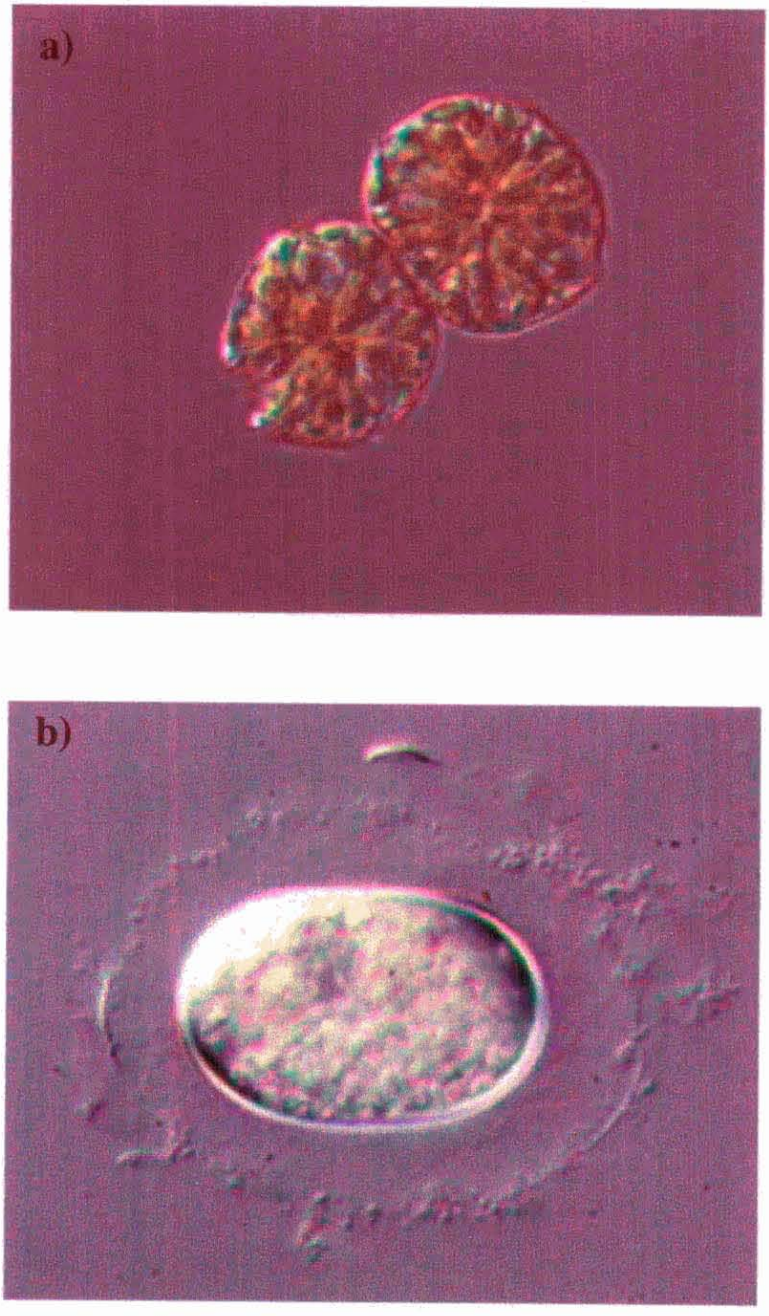

Figure 1.6: Light micrographs of *Alexandrium catenella* a) chain of two vegetative cells and b) resting cyst (resting cyst micrograph courtesy of S. Blackburn).

resting cyst dormancy period than Japanese strains, as short as 21 days compared with 3-6 months (Hallegraeff et al., 1998; Yoshimatsu, 1984 respectively).

1.8 *PROTOCERATIUM RETICULATUM*

Protoceratium reticulatum (Claparéde and Lachmann) Butschli (synonymous with *Gonyaulax grindleyi*) is a ubiquitous dinoflagellate that is known to form a resting cyst as part of its life cycle (Kobayashi et al., 1986) although the full sexual life cycle of *P. reticulatum* has not been described (Fig. 1.7). *Protoceratium reticulatum* is widely distributed in estuarine waters from cold temperate to subtropical waters (Steidinger and Tangen, 1997). Previous research on this species in Australian waters has been confined to taxonomy and distribution. *Protoceratium reticulatum* has been found to produce yessotoxin in some regions (Satake et al., 1997, 1999) although its toxicity in Australian waters is unknown: *Protoceratium reticulatum* is often abundant as resting cysts in sediments (e.g. Tasmania, Australia, Bolch and Hallegraeff, 1990; west coast of Sweden, Persson et al., 2000; southwest India, Godhe et al., 2000) although blooms are rarely observed (I. Jameson, personal communication).

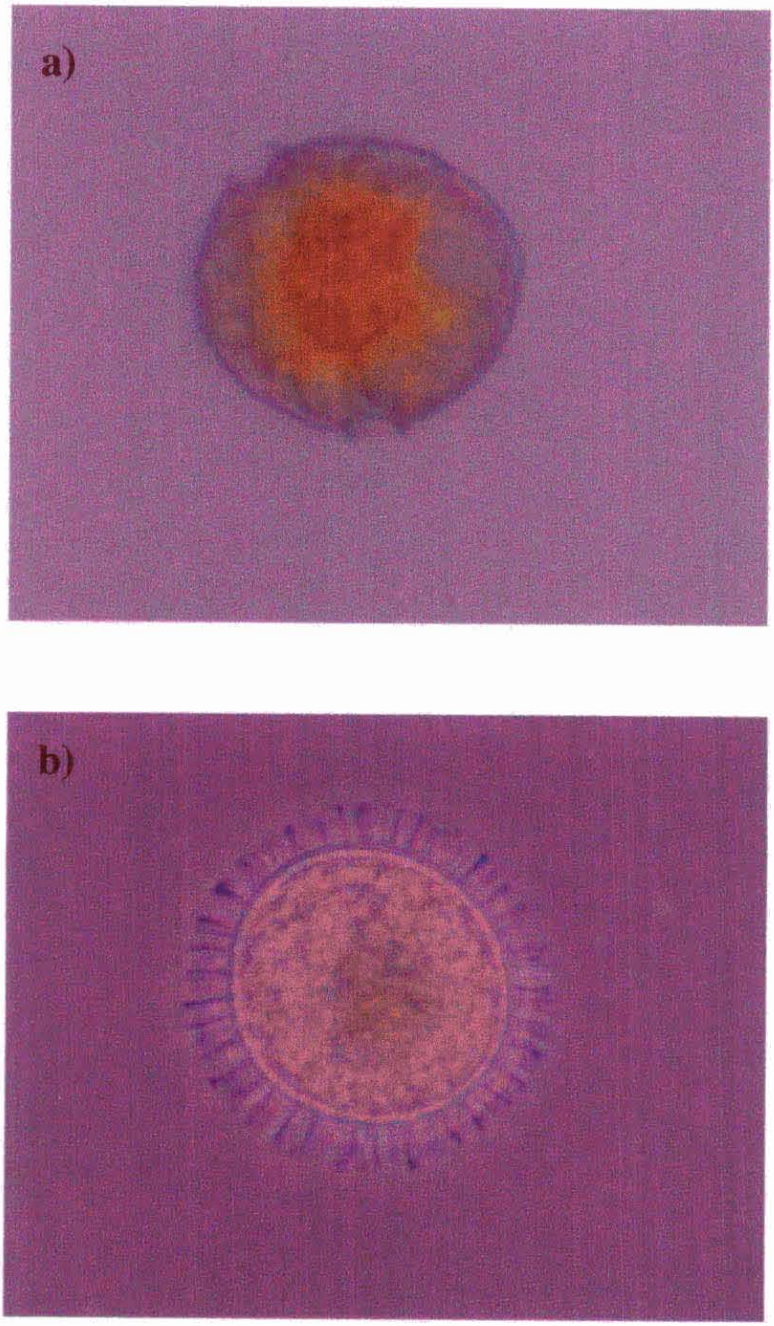

Figure 1.7: Light micrographs of *Protoceratium reticulatum* a) vegetative cell and b) resting cyst.

RESEARCH AIMS AND CHAPTER SUMMARY

The traditional approach to studies of 'planktonic' dinoflagellate ecology neglects the life history links between the plankton and the benthos (Boero et al., 1996). Reproduction is a key element of the ecology of any species. To ignore or separate one type of reproduction can lead to serious misunderstandings of the ecology of an organism. Sexual reproduction facilitates genetic exchange, leading to increased variation, which is important for species survival (Maynard Smith, 1976). In many dinoflagellates, sexual reproduction is not only a means for genetic recombination but also produces an environmentally resistant resting stage which has been demonstrated to be an important factor in their persistence in both time and space (Walker, 1984).

The aim of this thesis is to develop a greater understanding of the complex nature of sexual reproduction in four Australian toxic dinoflagellates, the stages in their life cycles, the frequency with which sexual reproduction occurs, their cues for sexual reproduction and the role of sexual reproduction in their bloom dynamics.

The specific research aims of this thesis are to:

1. Develop methods for the reliable production of resting cysts in culture of four ecologically significant dinoflagellates: *Alexandrium minutum*, *Alexandrium catenella*, *Gymnodinium catenatum* and *Protoceratium reticulatum*.

2. Elucidate the complete sexual life cycle of *Alexandrium minutum*.

3. Investigate the mating systems of the four dinoflagellates, and explore their ecological implications.

4. Determine whether high density cultivation of toxic dinoflagellates is feasible and whether or not life cycle transitions pose a problem for these techniques.

5. Determine the influence of various ecological parameters on encystment and excystment in *Gymnodinium catenatum* and *Alexandrium minutum*.

6. Study the autecology of *Gymnodinium catenatum* in the Huon Estuary, in southeast Tasmania, focusing on life cycle transitions and the physical, chemical and biological factors influencing bloom dynamics.

7. Develop a greater understanding of the role of resting cysts in the bloom dynamics and autecology of toxic dinoflagellates from Australian waters.

The importance and relevance of this work is several fold. Sexual reproduction of dinoflagellates is a key factor in their survival and also aids species range expansion. The research presented here will have significant benefit to management of Australia's estuarine and coastal waters. Understanding the key factors influencing algal bloom formation, particularly the role of resistant resting stages are essential to environmental management, aquaculture development, and management of ballast water and other import/transfer vectors.

Chapter 2 describes the sexual life cycle of *A. minutum* for the first time, identifying the life cycle stages and dormancy requirements. Chapter 3 details the mating systems of all four of the study species looking at intra-population and inter-population variation in mating systems, and explores the ecological implications of mating systems. Chapter 4 presents a feasibility study into high density cultivation of *A. minutum* using aerated systems and investigates whether life cycle transitions have any effect on the use of these technologies. Chapter 5 details results of laboratory experiments on the effect of various parameters on encystment and excystment in *A. minutum* and *G. catenatum*. Chapter 6 provides a detailed analysis of *G. catenatum* dynamics in the Huon Estuary in southeast Tasmania over a three-year period and details the impacts of physical, chemical and biological parameters on the growth, distribution and sexual reproduction of this species. Chapter 7 concludes this thesis with a summary of the major findings and a general discussion of the role of resting cysts in bloom dynamics of toxic dinoflagellates from Australian estuarine waters[1].

[1] Each chapter has been structured as a 'stand-alone' paper with a separate reference list; there may therefore be some overlap in introductory material.

REFERENCES

Anderson DM (1980) Effects of temperature conditioning on development and germination of *Gonyaulax tamarensis* (Dinophyceae) hypnozygotes. Journal of Phycology 16: 166–172.

Anderson DM (1984) Shellfish toxicity and dormant cysts in toxic dinoflagellate blooms. In Ragelis EP (ed), Seafood Toxins. American Chemical Society, Washington DC: 125–138.

Anderson DM, Keafer BA (1985) Dinoflagellate cyst dynamics in coastal and estuarine waters. In Anderson DM, White AW, Baden DG (eds), Toxic Dinoflagellates. Elsevier Science Publishing Co., New York: 219–224.

Anderson DM, Keafer BA (1987) An endogenous annual clock in the toxic marine dinoflagellate *Gonyaulax tamarensis*. Nature 325: 616–617.

Anderson DM, Wall D (1978) Potential importance of benthic cysts of *Gonyaulax tamarensis* and *G. excavata* in initiating toxic dinoflagellate blooms. Journal of Phycology 13: 224–234.

Anderson DM, White AW (1992) Marine biotoxins at the top of the food chain. Oceanus 35: 55–61.

Anderson DM, Chisholm SW, Watras CJ (1983) Importance of life cycle events in the population dynamics of *Gonyaulax tamarensis*. Marine Biology 76: 179–189.

Anderson DM, Jacobson D, Bravo I, Wrenn JH (1988) The unique, microreticulate cyst of the naked dinoflagellate *Gymnodinium catenatum*. Journal of Phycology 24: 255–262.

Barlow S, Triemer RE (1988) Alternate life history stages in *Amphidinium klebsii* (Dinophyceae, Pyrrophyta). Journal of Phycology 27: 413–420.

Beam CA, Himes M (1974) Evidence for sexual fusion and recombination in the dinoflagellate *Crypthecodinium cohnii*. Nature 250: 435.

Blackburn SI, Hallegraeff GM, Bolch CJ (1989) Vegetative reproduction and sexual life cycle of the toxic dinoflagellate *Gymnodinium catenatum* from Tasmania, Australia. Journal of Phycology 25: 577–590.

Blackburn SI, Bolch CJ, Haskard KA, Hallegraeff GM (2001) Reproductive compatibility among four global populations of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). Phycologia 40: 78–87.

Blanco J (1995) Cyst production in four species of neritic dinoflagellates. Journal of Plankton Research 17: 165–182.

Boero F, Belmonte G, Fanelli G, Piraino S, Rubino F (1996) The continuity of living matter and the discontinuities of its constituents: do plankton and benthos really exist? Trends in Ecology and Evolution 11: 177–180.

Bolch CJ, Hallegraeff GM (1990) Dinoflagellate cysts in recent marine sediments from Tasmania, Australia. Botanica Marina 33: 173–192.

Bolch CJ, Blackburn SI, Cannon JA, Hallegraeff GM (1991) The resting cyst of the red-tide dinoflagellate *Alexandrium minutum* (Dinophyceae). Phycologia 30: 215–219.

Bravo I (1986) Cyst germination, cultivation and encystment of *Gymnodinium catenatum* Graham. Investigación Pesquera 50: 313–321.

Bravo I, Anderson DM (1994) The effects of temperature, growth medium and darkness on excystment and growth of the toxic dinoflagellate *Gymnodinium catenatum* from northwest Spain. Journal of Plankton Research 16: 513–525.

Cannon JA (1990) Development and dispersal of red tides in the Port River, South Australia. In Graneli E, Sundstrom B, Edler L, Anderson DM (eds), Toxic Marine Phytoplankton. Elsevier, New York: 110–115.

Cannon JA (1993) Germination of the toxic dinoflagellate, *Alexandrium minutum*, from sediments in the Port River, South Australia. In Smayda TJ, Shimizu Y (eds), Toxic phytoplankton blooms in the sea. Elsevier, Amsterdam: 109–114.

Cao Vien M (1967) Sur l'existence de phénomenes sexuels chez un Péridinien libre, *l'Amphidinium carteri*. Compte rendu hebdomadaire des seances de l'Academie des Sciences. Paris, Series D 264: 1006–1008.

Cao Vien M (1968) Sur la germination du zygote et sur un mode particular de multiplication vegetative chez le peridinien libre, *l'Amphidinium carteri*. Compte rendu hebdomadaire des seances de l'Academie des Sciences. Paris, Series D 267: 701–703.

Chang FH, Chiswell SM, Uddstrom MJ (2001) Occurrence and distribution of *Karenia brevisulcata* (Dinophyceae) during the 1998 summer toxic outbreaks on the central east coast of New Zealand. Phycologia 40: 215–222.

Chesnick JM (1987) Ultrastructural investigations of three stages of the sexual life history of *Peridinium balticum* (Pyrrhophyta). PhD Dissertation, Texas A&M University. Dissertation Abstracts International Part B: Science and Engineering 47: 179 pp.

Chesnick JM, Cox ER (1989) Fertilization and zygote development in the binucleate dinoflagellate *Peridinium balticum* (Pyrrhophyta). American Journal of Botany 76: 1060–1072.

Coats DW, Tyler MA, Anderson DM (1984) Sexual processes in the life cycle of *Gyrodinium uncatenum* (Dinophyceae): A morphogenetic overview. Journal of Phycology 20: 351–361.

Corrales RA, Reyes M, Martin M (1995) Notes on the encystment and excystment of *Pyrodinium bahamense* var. compressum in vitro. In Lassus P, Arzul G, Erard-Le Denn E, Gentien P, Marcaillou-Le Baut C (eds), Harmful marine Algal Blooms. Lavoisier Publishing, Paris: 573–578.

Costas E, Gonzalez Gil S, Aguilera A, Lopez Rodas V (1993) An apparent growth factor modulation of marine dinoflagellate excystment. Journal of Experimental Marine Biology and Ecology 166: 241–249.

Dale B (1977) Cysts of the toxic red-tide dinoflagellate *Gonyaulax excavata* (Braarud) Balech from Oslofjorden, Norway. Sarsia 63: 29–34.

Dale B, Yentch CM, Hurst J (1978) Toxicity in resting cysts of the red tide dinoflagellate *Gonyaulax excavata* from deeper water coastal sediments. Science 201: 1223–1225.

Destombe C, Cembella A (1990) Mating-type determination, gametic recognition and reproductive success in *Alexandrium excavatum* (Gonyaulacales, Dinophyta), a toxic red-tide dinoflagellate. Phycologia 29: 316–325.

Diwald K (1937) Die ungeshlechtiliche und geschlechtiche Fortpflanzung von *Glenodinium lubiniensiforme* spec. nov. Flora 132: 174–192.

Ellegaard M, Moestrup O (1999) fine structure of the flagellar apparatus and morphological details of *Gymnodinium nolleri* sp. nov. (Dinophyceae), an unarmored dinoflagellate producing a microreticulate cyst. Phycologia 38: 289–300.

Ellegaard M, Kulis DM, Anderson DM (1998) Cysts of Danish *Gymnodinium nolleri* Ellegaard et Moestrup Sp. Ined. (Dinophyceae) – studies on encystment, excystment and toxicity. Journal of Plankton Research 20: 1743–1755.

Faust MA (1993) Sexuality in a toxic dinoflagellate, *Prorocentrum lima*. In Smayda TJ, Shimizu Y (eds), Toxic phytoplankton blooms in the sea. Elsevier, Amsterdam: 121–126.

Faust MA (1992) Observations on the morphology and sexual reproduction of *Coolia monotis* (Dinophyceae). Journal of Phycology 28: 94–104.

Furlani D (1996) A guide to the introduced marine species in Australian waters. Technical report (Centre for Research on Introduced Marine Pests (Australia)) no. 5. CSIRO Division of Fisheries, Hobart.

Gao X, Dodge JD (1991) The taxonomy and ultrastructure of a marine dinoflagellate, *Scrippsiella minima* sp. nov. British Phycological Journal 26: 21–31.

Garcés E, Delgado M, Maso M, Camp J (1998) Life history and in situ growth rates of *Alexandrium taylori* (Dinophyceae, Pyrrophyta). Journal of Phycology 34: 880–887.

Giacobbe MG, Gangemi ESO (1997) Vegetative and sexual aspects of *Dinophysis pavillardi* (Dinophyceae). Journal of Phycology 33: 73–80.

Giacobbe MG, Yang X (1999) The life history of *Alexandrium taylori* (Dinophyceae). Journal of Phycology 35: 331–338.

Godhe A, Karunasagar I, Karunasagar I, Karlson B (2000) Dinoflagellate cysts in recent marine sediments from SW India. Botanica Marina 43: 39–48.

Hallegraeff GM (1993) A review of harmful algal blooms and their apparent increase. Phycologia 32:79–99.

Hallegraeff GM, Fraga S (1998) Bloom dynamics of the toxic dinoflagellate *Gymnodinium catenatum*, with emphasis on Tasmanian and Spanish coastal waters. In Anderson DM, Cembella AD, Hallegraeff GM (eds) Physiological Ecology of Harmful Algal Blooms. Springer-Verlag, Berlin: 59–80.

Hallegraeff GM, Sumner C (1986) Toxic plankton blooms affect shellfish farms. Australian Fisheries 45: 15–18.

Hallegraeff GM, McCausland MA, and Brown RK (1995) Early warning of toxic dinoflagellate blooms of *Gymnodinium catenatum* in southern Tasmanian waters. Journal of Plankton Research 17: 1163–1176.

Hallegraeff GM, Steffenson DA, Wetherbee R (1988) Three estuarine Australian dinoflagellates that can produce paralytic shellfish toxins. Journal of Plankton Research 10: 533–541.

Hallegraeff GM, Bolch CJ, Blackburn SI, Oshima Y (1991) Species of the toxigenic dinoflagellate genus *Alexandrium* in southeastern Australian waters. Botanica Marina 34: 575–587.

Hallegraeff GM, Marshall JA, Valentine J, Hardiman S (1998) Short cyst-dormancy period of an Australian isolate of the toxic dinoflagellate *Alexandrium catenella*. Marine and Freshwater Research 49: 415–420.

Hallegraeff FM, Valentine JP, Marshall JA, Bolch CJ (1997) Temperature tolerances of toxic dinoflagellate cysts: application to the treatment of ships' ballast water. Aquatic Ecology 31: 47–52.

Hickel B, Pollingher U (1986) On the morphology and ecology of *Gonyaulax apiculata* (Penard) ENTZ from the Selenter See (West Germany). Archiv fuer Hydrobiologie 73: 227–232.

Hoefker I (1930) Uber *Noctiluca scintillans* (Macartney). Archiv fuer Protistenkunde 71:57–78

Ishikawa A, Tanuguchi A (1996) Contribution of benthic cysts to the population dynamics of *Scrippsiella* spp. (Dinophyceae) in Onagawa Bay, northeast Japan. Marine Ecology Progress Series 140: 169–178.

Jensen MO, Moestrup O (1997) Autecology of the toxic dinoflagellate *Alexandrium ostenfeldii*: Life history and growth at different temperatures and salinities. European Journal of Phycology 32: 9–18.

Kelley I (1989) Observations on the life cycle and ultrastructure of the freshwater dinoflagellate *Gloeodinium montanum* K (Dinophyceae). PhD Dissertation, University of Oklahoma, Norman. Dissertation Abstracts International Part B: Science and Engineering 49: 149 pp.

Kelley I, Pfiester LA (1990) Sexual reproduction in the freshwater dinoflagellate *Gloeodinium montanum*. Journal of Phycology 26: 167–173.

Kita T, Fukuyo Y, Tokuda H, Hirano R (1985) Life history of *Goniodoma pseudogoniaulax* (Pyrrophyta) in a rockpool. Bulletin of Marine Science, 37: 643–651.

Kita T, Fukuyo Y, Tokuda H, Hirano R (1993) Sexual reproduction of *Alexandrium hiranoi* (Dinophyceae). Bulletin of the Plankton Society of Japan 39: 79–85.

Kobayashi S, Matsuoka K, Lizuka S (1986) Distribution of dinoflagellate cysts in surface sediments of Japanese coastal waters. 1. Omura Bay, Kyushu. Bulletin of the Plankton Society of Japan 33: 81–93.

Kremp A, Anderson DM (2000) Factors regulating germination of resting cysts of the spring bloom dinoflagellate *Scrippsiella hangoei* from the northern Baltic Sea. Journal of Plankton Research 22: 1311–1327.

Kremp A, Heiskanen A-S (1999) Sexuality and cyst formation of the spring-bloom dinoflagellate *Scrippsiella hangoei* in the coastal northern Baltic Sea. Marine Biology 134: 771–777.

Maynard Smith J (1976) The Evolution of Sex. Cambridge University Press, Cambridge: 222pp.

Mee LD, Espinosa M, Diaz G (1986) Paralytic shellfish poisoning with a *Gymnodinium catenatum* red tide on the Pacific coast of Mexico. Marine Environmental Research 19: 77–92

Montresor M (1995) The life history of *Alexandrium pseudogonyaulax* (Gonyaulacales, Dinophyceae). Phycologia 34: 444–448.

Montresor M, Marino D (1996) Modulating effect of cold-dark storage on excystment in *Alexandrium pseudogonyaulax* (Dinophyceae). Marine Biology 127: 55–60.

Morey-Gaines G, Ruse RH (1980) Encystment and reproduction of the predatory dinoflagellate *Polykrikos kofoidii* Chatton (Gymnodiniales). Phycologia 19: 230–236.

Nuzzo L, Montresor M (1999) Different excystment patterns in two calcareous cyst-producing species of the dinoflagellate genus Scrippsiella. Journal of Plankton Research 21: 2009–2018.

Oshima Y, Hirota M, Yasumoto T, Hallegraeff GM, Blackburn SI, Steffensen DA (1989) Production of paralytic shellfish toxins by the dinoflagellate *Alexandrium minutum* Halim from Australia. Nippon Suisan Gakkaishi 55: 925.

Ouchi A, Aida S, Uchida T, Honjo T (1994) Sexual reproduction of a red tide dinoflagellate *Gymnodinium mikimotoi*. Fisheries Science 60:125–126.

Park JG, Jeong MK, Lee JA, Cho K-J, Kwon O-S (2001) Diurnal vertical migration of a harmful dinoflagellate, *Cochlodinium polykrikoides* (Dinophyceae) during a red tide in coastal waters of Namhae Island, Korea. Phycologia 40: 292–297.

Parker NS, Blackburn SI (in prep) The sexual life cycle of the toxic dinoflagellate *Alexandrium minutum* (Dinophyceae) from the Port River Estuary, Australia. To be submitted to Journal of Phycology.

Persson A, Godhe A, Karlson B (2000) Dinoflagellate cysts in recent marine sediments from the west coast of Sweden. Botanica Marina 43: 69–79.

Pfiester LA (1975) Sexual reproduction of *Peridinium cinctum* f. ovoplanum (Dinophyceae) Journal of Phycology 11: 259–265.

Pfiester LA (1976) Sexual reproduction of *Peridinium willei* (Dinophyceae) Journal of Phycology 12: 234–238.

Pfiester LA (1977) Sexual reproduction of *Peridinium gatunense* (Dinophyceae) Journal of Phycology 13: 92–95.

Pfiester LA (1989) Dinoflagellate sexuality. International Review of Cytology 114: 249–272.

Pfiester LA, Anderson DM (1987) Dinoflagellate reproduction. In Taylor FJR (ed), The biology of dinoflagellates. Blackwell Scientific Publications, Oxford: 611–648.

Pfiester LA, Lynch RA (1980) Amoeboid stages and sexual reproduction of *Cystodinium bataviense* and its similarity to Dinococcus (Dinophyceae). Phycologia 19: 178.

Pfiester LA, Skvarla JJ (1979) Heterothallism and thecal development in the sexual life history of *Peridinium volzii* (Dinophyceae). Phycologia 18:13–18.

Pfiester LA, Skvarla JJ (1980) Comparative ultrastructure of vegetative and sexual thecae of *Peridinium limbatum* and *Peridinium cinctum* (Dinophyceae). American Journal of Botany 67: 955–958.

Pfiester LA, Timpano P, Skvarla JJ, Holt JR (1984) Sexual reproduction and meiosis in *Peridinium inconspicuum* lemmermann (Dinophyceae). American Journal of Botany 71: 1121–1127.

Pholpunthin P, Fukuyo Y, Matsuoka K, Nimura Y (1999) Life history of a marine dinoflagellate *Pyrophacus steinii* (Schiller) Wall et Dale. Botanica Marina 42: 189–197.

Probert IP, Erard-le Denn E, Lewis J (1998) Intracellular nutrient status as a factor in the induction of sexual reproduction in a marine dinoflagellate. In Reguera B, Blanco J, Fernandez ML, Wyatt T (eds), Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO: 343–344.

Ragelis EP (ed) (1984) Seafood Toxins. American Chemical Society, Washington DC: 460pp.

Rengefors K, Anderson DM (1998) Environmental and endogenous regulation of cyst germination in two freshwater dinoflagellates. Journal of Phycology 34: 568–577.

Sako Y, Ishida Y, Kadota H, Hata Y (1984) Sexual reproduction and cyst formation in the freshwater dinoflagellate *Peridinium cunningtonii*. Bulletin of the Japanese Society of Fisheries 50: 743–750.

Sako Y, Ishida Y, Nishijima T, Hata Y (1987) Sexual reproduction and cyst formation in the freshwater dinoflagellate *Peridinium penardii*. Nippon Suisan Gakkaishi 53: 473–478.

Sampayo MA (1985) Encystment and excystment of a Portuguese isolate of *Amphidinium carterae* in cultures. In Anderson DM, White AW, Baden DG (eds), Toxic Dinoflagellates. Elsevier Science Publishing Co., New York: 125–130.

Satake M, MacKenzie L, Yasumoto T (1997) Identification of *Protoceratium reticulatum* as the biogenetic origin of yessotoxin. Natural Toxins 5: 164–167.

Satake M, Ichimura T, Sekiguchi K, Yoshimatsu S, Oshima Y (1999) Confirmation of yessotoxin and 45, 46, 47 – Trinoryessotoxin production by *Protoceratium reticulatum* collected in Japan. Natural Toxins 7: 147–150.

Schnepf E, Drebes G (1993) Anisogamy in the dinoflagellate Noctiluca? Helgolander Meeresuntersuchungen 47: 265–273.

Sgrosso S, Esposito F, Montresor M (2001) Temperature and daylength regulate encystment in calcareous cyst-forming dinoflagellates. Marine Ecology Progress Series 211: 77–87.

Silva ES, Faust MA (1995) Small cells in the life history of dinoflagellates (Dinophyceae): a review. Phycologia 34: 396–408.

Smayda TJ (1990) Novel and nuisance phytoplankton blooms in the sea: evidence for a global epidemic. In Graneli E, Sundstrom B, Edler L, Anderson DM (eds), Toxic Marine Phytoplankton. Elsevier, New York: 29–40.

Sonneborn TM (1957) Breeding systems, reproductive methods, and species problems in protozoa. In Mayr E (ed), The species problem: a symposium presented at the Atlanta meeting of the American Association for the Advancement of Science, December 28-29, 1955. The American association for the advancement of science, Washington D.C.: 155–324.

Sonnemann JA, Hill DRA (1996) A taxonomic survey of cyst-producing dinoflagellates from Recent sediments of Victorian coastal waters, Australia. Botanica Marina 40: 149–177.

Spero HJ, Morée MD, (1981) Phagotrophic feeding and its importance to the life cycle of the holozoic dinoflagellate, *Gymnodinium fungiforme*. Journal of Phycology 17: 43–51.

State of the Environment Advisory Council (1996) State of the Environment, Australia. CSIRO Publishing, Collingwood: 47pp.

Steidinger KA, Tangen K (1997) Dinoflagellates. In Tomas CR (ed), Identifying Marine Phytoplankton. Academic Press, San Diego: 387–584.

Steidinger KA, Burkholder JM, Glasgow HB Jr, Hobbs CW, Garrett JK, Truby EW, Noga EJ, Smith SA (1996) *Pfiesteria piscicida* gen. et sp. nov. (Pfiesteriaceae fam. nov.), a new toxic dinoflagellate with a complex life cycle and behavior. Journal of Phycology 32: 157–164.

Steidinger KA, Vargo GA, Tester PA, Tomas CR (1998) Bloom dynamics and physiology of *Gymnodinium breve* with emphasis on the Gulf of Mexico. In Anderson DM, Cembella AD, Hallegraeff GM (eds), Physiological ecology of harmful algal blooms. Springer-Verlag Berlin: 135–153.

Steidinger KA, Landsberg JH, Truby EW, Roberts BS (1998) First report of *Gymnodinium pulchellum* (Dinophyceae) in North America and associated fish kills in the Indian River, Florida. Journal of Phycology 34: 431–437.

Subba Rao DV (1995) Life cycle and reproduction of the dinoflagellate *Dinophysis norvegica*. Aquatic Microbial Ecology 9: 199–201.

Taylor DL (1973) The cellular interactions of algae-invertebrate symbiosis. Advances in Marine Biology 11: 1–56.

Taylor FJR (1979) A description of the benthic dinoflagellate associated with maitotoxin and ciguatoxin, including observations of Hawaiian material. In Taylor DL, Seliger HH (eds), Toxic Dinoflagellate Blooms. Elsevier/North Holland, New York: 71–76.

Taylor FJR (ed) (1987) The Biology of Dinoflagellates. Blackwell Scientific Publications, Oxford: 785pp.

Trainer VL, Baden DG (1999) High affinity binding of red tide neurotoxins to marine mammal brain. Aquatic Toxicology 46: 139–148.

Turpin DH, Dobell PER, Taylor FJR (1978) Sexuality and cyst formation in Pacific strains of the toxic dinoflagellate *Gonyaulax tamarensis*. Journal of Phycology 14: 235–238.

Tuttle RC, Loeblich AR III (1974) Genetic recombination in the dinoflagellate *Crypthecodinium cohnii*. Science 185: 1061–1062.

Tyler MA, Heinbokel JF (1985) Cycles of red water and encystment of *Gymnodinium pseudopalustre* in the Chesapeake Bay: Effects of hydrography and grazing. In Anderson DM, White AW, Baden DG (eds), Toxic Dinoflagellates. Elsevier Science Publishing Co., New York: 213–218.

Tyler MA, Coats DW, Anderson DM (1982) Encystment in a dynamic estuarine environment, selective deposition of dinoflagellate cysts by a frontal convergence. Marine Ecology Progress Series 7: 163–178.

Uchida T (1991) Sexual reproduction of *Scrippsiella trochoidea* isolated from Muroran Harbor, Hokkaido. Nippon Suisan Gakkaishi 57: 1215.

Uchida T, Matsuyama Y, Kamiyama T (1999) Cell fusion in *Dinophysis fortii*. Bulletin of Fisheries and Environment of the Inland Sea 1: 163–165.

van den Hoek C, Mann DG, Jahns HM (1997) Algae: an introduction to phycology. Cambridge University Press, Cambridge: 627pp.

Voltolina D (1993) The origin of recurrent blooms of *Gymnodinium sanguineum* Hirasaka in a shallow coastal region. Journal of Experimental Marine Biology and Ecology 168: 217–222.

von Stosch HA (1964) Zum problem der sexuellen Fortpflanzung in der Peridineengattung *Ceratium*. Helgol. Wiss. Meeresunters. 10: 140–152.

von Stosch HA (1965) Sexualitat bei *Ceratium cornutum* (Dinophyta). Naturwissenschaftern 52: 112–113.

von Stosch HA (1972) La signification cytologique de la 'cyclose nucléaire' dans le cycle de vie des Dinoflagalles. Mem. Soc. Bot. Fr. 201–212.

von Stosch HA (1973) Observations on vegetative reproduction and sexual life cycles of two freshwater dinoflagellates, *Gymnodinium pseudopalustre* Schiller and *Woloszynskia apiculata* sp. nov. British Phycological Journal 8: 105–134.

Walker LM (1982) Evidence for a sexual cycle in the Florida red tide dinoflagellate, *Ptychodiscus brevis* (= *Gymnodinium breve*). Transactions of the American Microscopy Society 101: 809–810.

Walker LM (1984) Life histories, dispersal and survival in marine, planktonic dinoflagellates. In Steidinger KA, Walker LM (eds), Marine Plankton Life Cycle Strategies. CRC Press Inc., Boca Raton, Florida: 19–34.

Walker LM, Steidinger KA (1979) Sexual reproduction in the toxic dinoflagellate *Gonyaulax monilata*, Journal of Phycology 15: 312–315.

Wall D (1971) Biological problems concerning fossilizable dinoflagellates. Geoscience and Man 3: 1–15.

Watanabe MM, Watanabe M, Fukuyo Y (1982) *Scrippsiella trochoidea*: encystment and excystment of red tide flagellates 1. Induction of encystment of *Scrippsiella trochoidea*. Res. Rep. Natl. Inst. Environ. Stud. (Jpn.) 30: 27–41.

Wear RG, Gardner JPA (2001) Biological effects of the toxic algal bloom of February and March 1998 on the benthos of Wellington Harbour, New Zealand. Marine Ecology Progress Series 218: 63–76.

Whyte JNC, Haigh N, Ginther NG, Keddy LJ (2001) First record of blooms of *Cochlodinium* sp. (Gymnodiniales, Dinophyceae) causing mortality to aquacultured salmon on the west coast of Canada. Phycologia 40: 298–304.

Wyatt T, Jenkinson IR (1997) Notes on *Alexandrium* dynamics. Journal of Plankton Research 19: 551–575.

Yentsch CM (1984) Paralytic Shellfish Poisoning: An emerging perspective. In Ragelis EP (ed), Seafood Toxins. American Chemical Society Washington DC: 9–23.

Yoshimatsu S (1981) Sexual reproduction of *Protogonyaulax catenella* in culture. I. Heterothallism. Bulletin of the Plankton Society of Japan 28: 131–139.

Yoshimatsu S (1984) Sexual reproduction of *Protogonyaulax catenella* in culture. II. Determination of mating type. Bulletin of the Plankton Society of Japan 31: 107–111.

Zhang S, Li S (1990) Sexual reproduction and hypnozygote formation in the freshwater dinoflagellate *Peridinium bipes* Stein. Acta Hydrobiologica Sinica 14: 1–9.

Zingmark RG (1970) Sexual reproduction in the dinoflagellate *Noctiluca miliaris* Suriray. Journal of Phycology 6: 122–126.

CHAPTER 2

THE SEXUAL LIFE CYCLE OF THE TOXIC DINOFLAGELLATE *ALEXANDRIUM MINUTUM* (DINOPHYCEAE) FROM THE PORT RIVER ESTUARY, SOUTH AUSTRALIA

INTRODUCTION

The primary means of reproduction in dinoflagellates is through vegetative division (Pfiester and Anderson, 1987). However, since the 1960s many species have also been found to have a sexual life cycle (Walker, 1984). With improved methods in culturing and microscopy, the number of dinoflagellate species for which sexual life histories have been defined has increased from approximately 22 species in 1987 (Pfiester and Anderson, 1987) to over 50 today (Chapter 1, Table 1.1). However, this still represents only about 2.5% of the more than 2000 extant species of dinoflagellates (Steidinger and Tangen, 1997). Most of these 50 species have a confirmed resting stage and life cycles range from relatively simple ones such as that observed in some *Alexandrium* species (Turpin et al., 1978) to extremely complex life cycles of species such as *Pfiesteria piscicida* (Steidinger et al., 1996). In some dinoflagellates, sexual reproduction occurs within clonal culture and such species are defined as being homothallic while in other species compatible clones of differing mating types must be combined in order for sexual reproduction to occur (heterothallism) (Table 2.1 and Chapter 3).

Knowledge of life cycles is important in increasing our understanding of the ecology of dinoflagellates, including the many economically and environmentally significant species. Life cycle dynamics can play an important role in the development and decline of blooms and in the persistence of a species within a given region (Anderson et al., 1983). The life cycles of toxic and red-tide forming species have received particular attention with the elucidation of these life cycles aiding in the prediction and management of blooms. The sexually produced resting cyst or hypnozygote that forms a part of many dinoflagellate life cycles is particularly important in management. The ecological roles that resting cysts may play are varied and include: acting as a "seed" population to inoculate overlying waters and initiate blooms; a survival mechanism during environmental extremes; a population dispersal mechanism (through entrainment in currents); a means of genetic recombination; a direct source of toxicity (through ingestion by shellfish); and enhancement of bloom decline (Wall, 1971; Anderson, 1984).

Alexandrium minutum is a 'red tide' producing dinoflagellate and a causative agent of paralytic shellfish poisoning (PSP). It is the type species of the genus *Alexandrium* and was first described by Halim (1960) from Alexandria Harbour in Egypt and re-described by Balech (1989). It is considered to be synonymous with *A. ibericum* (Balech, 1989). Since *A. minutum* was first described it has also been reported from South Australia (Hallegraeff et al., 1988), Spain and Portugal (Balech 1985; as *A. ibericum*), Italy (Montresor, et al., 1990), Turkey (Koray and Buyukisik, 1988), and France (Nezan et al., 1989). Red tides of *A. minutum* concurrent with PSP events have resulted in investigations focusing on its toxicity,

physiology, distribution, and ecology (e.g. Flynn et al., 1994; Chang and McClean, 1997; Belin, 1993; Marasovic et al., 1995; Cannon, 1990, 1993a, 1993b). Toxin profiles of *A. minutum* have been determined for Australian, Spanish and New Zealand populations (Oshima et al., 1989; Franco et al., 1994; MacKenzie and Berkett, 1997).

In Australia, blooms of *A. minutum* develop regularly in the Port River, South Australia in spring and autumn, with cell densities of 10^6 to 10^7 cells L^{-1} (Cannon, 1990; Cannon, 1993b). It has been suggested that the resting cyst of *A. minutum* plays an important role in maintaining the vegetative population in the Port River, although accumulation and in situ growth are thought to be the primary factors facilitating bloom development (Cannon, 1993a).

Although the complete life cycle of *Alexandrium minutum* has not been observed and described it is known to form a resting cyst as part of its life cycle (Bolch et al., 1991). An understanding of the full life cycle of *A. minutum* is required in order to determine conclusively the role of life cycle dynamics in blooms of this species. In this chapter the life cycle and dormancy requirements for *A. minutum* are described, as investigated through observations of crosses of clonal strains isolated from the Port River population in South Australia. The life cycle of *A. minutum* is contrasted with that of other *Alexandrium* species and the ecological implications of its life cycle dynamics are discussed.

METHODS

2.1 STRAIN DETAILS

Two strains of *Alexandrium minutum* were obtained from the CSIRO Collection of Living Microalgae (CSIRO Microalgae Research Centre) AMAD06, (isolated by S. Blackburn, 27/10/87) and AMAD16 (isolated by S. Blackburn and J. Cannon, 6/4/89). Both strains are clonal, non-axenic and isolated from vegetative cells originating from the Port River Estuary in Adelaide, South Australia. *Alexandrium minutum* has a primarily heterothallic mating system (compatible strains of different mating types required for sexual reproduction) and the two strains used are known to be sexually compatible (see Chapter 3). Strains were maintained at 18°C ± 0.5 °C at 80 µmol photons PAR m^{-2} s^{-1} (cool white fluorescent lights) on a 12:12 light:dark cycle in 50 mL glass Erlenmeyer flasks in 40 mL GSe medium (Blackburn et al., 2001 and Appendix A).

2.2 LIFE CYCLE OBSERVATIONS

2.2.1 RESTING CYST FORMATION

Crosses of AMAD06 and AMAD16 were made using a modification of the resting cyst formation methods of Oshima et al. (1993) as follows. Culture volumes were first increased from 30 to 70 mL under the maintenance conditions described above. After two weeks, 10 mL of each culture was transferred into 50 mL of GSe/20 (GSe medium with $^1/_{20}$ of the nutrients and soil extract) in a 125 mL glass Erlenmeyer flask. After a further week, ten crosses were made by transferring 1 mL of each of the two strains, and 9 mL of GSe/20 into 50 mm diameter, presterilized polystyrene petri dishes.

Crosses in petri dishes were examined with an inverted microscope (Nikon Diaphot or Leitz Labovert) at up to 400x magnification for evidence of sexual activity (gametes, planozygotes, and resting cysts), every 2 days for 3 weeks and weekly thereafter. The time at which various life cycle stages were first observed was noted. Cell sizes of all life cycle stages were measured using a Leitz Labovert inverted microscope fitted with an eyepiece graticule at 320x magnification. Twenty cells from a single cross were measured for each life cycle stage. Different stages of the life cycle were recorded by micrography using a Nikon inverted microscope (phase contrast optics) with an attached camera or a Zeiss Axioplan compound microscope (Nomaski optics) fitted with a Zeiss Axiocam digital camera. Vegetative *A. minutum* cells are fast swimming and somewhat fragile and therefore, in order to obtain micrographs of the different stages without loss of flagella or cell lysis, very dilute glutaraldehyde (1:1000) was added drop by drop until cells slowed enough to be photographed. This technique was found not to distort shape and size of the various life cycle stages.

2.2.2 RESTING CYST GERMINATION

When several hundred resting cysts had been produced in each petri dish (after approximately 3 weeks) the petri dishes were washed three times with fresh medium. By pouring off the old medium and rinsing three times with fresh GSe medium the vegetative cells were completely removed with minimal loss of resting cysts (personal observation). The resting cysts have a mucilaginous sheath that adheres to the polystyrene petri dish surface. The resting cysts were then incubated under the light and temperature conditions described in section 2.1. Cultures were then observed every few days for a further three months for evidence of germination, such as germlings or planomeiocytes, vegetative cells and the empty cell walls of germinated resting cysts. Dormancy requirements and the viability of the resting cysts were also determined through these observations.

RESULTS

2.3 LIFE CYCLE OBSERVATIONS

The life cycle of *A. minutum* consists of six different and readily distinguishable stages. A diagrammatic summary of this life cycle is presented in Figure 2.1. The sexually compatible strains AMAD06 and AMAD16 used in the present study readily reproduced sexually. 'Dancing gametes' (cf. Von Stosch, 1973) were observed within 24 hours of crossing the compatible strains. The gametes were isogamous (of identical morphology) and slightly smaller than the vegetative cells (Fig. 2.2a) (average length of 15μm (n=20, stdev=1.1) compared with an average length for vegetative cells of 19μm (n=20, stdev=1.3)) and were difficult to distinguish from vegetative cells unless they were 'dancing' around one another. Fusion of gametes (Fig. 2.2b) to form planozygotes was first observed 24 hours after crossing: the gametes fused laterally, in contrast with oblique vegetative division, and swam in a helical pattern, also distinguishable from vegetatively dividing cells. The planozygotes which resulted from gamete fusion (Fig. 2.2c) were relatively large cells (average length of 25μm (n=20, stdev=1.7) and were more heavily pigmented than vegetative cells, as they were darker brown in colour. Planozygotes were most commonly observed to have two longitudinal flagella although planozygotes with one flagellum were also observed, suggesting that one flagellum is lost before the other. A single transverse flagellum was observed but the presence or absence of a second transverse flagella could not be confirmed using light microscopy. Planozygotes were observed within 48 hours after crossing.

The first hypnozygotes or resting cysts (Fig. 2.2d) were observed within 5 days of crossing and continued to form for the duration of observations (three months). The hypnozygotes were hemispherical in shape, almost circular when seen from above (average 25μm in diameter (n=20, stdev=2.4) and reniform (kidney shaped) when viewed from the side (16μm average height (n=20, stdev=1.8). The resting cyst wall was clear and an orange-red accumulation body could usually be seen. A mucoid sheath surrounded the resting cyst. Resting cysts commonly formed large clusters of hundreds and often thousands of cysts, with the mucoid sheathes adhering to one another (Figs. 2.2i and 2.2j).

The obligate endogenous resting cyst dormancy period, during which cysts are unable to germinate, was a minimum of approximately 4 weeks after encystment. After four weeks resting cysts were able to germinate under the normal growth conditions (18°C, 12:12 light/dark cycle, nutrient replete media). Resting cysts in one petri dish did not germinate synchronously but continued to excyst for many weeks after the first excystment. Although the minimum dormancy requirement is approximately four weeks, some resting cysts had

not germinated at the termination of the observations (after three months), suggesting that dormancy requirements may be variable. The archeopyle (opening in the cyst wall through which the cell exits upon germination) of the *A. minutum* resting cyst was cryptopylic and chasmic (does not reflect any plate boundary and has a slit-like opening; sensu Matsuoka and Fukuyo, 1995), in most cases the slit was very small (Fig. 2.2e). No opercula (the piece or pieces which separate off the resting cyst during germination) were observed. A membrane, which appears to detach from the germling cell on germination, was observed within most empty cyst walls (Fig. 2.2f). The germling was similar in appearance to the planozygote, averaging 26 μm in length (n=20, stdev=1.9) and appeared relatively heavily pigmented, with two longitudinal flagella (Fig. 2.2g). The germling divided into two cells with typical vegetative cell morphology after 2-3 days under the normal growth conditions (Fig. 1h). These cells separated after a short period and the two single cells then further divided after another 2-3 days. Asexually produced temporary resting cysts were also observed. These formed from single cells in cultures exposed to a cold shock (4 °C); they were thin walled and did not have a mucoid sheath (Fig. 2.2k and 2.2l).

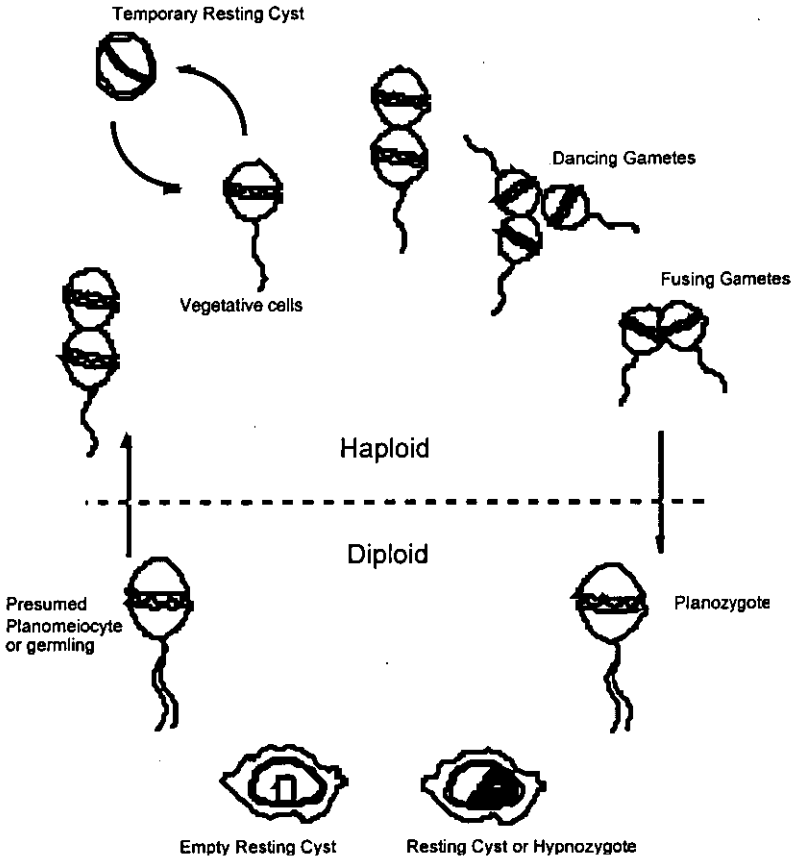

Figure 2.1. Diagrammatic summary of the sexual life cycle of *Alexandrium minutum* determined from culture studies of strains from the Port River Estuary, South Australia.

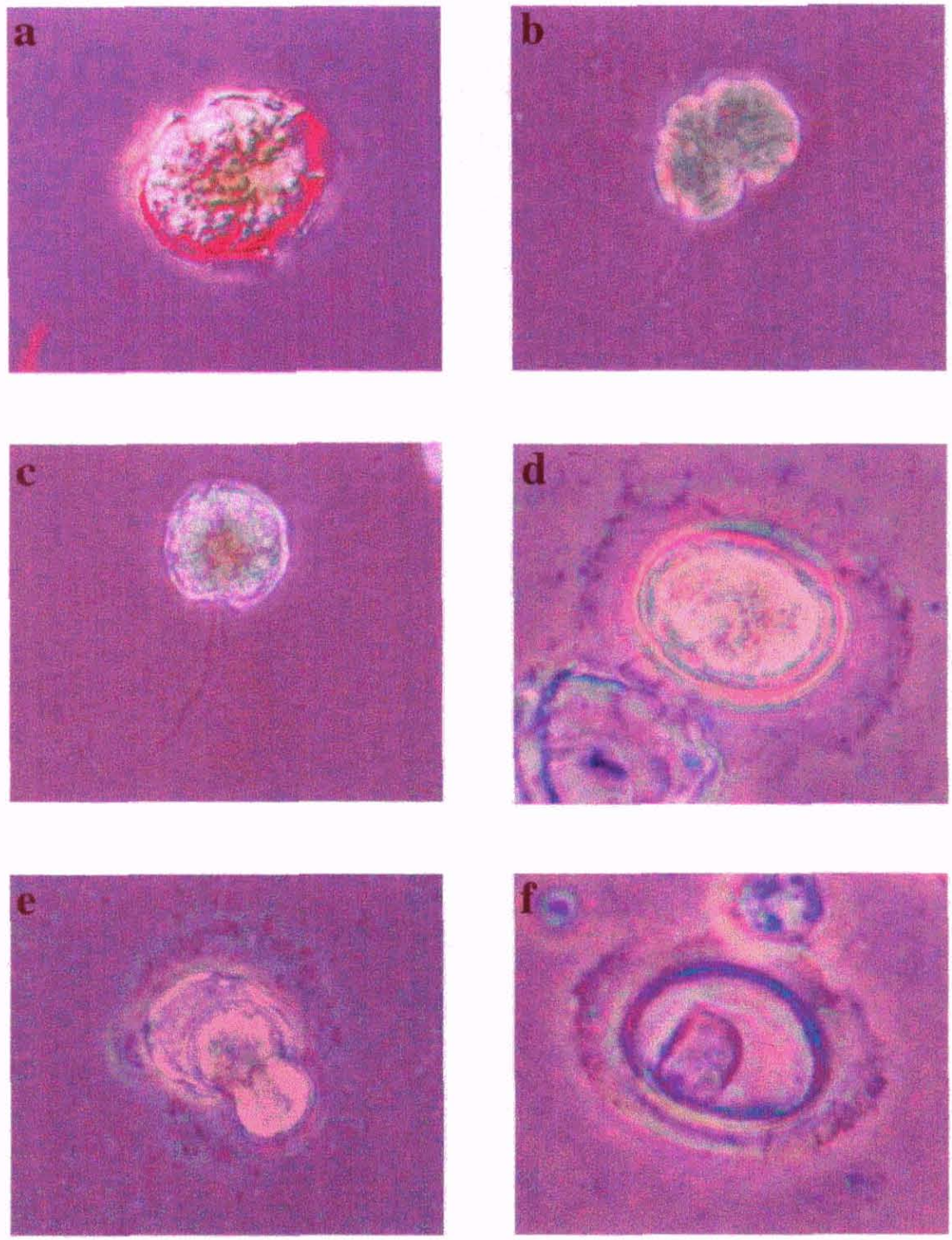

Figure 2.2a-f. Life cycle stages of *Alexandrium minutum* a) vegetative cell, b) fusing gametes, c) planozygote, d) resting cyst or hypnozygote, e) germinating resting cyst, f) empty resting cyst showing membrane left behind by germinating cell.

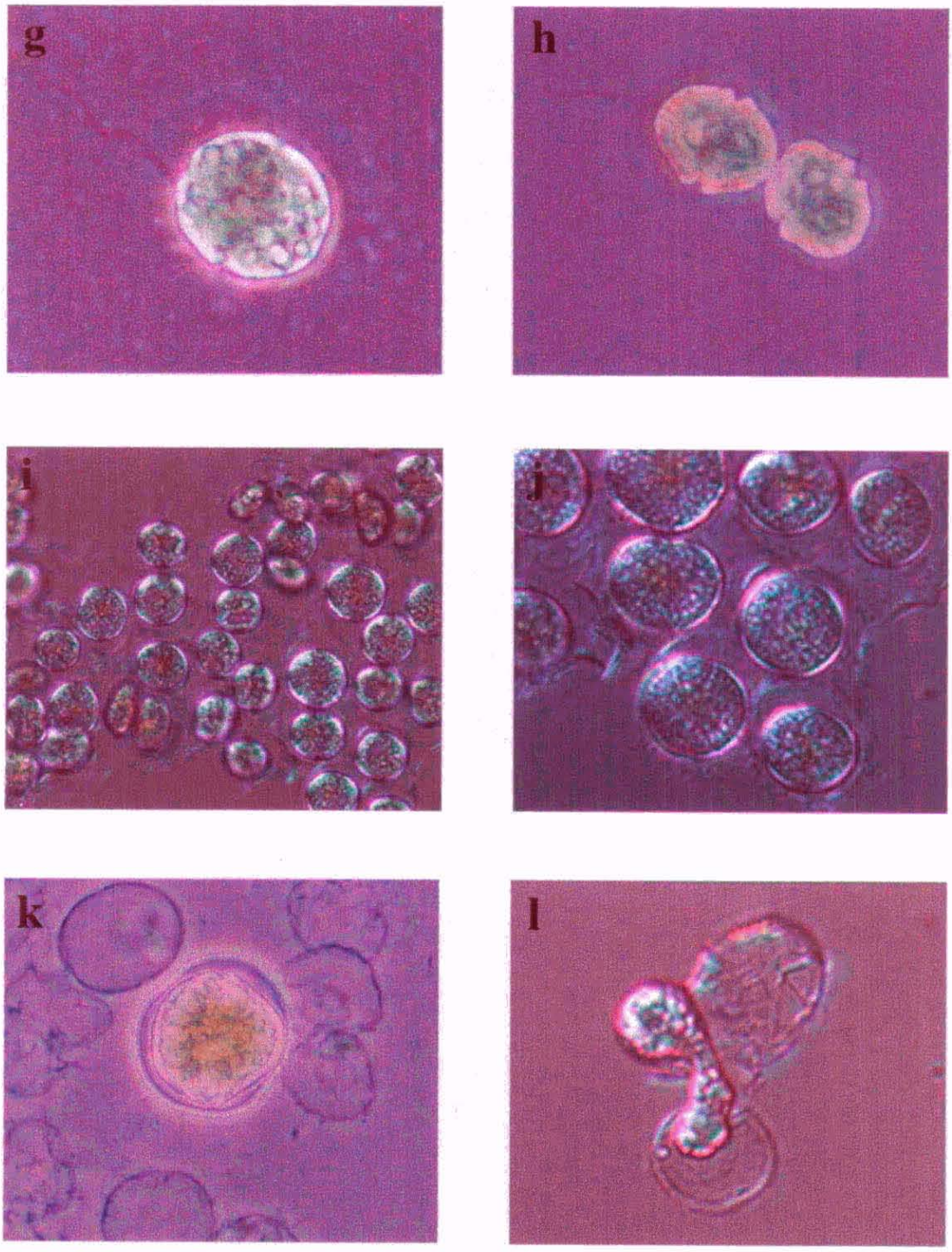

Figure 2.2g-l. Life cycle stages of *Alexandrium minutum* g) germling or planomeiocyte, h) vegetatively dividing cells, i) cluster of resting cysts j) higher magnification view of resting cyst cluster clearly showing fusion of mucoid sheaths, k) temporary resting cyst, l) germinating temporary resting cyst.

DISCUSSION

The life cycle characteristics of *A. minutum* are similar to those of other dinoflagellates and particularly similar to the other eight species of the *Alexandrium* genus for which life cycles have been described (Table 2.1). Gametes in all nine species are isogamous, with the exception that both anisogamy and isogamy have been reported for *A. tamarense* (Turpin et al., 1978; Destombe and Cembella, 1990). A planozygote that is larger and darker than the vegetative cell and with two longitudinal flagella has also been found in all *Alexandrium* species that have been studied. The morphology of the *A. minutum* resting cyst is similar to many other species in this genus, with a thick, smooth cell wall encased in a mucoid sheath (Table 2.1). Exceptions are *A. pseudogonyaulax* which has a paratabulate cyst (with markings reflecting the plates of the vegetative cell, – Montresor, 1995) and *A. hiranoi* which has a highly pigmented sexually produced temporary cyst with a thin cell wall (Kita et al., 1993). The archeopyle structure of *Alexandrium* species has not been studied in detail although it has been described as chasmic (a slit like opening) in some species (*A. catenella*, *A. affine*, (Matsuoka and Fukuyo, 1995), *A. ostenfeldii* (MacKenzie et al., 1996)). This type of archeopyle was also observed for *A. minutum* (Bolch et al., 1991 and this study). Another obvious feature of the *A. minutum* resting cyst was the presence of a membrane within empty resting cysts which appears to detach from the germling cell upon germination (Fig 2.2f). This membrane has also been observed in *A. ostenfeldii* by MacKenzie et al. (1996) who suggest that it is the pellicle discarded by the emerging cell. The germling cell is similar in morphology to the planozygote and this too has been observed in all *Alexandrium* species studied. Based on the observations presented here, the germling cell which emerges from the hypnozygote or resting cyst can only be presumed to be a planomeiocyte as the point at which meiosis occurs has not been determined.

Temporary cysts similar to those observed for *A. minutum* in response to cold shock are also common within the genus *Alexandrium* and have been observed for all but a few species (Table 2.1).

An interesting aspect of encystment in *A. minutum* is the formation of large clusters of hundreds to thousands of cysts in culture (Figs. 2.2i and 2.2j). Cyst deposition was clearly non-random in the small culture volumes which were studied (5-50 mL), with cysts tending to aggregate near the edges of petri dishes or flasks or in definite clusters away from the edges. Whether this aggregation of cysts occurs in nature or is an artifact of culture conditions was not determined in the present study. There is evidence that some freshwater dinoflagellate planozygotes are able to carry out substrate selection to a certain degree, rather

Table 2.1: Life cycle characteristics of species of the genus *Alexandrium*.

Species	Mating Characteristics	Temporary Cyst	Resting Cyst	Induction of Sexuality	Dormancy	References
Alexandrium catenella	heterothallic isogamous	not observed	yes	Nutrient limitation or + GeO_2	3-4 months - Japanese population; 4-8 wks - Australian population	Yoshimatsu 1981, Hallegraeff et al. 1998
Alexandrium hiranoi	homothallic, isogamous	veg. and sexual	no	no cue required	none	Kita et al. 1993
Alexandrium lusitanicum	isogamous, homothallic	unknown	yes	N & Fe limitation	unknown	Silva & Faust 1995, Blanco 1995
Alexandrium minutum	heterothallic isogamous	yes	yes	no cue required	3-4 wks	Parker–this study.
Alexandrium monilatum	isogamous, thallism unknown	not observed	yes	N limitation	2-4 wks	Walker & Steidinger 1979
Alexandrium ostenfeldii	heterothallic, isogamous	yes	yes - known only from field	nutrient limitation	unknown	Mackenzie et al. 1996, Jensen & Moestrup 1997
Alexandrium pseudogonyaulax	isogamous, thallism unknown	yes	yes	nutrient limitation	minimum 77 days	Montresor 1995, Montresor & Marino 1996
Alexandrium tamarense	anisogamous/ isogamous, homothallic/ heterothallic	yes	yes	nutrient limitation	up to 6 months	Turpin et al, 1978, Destombe & Cembella 1990
Alexandrium taylori	homothallic, isogamous	yes	yes	N limitation	15 days	Garces et al. 1998, Giaccobe & Yang 1999

than resting cyst deposition being a purely random process (Holt et al., 1994). If this is the case for *A. minutum*, active preferential settling of planozygotes in regions where other resting cysts are already present may occur, resulting in resting cyst aggregations in the natural environment. Resting cyst aggregation could potentially increase the sinking rate of resting cysts or inhibit grazing of resting cysts in the sediments by increasing particle size. One function of the mucoid sheath of *A. minutum* and other *Alexandrium* species may be to stick together such aggregations of resting cysts.

Both temporary cysts and sexually produced resting cysts have the potential to be ecologically important for the persistence of *A. minutum* in the phytoplankton assemblage of a given area. Resting cysts are more environmentally tolerant and robust than vegetative cells (Anderson, 1980) and may therefore enable *A. minutum* to survive environmental fluctuations on both short time scales (e.g. temporary cyst formation in response to sudden temperature changes, deep mixing or perhaps unusual circulation patterns) and long time scales (e.g. sexual resting cyst formation as a means to survive seasonal changes in light and temperature). There is also evidence that resting cysts are resistant to algicidal bacteria, which cause cell lysis of vegetative cells (Chapter 5). Through resting cyst formation *A. minutum* can be completely absent from the water column in a given region but be present as viable resting cysts in the sediments. These cysts can then inoculate overlying waters when conditions become suitable for germination. The long-term viability of *A. minutum* resting cysts is unknown. However, Keafer et al. (1992) suggested that *A. tamarense* resting cysts have half lives on the order of years to decades and cysts of the freshwater dinoflagellate *Peridinium cinctum* are believed to be viable for up to 16 years (Huber and Nipkow, 1922). Therefore the production of resting cysts is potentially a long-term survival strategy.

The minimum dormancy period for the *A. minutum* resting cysts in the present study (approximately 4 weeks) is similar to that of *A. monilata* and *A. taylori*, but short compared to other *Alexandrium* species (Table 2.1). Resting cysts of *A. minutum* should be able to germinate whenever conditions are appropriate for germination after this short dormancy period. However, many resting cysts in this study did not germinate at the end of 4 weeks. There are two possible explanations for this. The first is that resting cyst dormancy varies between resting cysts. The second is that those resting cysts that do not germinate after four weeks are not dormant but quiescent, that is, they are able to germinate but for some external reason do not. Quiescence is less likely given that all cysts were in the same environmental conditions. Determination of the 'median resting cyst dormancy period' of a species may be a more ecologically meaningful parameter than minimum resting cyst dormancy (Binder and Anderson, 1987; Blackburn et al., 2001). Median resting cyst dormancy is the number of days from cross inoculation to 50% resting cyst germination. The minimum dormancy

requirements of *A. minutum* suggest that resting cysts formed in a bloom may contribute new cells to that same bloom, but if the median dormancy is much higher this may be a rare event. A higher median dormancy may also increase the chance that resting cysts will be buried in the sediments, in an environment that will keep them quiescent, before the dormancy period ends. The quantification of median resting cyst dormancy for *A. minutum* is important for identifying the role of resting cysts in bloom dynamics.

It has been previously suggested by Bolch et al. (1991) that *A. minutum* may have a long requisite dormancy period similar to *A. tamarense* as determined by Anderson (1980; up to 6 months depending on storage temperature). This suggestion was based on the failure of cysts collected from sediments in September of 1988 to germinate in June/July 1989 that then readily germinated in November/December 1989. In the present study resting cysts of *A. minutum* had a minimum dormancy period of approximately 4 weeks at different times of the year. The reason for the discrepancy between minimum dormancy requirements of resting cysts in this study and those suggested by Bolch et al. (1991) is uncertain but the environmental history of their field obtained cysts was very different to those in the laboratory. Resting cysts in this study were maintained at 18 °C, while Bolch et al. (1991) stored resting cysts at 4 °C. Based on field observations European populations of *A. minutum* have also been hypothesised to have a long dormancy period of several months (Marasovic et al., 1995) but this has not been confirmed by laboratory studies and again may be a result of environmental conditions rather than dormancy requirements. The interaction between dormancy, quiescence and the resting cyst environment is complex. Cysts from an Australian population of *Alexandrium catenella* were found to have shorter dormancy periods than those from northern hemisphere (Japanese) populations (4-8 weeks, Hallegraeff et al., 1998; compared with 3-4 months Yoshimatsu, 1981). These observations indicate that resting cysts from different populations of the same dinoflagellate species can have different required dormancy periods. If dormancy requirement is a genetically determined trait, change may occur due to selection over time in response to a different environment, for example, longer dormancy periods may be selected for as a better strategy for temperate than sub-tropical waters. A second possibility which has been suggested and investigated by various authors (Huber and Nipkow, 1923; Anderson, 1980; Dale, 1983; Perez et al., 1998) is that temperature may influence or condition dormancy through a time-temperature relationship in which lower temperatures result in longer maturation times or dormancy. This would help explain the different minimum dormancy requirements for *A. minutum* resting cysts in this study compared with that of Bolch et al. (1991). The adjustment of dormancy in response to environment may enhance invasive success of a species after introduction into new regions through either natural or anthropogenic means (Hallegraeff et al., 1998). Environmental conditioning of dormancy makes the comparison of reported

dormancy periods for different species somewhat difficult. To accurately determine differences in either mean or minimum resting cyst dormancy requirements between populations or species, comparisons need to be made under the same conditions and the effect of different environmental factors, especially temperature, tested.

The observation and description of the complete sexual life cycle of *A. minutum* and determination of its minimum dormancy requirements is a critical step towards understanding the role of resting cysts in the ecology of this important harmful algal bloom forming species. The next challenges are to determine the frequency with which sexual reproduction occurs in natural systems, identify the median dormancy requirements and the impact that environmental factors may have on dormancy, and to determine if aggregation of resting cysts occurs in nature.

REFERENCES

Anderson DM (1980) Effects of temperature conditioning on development and germination of *Gonyaulax tamarensis* (Dinophyceae) hypnozygotes. Journal of Phycology 16: 166–172.

Anderson DM (1984) Shellfish toxicity and dormant cysts in toxic dinoflagellate blooms. In Ragelis EP (ed), Seafood Toxins. American Chemical Society, Washington DC: 125–138.

Anderson DM, Chisholm SW, Watras CJ (1983) Importance of life cycle events in the population dynamics of *Gonyaulax tamarensis*. Marine Biology 76: 179–189.

Balech E (1985) The genus *Alexandrium* or *Gonyaulax* of the tamarense group. In Anderson DM, White AW, Baden DG (eds), Toxic Dinoflagellates. Elsevier/North Holland, Amsterdam: 33–38.

Balech E (1989) Redescription of *Alexandrium minutum* Halim (Dinophyceae) type species of the genus *Alexandrium*. Phycologia 28: 206–211.

Belin C (1993) Distribution of Dinophysis spp. and *Alexandrium minutum* along French coasts since 1984 and their DSP and PSP toxicity levels. In Smayda TJ, Shimizu Y (eds), Toxic Phytoplankton Blooms in the Sea. Elsevier Science Publishers BV: 469–474.

Binder BJ, Anderson DM (1987) Physiological and environmental control of germination in *Scrippsiella trochoidea* (Dinophyceae) resting cysts. Journal of Phycology 25: 577–590.

Blackburn SI, Bolch CJ, Haskard KA, Hallegraeff GM (2001) Reproductive compatibility among four global populations of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). Phycologia 40: 78–87.

Blanco J (1995) Cyst production in four species of neritic dinoflagellates. Journal of Plankton Research 17: 165–182.

Bolch CJ, Blackburn SI, Cannon JA, Hallegraeff GM (1991) The resting cyst of the red-tide dinoflagellate *Alexandrium minutum* (Dinophyceae). Phycologia 30: 215–219.

Cannon JA (1990) Development and dispersal of red tides in the Port River, South Australia. In Graneli E, Sundstrom B, Edler L, Anderson DM (eds), Toxic Marine Phytoplankton. Elsevier, New York: 110–115.

Cannon JA (1993a) Germination of the toxic dinoflagellate, *Alexandrium minutum*, from sediments in the Port River, South Australia. In Smayda TJ, Shimizu Y (eds), Toxic phytoplankton blooms in the sea. Elsevier, Amsterdam: 109–114.

Cannon JA (1993b) Growth in culture of the toxic dinoflagellate *Alexandrium minutum* from the Port River, South Australia. In Smayda TJ, Shimizu Y (eds), Toxic Phytoplankton Blooms in the Sea. Elsevier, New York: 741–745.

Chang FH, McClean M (1997) Growth responses of *Alexandrium minutum* (Dinophyceae) as a growth function of three different nitrogen sources and irradiance. New Zealand Journal of Marine and Freshwater Research 31: 1–7.

Destombe C, Cembella A (1990) Mating-type determination, gametic recognition and reproductive success in *Alexandrium excavatum* (Gonyaulacales, Dinophyta), a toxic red-tide dinoflagellate. Phycologia 29: 316–325.

Flynn K, Franco JM, Fernandez P, Reguera B, Zapata M, Wood G, Flynn KJ (1994) Changes in toxin content, biomass and pigments of the dinoflagellate *Alexandrium minutum* during nitrogen refeeding and growth into nitrogen or phosphorus stress. Marine Ecology Progress Series 111: 99–109.

Franco JM, Fernandez P, Reguera B (1994) Toxin profiles of natural populations and cultures of *Alexandrium minutum* Halim from Galician (Spain) coastal waters. Journal of Applied Phycology 6: 275–279.

Garcés E, Delgado M, Maso M, Camp J (1998) Life history and in situ growth rates of *Alexandrium taylori* (Dinophyceae, Pyrrophyta). Journal of Phycology 34: 880–887.

Giacobbe MG, Gangemi ESO (1997) Vegetative and sexual aspects of *Dinophysis pavillardi* (Dinophyceae). Journal of Phycology 33: 73–80.

Giacobbe MG, Yang X (1999) The life history of *Alexandrium taylori* (Dinophyceae). Journal of Phycology 35: 331–338.

Halim Y (1960) *Alexandrium minutum* n. gen. s. sp., dinoflagelle provocant "des eaux rouges". Vie Milieu 11: 102–105.

Hallegraeff GM, Steffenson DA, Wetherbee R (1988) Three estuarine Australian dinoflagellates that can produce paralytic shellfish toxins. Journal of Plankton Research 10: 533–541.

Hallegraeff GM, Marshall JA, Valentine J, Hardiman S (1998) Short cyst-dormancy period of an Australian isolate of the toxic dinoflagellate *Alexandrium catenella*. Marine and Freshwater Research 49: 415–420.

Holt JR, Merrell JR, Seaborn DW, Hartranft JL (1994) Population dynamics and substrate selection by three Peridinium species. Journal of Freshwater Ecology 9: 17–128.

Huber G, Nipkow F (1922) Experimentelle Untersuchungen uber die Entwicklung *von Ceratium hirundinella*. Zeitschrift fur Botanik 14: 337–371.

Huber G, Nipkow F (1923) Experimentelle Untersuchungen uber die Entwicklung und Formbildung *von Ceratium hirundinella* O. Fr. Mull. Flora New Ser. 16: 114–215.

Jensen MO, Moestrup O (1997) Autecology of the toxic dinoflagellate *Alexandrium ostenfeldii*: Life history and growth at different temperatures and salinities. European Journal of Phycology 32: 9–18.

Keafer BA, Buesseler KO, Anderson DM (1992) Burial of living dinoflagellate cysts in estuarine and nearshore sediments. Marine Micropaleontology 20: 147–161.

Kita T, Fukuyo Y, Tokuda H, Hirano R (1993) Sexual reproduction of *Alexandrium hiranoi* (Dinophyceae). Bulletin of the Plankton Society of Japan 39: 79–85.

Koray T, Buyukisik B (1988) Toxic dinoflagellate blooms in the harbour region of Izmir Bay (Aegean Sea). Revue Internationale d'Oceanographie Medicale. 91–92, 25–42.

MacKenzie LM and Berkett N (1997) Cell morphology and PSP-toxin profiles of *Alexandrium minutum* in the Marlborough Sounds, New Zealand. New Zealand Journal of Marine and Freshwater Research 31: 403–409.

MacKenzie L, White D, Oshima Y, Kapa J (1996) The resting cyst and toxicity of *Alexandrium ostenfeldii* (Dinophyceae) in New Zealand. Phycologia 35: 148–155.

Marasovic I, Nincevic Z, Odzak N (1995) The effect of temperature on blooms of *Lingulodinium poyedra* and *Alexandrium minutum* in Kastela Bay. In Lassus P, Arzul E, Erard P, Gentien P, Marcaillou C (eds), Harmful Marine Algal Blooms, Lavoisier, Intercept Ltd.: 187–192.

Matsuoka K, Fukuyo Y (1995) Taxonomy of cysts. In Hallegraeff GM, Anderson DM, Cembella AD (eds), Manual on harmful marine microalgae. IOC Manuals and Guides No 33. UNESCO, France: 381–401.

Montresor M, Marino D, Zingone A, Dafnis G (1990) Three *Alexandrium* species from coastal Tyrrhenian waters (Mediterranean Sea). In Graneli E, Sundstroem B, Edler L, Anderson DM (eds), Toxic Marine Phytoplankton. Elsevier, New York: 82–87.

Montresor M (1995) The life history of *Alexandrium pseudogonyaulax* (Gonyaulacales, Dinophyceae). Phycologia 34: 444–448.

Montresor M, Marino D (1996) Modulating effect of cold-dark storage on excystment in *Alexandrium pseudogonyaulax* (Dinophyceae). Marine Biology 127: 55–60.

Nezan E, Belin C, Lassus P, Piclet G, Berthome JP (1989) *Alexandrium minutum*: first PSP occurrence in France. In Fourth international conference on Toxic Marine Phytoplankton: Abstracts. Lund. p. 111

Oshima Y, Hirota M, Yasumoto T, Hallegraeff GM, Blackburn SI, Steffensen DA (1989) Production of paralytic shellfish toxins by the dinoflagellate *Alexandrium minutum* Halim from Australia. Nippon Suisan Gakkaishi 55: 925.

Oshima Y, Itakura H, Lee KC, Yasumoto T, Blackburn S, Hallegraeff G (1993) Toxin production by the dinoflagellate *Gymnodinium catenatum*. In Smayda TJ, Shimizu Y (eds), Toxic Phytoplankton Blooms in the Sea. Elsevier, New York: 907–912.

Perez CC, Roy S, Levasseur M, Anderson DM (1998) Control of germination of *Alexandrium tamarense* (Dinophyceae) cysts from the lower St. Lawrence Estuary (Canada). Journal of Phycology 34: 242–249.

Pfiester LA, Anderson DM (1987) Dinoflagellate reproduction. In Taylor FJR (ed), The biology of dinoflagellates. Blackwell Scientific Publications, Oxford: 611–648.

Silva ES, Faust MA (1995) Small cells in the life history of dinoflagellates (Dinophyceae): a review. Phycologia 34: 396–408.

Steidinger KA, Tangen K (1997) Dinoflagellates. In Tomas CR (ed), Identifying Marine Phytoplankton. Academic Press, San Diego: 387–584

Steidinger KA, Burkholder JM, Glasgow HB Jr, Hobbs CW, Garrett JK, Truby EW, Noga EJ, Smith SA (1996) *Pfiesteria piscicida* gen. et sp. nov. (Pfiesteriaceae fam. nov.), a new toxic dinoflagellate with a complex life cycle and behavior. Journal of Phycology 32: 157–164.

Turpin DH, Dobell PER, Taylor FJR (1978) Sexuality and cyst formation in Pacific strains of the toxic dinoflagellate *Gonyaulax tamarensis*. Journal of Phycology 14: 235–238.

von Stosch HA (1973) Observations on vegetative reproduction and sexual life cycles of two freshwater dinoflagellates, *Gymnodinium pseudopalustre* Schiller and *Woloszynskia apiculata* sp. nov. British Phycological Journal 8: 105–134.

Walker LM (1984) Life histories, dispersal and survival in marine, planktonic dinoflagellates. In Steidinger KA, Walker LM (eds), Marine Plankton Life Cycle Strategies. CRC Press Inc., Boca Raton, Florida: 19–34.

Walker LM, Steidinger KA (1979) Sexual reproduction in the toxic dinoflagellate *Gonyaulax monilata*, Journal of Phycology 15: 312–315.

Wall D (1971) Biological problems concerning fossilizable dinoflagellates. Geoscience and Man 3: 1–15.

Yoshimatsu S (1981) Sexual reproduction of *Protogonyaulax catenella* in culture. I. Heterothallism. Bulletin of the Plankton Society of Japan 28: 131–139.

CHAPTER 3

DINOFLAGELLATE MATING SYSTEMS

INTRODUCTION

Sexual reproduction has been described in all major classes of algae except the Euglenophyta and the Cyanophyta (Coleman, 1962; van den Hoek et al., 1995) and in many other protists, including the ciliates. Protist mating systems are highly variable. Some protist species are self-fertile, i.e. sexual reproduction occurs within clonal culture (homothallism) while other species are outcrossing i.e. reproductively compatible strains of different 'mating types' must be combined in order for sexual reproduction to occur (heterothallic or self-infertile). Mating type is a genetically determined physiological difference between individuals that determines which other individuals they are sexually compatible with (Sonneborn, 1957). In many algae, fungi and protozoa there are multiple genetically determined mating types within a single species, which can be identified by the specificity they express in sexual reproduction (Coleman, 1977).

Mating systems have been studied in various algal groups, as well as in other protists. Mating systems of algae and other protists describe how and when, under what intrinsic and extrinsic factors, individuals shift to a sexual reproductive mode and accomplish sexual reproduction rather than continuing multiplication of vegetative individuals (Ichimura and Kasai, 1990). Mating systems vary in complexity. The simplest possible mating system is a homothallic one. The simplest heterothallic mating system is a binary system in which all individuals are either mating type (+) or mating type (–) with mt(+) mt(–) combinations resulting in sexual reproduction but not mt(+) mt(+) or mt(–) mt(–) combinations (Yoshimatsu, 1981, 1984). Other mating systems of greater complexity have been identified as having multiple fixed mating types e.g. seven mating types in the ciliate *Tetrahymena thermophila* (Doerder et al., 1995). Also described are mating systems which include strains that are both homothallic and heterothallic e.g. the desmid *Triploceras gracile* (Ichimura and Kasai, 1989) and multiple mating type systems with varying levels of compatibility between groups e.g. the ciliate *Euplotes octocarinatus* (Kuhlmann et al., 1997). Within the algae, significant investigations of mating types and systems have been carried out on the Chlorophytes (Coleman et al., 1994) and Chrysophytes (e.g. Sandgren, 1981; Sandgren and Flanagin, 1986). The Chlorophyte genus *Chlamydomonas* is particularly well understood, including the genetic basis of mating types, and contains species with both homothallic (*C. monoica*) and heterothallic (*C. reinhardtii*) mating systems (van den Ende and van Winkle-Swift, 1994).

Sexual reproduction in dinoflagellates (Phylum Dinophyta, Class Dinophyceae) has been documented since the 1960s but in only a small proportion (53 – Chapter 1, Table 1.1) of the 2000+ extant species of dinoflagellate (Steidinger and Tangen, 1997). The genetic basis of mating types and therefore mating systems has only been explored in a few dinoflagellate

species and detailed information on the position in the genome of genes coding for mating type is unknown (Beam and Himes, 1984). The genetic study of dinoflagellates has been hindered by various unique characteristics of this group of organisms. The dinoflagellate nucleus contains many chromosomes, ranging from 4 (*Syndinium borgerti*) to 325 (*Endodinium chattoni*) and the chromosomes remain condensed throughout the cell cycle (Spector, 1984). The nuclear DNA content of dinoflagellates is also very high, ranging from 3 pg in the *Amphidinium carterae* nucleus to 200 pg in the nucleus of *Gonyaulax polyedra* (Rizzo, 1987) and has been found to vary with growth phase (Allen et al., 1975). The initial difficulties with culturing dinoflagellates and inability to grow most on agar has also hindered the genetic study of this group of organisms (Beam and Himes, 1984). Mating type is the phenotypic expression of what is believed to be a single pair of alleles in some species (e.g. *Peridinium volzii*, and *Ceratium cornutum*, Beam and Himes, 1984). Mating systems of increasing complexity seem to support the idea that mating type is influenced by multiple genetic factors (a gene complex) segregating at meiosis, as in some fungal systems (Blackburn et al., 2001), rather than by a single pair of alleles. A gene complex would result in a greater range of possible mating types in the progeny of crosses than would a single pair of alleles. The observation that *G. catenatum* cultures established from resting cysts are rarely self-compatible also supports the idea that mating type is influenced by multiple genetic factors (C.J. Bolch and M. van Emmerik, unpublished data – Blackburn et al., 2001).

Despite the increased knowledge of sexual life cycles in dinoflagellates (see Chapter 2 and Table 1.1), there are still relatively few studies of the mating systems of this group of organisms. Many species with known sexual life cycles have been identified as either homothallic or heterothallic (see Table 1.1) but of those that are heterothallic, few have been studied in further detail. The number of species of dinoflagellates in which heterothallic mating systems have been investigated in any detail is low, (less than 10) even by comparison to the number for which sexual life cycles have been defined (53, Table 1.1).

Much of the early work on dinoflagellate mating systems was carried out on either homothallic dinoflagellate species such as *Crypthecodinium cohnii* (Beam and Himes, 1974) or on those with simple, binary heterothallic mating systems with just two mating types (mt(+) and mt(–)), such as *Peridinium volzii* (Pfiester and Skvarla, 1979) and *Alexandrium catenella* (Yoshimatsu, 1981). Subsequent work has identified mating systems of increasing complexity. Blackburn et al. (1989) described the mating system of *Gymnodinium catenatum*; they found that most strains (of 13 tested) could be classified as either mt(+) or mt(–) (i.e. a two mating type or binary system), but also found that not all mt(+) and mt(–) strains showed compatibility under the conditions tested. Destombe and Cembella (1990) introduced the concept of a continuum of mating compatibility in the dinoflagellates in

which some strains may be partially compatible but do not produce viable hypnozygotes. Their study of 21 strains of Canadian *Alexandrium tamarense*, showed that *A. tamarense* could not be classified as having a binary heterothallic mating system. The mating system of this species was described as a multiple mating-type system with gradients of gametic affinity and zygote viability resulting from sexual fusion. A similar mating system was found in a follow-up of the Blackburn et al. (1989) study of *Gymnodinium catenatum*; between three and seven mating types were identified within 21 strains from four global populations (Blackburn et al., 2001). In summary, the known dinoflagellate mating systems can be classified into three general categories, homothallic, binary heterothallic and complex heterothallic mating systems with gradients of affinity that may also include homothallic strains.

The ecological significance and impact, and evolutionary benefit of different mating systems has not been addressed for dinoflagellates, and is also largely unexplored for protists in general aside from a small body of literature on *Chlamydomonas*, desmids and ciliates (e.g. Ferris et al., 2001; Ichimura and Kasai, 1995; Doerder et al., 1996; Vallesi et al., 1995). There is little known of even the frequency of sexual reproduction in natural dinoflagellate populations (Kremp and Heiskanen, 1999). To obtain a greater understanding of the role of sexual reproduction in the ecology and bloom dynamics of Australian populations of dinoflagellates, the mating systems of four species were investigated; the toxic species *Alexandrium minutum*, *Alexandrium catenella* and *Gymnodinium catenatum* and the potentially toxic *Protoceratium reticulatum* (found to produce yessotoxin in some Japanese strains, Satake et al., 1997, 1999). Their mating systems are presented in this chapter. The mating systems of *A. minutum* and *P. reticulatum* are described for the first time and *A. catenella* for the first time from Australian populations. The mating system of Australian *G. catenatum* is explored further, focussing on the long-term stability of mating types, and spatial and temporal variability in reproductive compatibility of strains isolated from the Huon Estuary in south east Tasmania. The ecological implications of the different observed mating systems are discussed.

MATERIALS AND METHODS

3.1 STRAIN DETAILS

Twelve strains of *Alexandrium minutum*, fourteen strains of *Alexandrium catenella*, and seven strains of *Gymnodinium catenatum* were obtained from the CSIRO Collection of Living Microalgae (http://www.marine.csiro.au/microalgae). A further six strains of *Alexandrium catenella* were isolated from germinated resting cysts obtained from the Port River Estuary in South Australia (all Port River *A. catenella* strains were toxic, see Appendix

B). Thirty strains of *Gymnodinium catenatum* were isolated from blooms in the Huon Estuary in southeast Tasmania, and ten strains of *Protoceratium reticulatum* were isolated from a bloom in the Derwent Estuary, also in southeast Tasmania. Strain details for all strains are given in Table 3.1. Strain stock cultures were maintained at 18°C ± 0.5 °C at 80μmol photons PAR m^{-2} s^{-1} (cool white fluorescent lamps) on a 12:12 light:dark cycle in 50 mL Erlenmeyer flasks in 40 mL GSe medium (Blackburn et al., 2001, Appendix A).

3.2 MATING SYSTEM DETERMINATION

Crosses of strains of each species were made using a modification of the resting cyst formation methods of Oshima et al. (1993). Culture volumes were first increased from 40 to 70 mL under the same environmental conditions as indicated above. After 2 weeks, 10 mL was transferred into 50 mL of $GSe/_{20}$ i.e. GSe medium with $^1/_{20}$ nutrients and soil extract (GSe^{-N-P} i.e. GSe with no added nitrate or phosphate, and $Gse/_{10}$ were also used in some cases as specified below). After a further week under these reduced nutrient conditions, pair-wise crosses of groups of strains (i.e. crosses of strains in all combinations) for a particular species or population were made. These were either carried out in 50 mm diameter petri dishes (1- 2 mL of each of two strains and 6 - 9 mL of $GSe/_{20}$) or 32 mm diameter petri dishes (1 mL of each of two strains and 3 mL of $GSe/_{20}$) with initial cell concentrations on the order of 2 x 10^2 cells mL^{-1} for *G. catenatum* and 2 x 10^3 cells mL^{-1} for *A. minutum*, *A. catenella* and *P. reticulatum*. Control crosses, to determine selfing (homothallic) potential were made by adding only one strain (at double the volume) into the petri dish. Crosses were observed weekly for 2-3 months for any sexual activity (i.e. gametes, planozygotes, and resting cysts) and the appearance of these sexual stages recorded. Petri dish cultures were observed directly using either a Leitz Labovert or a Nikon Diaphot inverted microscope. Ten experimental crosses or crossing matrices were carried out to investigate differences in mating systems between species, and between populations within species. Details of the ten experiments are outlined below. Experimental cultures were maintained under the same environmental conditions as those outlined in Section 3.1. for stock cultures.

Resting cyst formation was used as the criterion for reproductive compatibility and determination of the mating system. To assess the variability in reproductive compatibility between strains within a crossing matrix, each compatible pair of strains was scored from 1 to 4. This scoring was based on the number of resting cysts produced per cross (i.e. a score of 1 represents < 10 resting cysts per cross and a score of 4 represents >1000 resting cysts per cross - see Table 3.2). A total compatibility index (TCI) was calculated for each group of strains. The TCI is the percentage of pairs of strains that produced viable resting cysts out of the total number of pairs of strains (including self-crosses). For each individual strain,

Table 3.1: Isolation details of strains used in mating system experiments. ND= not determined, UN = unknown, ex RC = germinated resting cyst

SPECIES	STRAIN I.D.	SOURCE LOCATION	ISOLATION HISTORY	ISOLATOR	DATE ISOLATED	CLONAL	TOXIC
Gymnodinium catenatum	GCHU02	Australia (Huon Estuary - Tas)	chain of 8 cells	S. Blackburn	6/6/1986	yes	yes
	GCHU09	Australia (Huon Estuary - Tas)	one of four 8-cell chains, the products of germinations of a GCHU05 resting cyst	S. Blackburn	15/6/1988	ND	No
	GCHU11	Australia (Huon Estuary - Tas)	The other 4-cell chain isolated with GCHU10	S. Blackburn	20/6/1988	ND	ND
	GCDE02	Australia (Derwent Estuary - Tas)	chain of 8 cells	S. Blackburn	23/1/1986	yes	yes
	GCDE06	Australia (Derwent Estuary - Tas)	chain of 10 cells	S. Blackburn	8/1/1987	yes	yes
	GCDE08	Australia (Derwent Estuary - Tas)	chain of 8 cells	S. Blackburn	15/6/1987	yes	yes
	GCJP10	Japan		UN	UN	ND	ND
	GCW1-97	Australia (Huon Estuary - Tas - W)	16 cell chain	C. Bolch	18/12/1997	yes	ND
	GCW5-97	Australia (Huon Estuary - Tas - W)	12 cell chain	C. Bolch	18/12/1997	yes	ND
	GCW7-97	Australia (Huon Estuary - Tas - W)	12 cell chain	C. Bolch	18/12/1997	yes	ND
	GCBP01-97	Australia (Huon Estuary - Tas - BPk)	10 cell chain	C. Bolch	18/12/1997	yes	ND
	GCBP04-97	Australia (Huon Estuary - Tas - BPk)	8 cell chain	C. Bolch	18/12/1997	yes	ND
	GCBP05-97	Australia (Huon Estuary - Tas - BPk)	8 cell chain	C. Bolch	18/12/1997	yes	ND
	GCBP06-97	Australia (Huon Estuary - Tas - BPk)	6 cell chain	C. Bolch	18/12/1997	yes	ND
	GCBP08-97	Australia (Huon Estuary - Tas - BPk)	6 cell chain	C. Bolch	18/12/1997	yes	ND
	GCBP10-97	Australia (Huon Estuary - Tas - BPk)	16 cell chain	C. Bolch	18/12/1997	yes	ND
	GCBP12-97	Australia (Huon Estuary - Tas - BPk)	4 cell chain	C. Bolch	18/12/1997	yes	ND
	GCBP13-97	Australia (Huon Estuary - Tas - BPk)	8 cell chain	C. Bolch	18/12/1997	yes	ND
	GCHUN1-97	Australia (Huon Estuary - Tas - KB)	13 cell chain	N. Parker	5/12/1997	yes	ND
	GCHUN2-97	Australia (Huon Estuary - Tas - KB)	15 cell chain	N. Parker	5/12/1997	yes	ND
	GCHUN3-97	Australia (Huon Estuary - Tas - BPt)	8 cell chain	N. Parker	5/12/1997	yes	ND
	GCHUN4-97	Australia (Huon Estuary - Tas - W)	8 cell chain	N. Parker	5/12/1997	yes	ND

Table 3.1 Continued: Isolation details of strains used in mating system experiments. ND= not determined, UN = unknown, ex RC = germinated resting cyst

SPECIES	STRAIN I.D.	SOURCE LOCATION	ISOLATION HISTORY	ISOLATOR	DATE ISOLATED	CLONAL	TOXIC
Gymnodinium catenatum	PC9802	Australia (Huon Estuary - Tas - PC)	6 cell chain	N. Parker	23/4/1998	yes	ND
	PC9803	Australia (Huon Estuary - Tas - PC)	16 cell chain	N. Parker	23/4/1998	yes	ND
	PC9806	Australia (Huon Estuary - Tas - PC)	16 cell chain	N. Parker	23/4/1998	yes	ND
	K9801	Australia (Huon Estuary - Tas - KB)	8 cell chain	N. Parker	23/4/1998	yes	ND
	K9804	Australia (Huon Estuary - Tas - KB)	32 cell chain	N. Parker	23/4/1998	yes	ND
	K9808	Australia (Huon Estuary - Tas - KB)	12 cell chain	N. Parker	23/4/1998	yes	ND
	K9811	Australia (Huon Estuary - Tas - KB)	12 cell chain	N. Parker	23/4/1998	yes	ND
	K9815	Australia (Huon Estuary - Tas - KB)	8 cell chain	N. Parker	23/4/1998	yes	ND
	BP9803	Australia (Huon Estuary - Tas - BPk)	20 cell chain	N. Parker	23/4/1998	yes	ND
	BP9815	Australia (Huon Estuary - Tas - BPk)	32 cell chain	N. Parker	23/4/1998	yes	ND
	BP9818	Australia (Huon Estuary - Tas - BPk)	28 cell chain	N. Parker	23/4/1998	yes	ND
	HU9813	Australia (Huon Estuary - Tas - W)	12 cell chain	C. Bolch	22/4/1998	yes	ND
	HU9820	Australia (Huon Estuary - Tas - W)	12 cell chain	C. Bolch	22/4/1998	yes	ND
	HU9828	Australia (Huon Estuary - Tas - W)	16 cell chain	C. Bolch	22/4/1998	yes	ND
	HU9833	Australia (Huon Estuary - Tas - W)	12 cell chain	C. Bolch	22/4/1998	yes	ND
Alexandrium minutum	AMAD01	Australia (Port River - Adelaide)		S. Blackburn	27/10/1987	yes	yes
	AMAD02	Australia (Port River - Adelaide)		S. Blackburn	27/10/1987	yes	yes
	AMAD03	Australia (Port River - Adelaide)		S. Blackburn	27/10/1987	yes	yes
	AMAD06	Australia (Port River - Adelaide)		S. Blackburn	27/10/1987	yes	yes
	AMAD08	Australia (Port River - Adelaide)		S. Blackburn	27/10/1987	yes	yes
	AMAD09	Australia (Port River - Adelaide)		S. Blackburn	27/10/1987	yes	yes
	AMAD11	Australia (Port River - Adelaide)		S. Blackburn	27/10/1987	yes	yes
	AMAD12	Australia (Port River, Adelaide - no.1 dock)	collected 11/11/88	S. Blackburn/J. cannon	6/4/1989	yes	yes
	AMAD13	Australia (Port River, Adelaide - no.1 dock)	collected 11/11/88	S. Blackburn/J. cannon	6/4/1989	yes	yes
	AMAD14	Australia (Port River, Adelaide - no.1 dock)	collected 11/11/88	S. Blackburn/J. cannon	6/4/1989	yes	yes
	AMAD15	Australia (Port River, Adelaide - no.1 dock)	collected 11/11/88	S. Blackburn/J. cannon	6/4/1989	yes	yes
	AMAD16	Australia (Port River, Adelaide - no.1 dock)	collected 11/11/88	S. Blackburn/J. cannon	6/4/1989	yes	yes
	AMAD22	Australia (Port River, Adelaide, Jervois Bridge)	collected 8/9/88 germ 12/1/90	C. Bolch	13/6/1909	ex RC	yes
	AMAD25	Australia (Port River, Adelaide, Jervois Bridge)	collected 8/9/88 germ 12/1/90	C. Bolch	13/6/1909	ex RC	yes
	AMAD26	Australia (Port River, Adelaide, Jervois Bridge)	collected 8/9/88 germ 12/1/90	C. Bolch	13/6/1909	ex RC	yes
	AMAD27	Australia (Port River, Adelaide, Jervois Bridge)	collected 8/9/88 germ 12/1/90	C. Bolch	13/6/1909	ex RC	yes

Table 3.1 Continued: Isolation details of strains used in mating system experiments. ND= not determined, UN = unknown, ex RC = germinated resting cyst

SPECIES	STRAIN I.D.	SOURCE LOCATION	ISOLATION HISTORY	ISOLATOR	DATE ISOLATED	CLONAL	TOXIC
Alexandrium catenella	ACPP01	Australia (Port Phillip Bay - Vic)	03/03/1988	S. Blackburn	3/3/1988	yes	yes
	ACPP02	Australia (Port Phillip Bay - Vic)	03/03/1988	S. Blackburn	3/3/1988	yes	yes
	ACPP03	Australia (Port Phillip Bay - Vic)	03/03/1988	S. Blackburn	3/3/1988	yes	yes
	ACPP04	Australia (Port Phillip Bay - Vic)	03/03/1988	S. Blackburn	3/3/1988	yes	yes
	ACPP05	Australia (Port Phillip Bay - Vic)	03/03/1988	S. Blackburn	3/3/1988	yes	yes
	ACPP06	Australia (Port Phillip Bay - Vic)	03/03/1988	S. Blackburn	3/3/1988	yes	yes
	ACPP08	Australia (Port Phillip Bay - Vic)	10/03/1988	O Moestrup	10/3/1988	yes	yes
	ACPP10	Australia (Port Phillip Bay - Vic)	10/03/1988	O Moestrup	10/3/1988	yes	yes
	ACSH02	Australia (Sydney Harbor - NSW)	1 cell	C.Bolch	5/7/1994	yes	ND
	ACSH03	Australia (Sydney Harbor - NSW)	1 cell	C.Bolch	5/7/1994	yes	ND
	ACSH04	Australia (Sydney Harbor - NSW)	1 cell	C.Bolch	5/7/1994		
	ACSH05	Australia (Sydney Harbor - NSW)	1 cell	C.Bolch	5/7/1994	yes	ND
	ACSH06	Australia (Sydney Harbor - NSW)	1 cell	C.Bolch	5/7/1994	yes	ND
	ACSH07	Australia (Sydney Harbor - NSW)	1 cell	C.Bolch	5/7/1994	yes	
	ACAD01	Australia (Port River - Adelaide)	ex resting cyst, several chains	N. Parker	14/10/1997	no	yes
	ACAD02	Australia (Port River - Adelaide)	ex resting cyst, several chains	N. Parker	14/10/1997	no	yes
	ACAD03	Australia (Port River - Adelaide)	3-cell chain	N. Parker	14/10/1997	yes	yes
	ACAD04	Australia (Port River - Adelaide)	2-cell chain	N. Parker	14/10/1997	yes	yes
	ACAD05	Australia (Port River - Adelaide)	a single chain	N. Parker	14/10/1997	yes	yes
	ACAD06	Australia (Port River - Adelaide)	a single chain	N. Parker	14/10/1997	yes	yes
Protoceratium reticulatum	DPR02	Australia (Derwent Estuary - CSIRO wharf)	1 cell	N. Parker	1/12/1999	yes	ND
	DPR04	Australia (Derwent Estuary - CSIRO wharf)	1 cell	N. Parker	1/12/1999	yes	ND
	DPR05	Australia (Derwent Estuary - CSIRO wharf)	1 cell	N. Parker	1/12/1999	yes	ND
	DPR11	Australia (Derwent Estuary - CSIRO wharf)	1 cell	N. Parker	1/12/1999	yes	ND
	DPR12	Australia (Derwent Estuary - CSIRO wharf)	1 cell	N. Parker	1/12/1999	yes	ND
	DPR13	Australia (Derwent Estuary - CSIRO wharf)	1 cell	N. Parker	1/12/1999	yes	ND
	DPR20	Australia (Derwent Estuary - CSIRO wharf)	1 cell	N. Parker	1/12/1999	yes	ND
	DPR21	Australia (Derwent Estuary - CSIRO wharf)	1 cell	N. Parker	1/12/1999	yes	ND
	DPR22	Australia (Derwent Estuary - CSIRO wharf)	1 cell	N. Parker	1/12/1999	yes	ND
	DPR24	Australia (Derwent Estuary - CSIRO wharf)	1 cell	N. Parker	1/12/1999	yes	ND

three different measures of compatibility were calculated: a strain compatibility index, CI_s = the number of compatible pairings resulting in resting cyst production divided by the number of possible crosses other than self-crosses (a maximum of 1); strain average vigour, AV_s = an average measure of resting cysts produced per cross in successful crosses based on the 1-4 scoring described above and in Table 3.2 (a maximum of 4); and strain reproductive compatibility, RC_s= the product of the CI_s and the AV_s (a maximum of 4) (Blackburn et al., 2001).

Table 3.2: Criteria for scoring of compatibility between strains based on the numbers of resting cysts produced per cross. For *A. minutum* the presence of planozygotes and apparently non-viable resting cysts were also assessed.

Number of resting cysts produced	Resting cyst production score	Compatibility level * (*A. minutum*)
No sexual stages observed	0	
Planozygotes only observed	0	PZ
< 10 resting cysts per cross (or for *A. minutum* the presence of apparently non-viable resting cysts)	1	+?
10 - 100 resting cysts per cross	2	+
101 - 1000 resting cysts per cross	3	++
>1001 resting cysts per cross	4	+++

* Notation used in *A. minutum* crossing matrix - Figure 3.3

To evaluate mating system structure, the crossing results were simplified into a binary notation such that any combinations forming resting cysts were designated 1 and those that did not form resting cysts were designated as 0. When a simple mating system (e.g. a homothallic or binary heterothallic mating system) was present, mating types could be easily allocated to groups of strains. However, in three of the four species, mating systems were complex, with multiple mating types and gradients of mating affinity or reproductive compatibility between strains. Therefore, to investigate the mating systems, each crossing matrix was analysed by hierarchical cluster analysis with average linkage, using the statistical package JMP (Version 4.0; SAS Institute Inc.). Using average linkage, the numerical distance between two clusters was the average distance between two pairs of observations, or one in each cluster. This analysis therefore clustered strains whose reproductive compatibilities were close to each other relative to strains of other clusters. The subsequent arrangement of strains (presented here as dendrograms) and the numerical distances between groups were used to determine the potential number of mating types identifiable for each group of strains, and to describe the mating system (all linkage distances are included in Appendix C). Strains separated by a distance of less than 1 had identical reproductive compatibility and were considered to be of the same mating type. In the complex mating systems there was the potential for a mating type to consist of multiple strains whose mating compatibilities were not identical (which therefore had distances of

greater than 1). By moving the acceptable distance cut-off it was possible to identify a variable number of mating types containing strains of variable closeness in reproductive compatibility.

Cluster analysis also provided a maximum distance between strains which was indicative of the amount of variability in reproductive compatibility within a crossing matrix. Selfing strains were not included in cluster analysis, as the combination of any other strain with a selfing strain which produces resting cysts does not give a true indication of compatibility between those two strains, as some or all resting cysts produced may be the result of selfing. Differences in means of compatibility measures for each crossing matrix or species were tested for significance using Mann Whitney Rank Sum Tests using SigmaStat (version 2.0, SPSS Inc.).

3.3 CROSSING MATRIX DETAILS

3.3.1 *PROTOCERATIUM RETICULATUM*

Matrix 1: *Protoceratium reticulatum*

Ten strains of *Protoceratium reticulatum* were crossed in a complete pair-wise crossing matrix (i.e. all combinations of strains) to determine the mating compatibility of the strains. Crosses were made in 55 mm diameter pre-sterilised polystyrene petri dishes with 2 mL of each strain (or 4 mL of a single strain as a control to check for selfing) and 8 mL of $GSe/_{20}$. Strains were observed weekly for 6 weeks for evidence of sexual reproduction.

3.3.2 *ALEXANDRIUM MINUTUM*

***Alexandrium minutum* germinated resting cyst cultures**

Four strains of *Alexandrium minutum* (AMAD22, 25, 26 and 27), which were each initiated from a wild resting cyst germinated in the laboratory, were transferred into 55 mm diameter pre-sterilised polystyrene petri dishes containing nutrient deficient medium (either GSe^{-N-P} or $GSe/_{10}$) in order to determine whether they were able to sexually reproduce. In other dinoflagellate species, mating type has been found to segregate in a regular manner such that half the products of meiosis (either 2 or 4 cells depending on the species) will be of one mating type e.g. mt(+) and the other half will be of the other mating type e.g. mt(–) (Beam and Himes, 1984; Yoshimatsu, 1984). Because these strains originated from resting cysts they are therefore likely to be non-clonal if all products of meiosis survive to reproduce; this is not always the case e.g. for *Peridinium volzii* three of the four nuclei produced by meiosis abort (Pfiester and Skvarla, 1979). After 3 weeks, subsamples of the cultures were transferred again into the same nutrient deficient media (either GSe^{-N-P} or $GSe/_{10}$). Observations on both the original (parent) and new (daughter) cultures for evidence of sexual reproduction continued weekly for a further 8 weeks.

Matrix 2: *Alexandrium minutum* clonal strains

Complete pair-wise crosses were carried out to determine the sexual compatibility of 12 clonal strains of *A. minutum* (AMAD01, 02, 03, 06, 08, 09, 11, 12, 13, 14, 15 and 16) with controls for selfing. Crosses were set up in 30 mm diameter pre-sterilised petri dishes in which 1 mL of each strain or 2 mL of a single strain (selfing controls) was added along with 3 mL of $GSe/_{20}$. Crosses were checked regularly for evidence of sexual reproduction for a period of 12 weeks.

3.3.3 ALEXANDRIUM CATENELLA

Matrices 3, 4 and 5: *Alexandrium catenella* – Clonal strains from three populations

Three complete pairwise crossing matrices of strains from three different Australian populations of *Alexandrium catenella* were made: a 6x6 matrix of strains from Sydney Harbour in New South Wales, an 8x8 matrix of strains from Port Phillip Bay, Victoria and a 6x6 matrix of strains from the Port River, South Australia. Crosses were set up in $GSe/_{20}$ in 50 mm diameter pre-sterilised petri dishes with controls for selfing and checked regularly for 2-3 months for evidence of sexual reproduction.

3.3.4 *GYMNODINIUM CATENATUM*

Matrix 6: *Gymnodinium catenatum* Long term cultured strains

Seven strains which had been maintained in culture in the CSIRO Collection of Living Microalgae for up to 11 years were crossed in a complete pairwise crossing matrix. Six of the strains were of Tasmanian origin and one was originally isolated from a Japanese population (GCJP10). These crosses were carried out to determine long term stability of reproductive compatibility, as all these strains had been crossed 7 years previously (Blackburn et al, 2001). Crosses were carried out in $GSe/_{20}$ in 50 mm pre-sterilised petri dishes with controls for selfing and checked regularly over 12 weeks for evidence of sexual reproduction. Results from these crosses were compared with those of Blackburn et al. (2001). The scoring system generally adopted for this study (Table 3.2) is slightly different to that used by Blackburn et al. (2001). For this experiment (matrix 6), the Blackburn et al. (2001) scoring method was adopted so that the results could be more directly compared (see Table 3.3).

Matrices 7, 8 and 9: *Gymnodinium catenatum* bloom populations

Thirty *Gymnodinium catenatum* strains were isolated from two separate blooms in the Huon Estuary, southeast Tasmania; summer of 1997/98 (15 strains), and autumn of 1998 (15 strains). In each case strains were obtained from several sites in the Huon estuary (see Chapter 6 and Table 3.1). Complete pair-wise crosses of each group of strains were carried out in $GSe/_{20}$ in 50 mm pre-sterilised petri dishes with controls for selfing. A subset of these strains (8 from each of the summer and autumn blooms) was then combined in another

complete pair-wise matrix without controls for selfing, (selfing had already been investigated in the crosses of strains from each bloom). The summer and autumn blooms had quite different characteristics and dynamics. In particular, resting cyst formation was only detected in the summer bloom (see Chapter 6). The reproductive compatibility of the two groups of strains (summer and autumn) and the combination of a subset of the two groups (summer x autumn) were compared. This was done in order to determine if the differences observed in naturally occurring sexual reproduction were due to differences in reproductive compatibility between bloom populations from the same geographic region, or if the observed differences in sexual reproduction were more likely due to environmental conditions. For the summer x autumn crossing matrix (matrix 9) compatibility measures were calculated from the summer x autumn bloom strains only (i.e. not summer x summer or autumn x autumn). This is a subset of the total crosses with the remainder of the crosses representing duplicates of crosses from matrices 7 and 8. Consistency of reproductive compatibility between the duplicate crosses was also examined.

Table 3.3: Criteria for scoring of compatibility between strains based on the numbers of resting cysts produced per cross, used for long-term cultured *G. catenatum* strains (reproduced from Blackburn et al., 2001).

Resting Cysts per Cross	Resting cyst production score
<4	0
4-24	1
25-124	2
125-1200	3
>1200	4

RESULTS

All experiments showed some reproductive compatibility. However, within several of the crossing matrices there were some strains that were not reproductively compatible with any other strains in the group. The four species each had different mating systems and considerable variation in the number of resting cysts produced per cross.

3.4 *PROTOCERATIUM RETICULATUM*

3.4.1 MATRIX 1: PROTOCERATIUM RETICULATUM

Based on the ten strains examined in this study, *Protoceratium reticulatum* is heterothallic as no resting cysts formed in any of the self-crosses (Fig. 3.1). The complete sexual life cycle of *P. reticulatum* has not been documented but observations during this experiment suggest that *P. reticulatum* is anisogamous, as the fusion of large and small cells were observed. The crossing matrix for *P. reticulatum* is given in Figure 3.1 and compatibility measures for each

strain are presented in Table 3.4. Resting cysts were formed in relatively few strain combinations, resulting in a TCI for this group of strains of only 16.4%. Only one strain combination produced > 1000 resting cysts (score of 4 - DPR02 x DPR22) and two strains (DPR11 and DPR24) did not form resting cysts in any combination (RC_s=0). The most reproductive strain was strain DPR02 (RC_s =1.44) and all successful crosses contained either strain DPR02 or strain DPR13.

Based on cluster analysis of strain compatibility, an overall distance between strains of 2.4 was found (Fig. 3.2). A maximum of five mating types was identified (i.e. at a distance <1.0 which clusters only those strains with identical reproductive compatibility) (Fig. 3.2). Strains DPR04, DPR05 and DPR21 had identical reproductive compatibility, as did strains DPR12, DPR20 and DPR22, and strains DPR11 and DPR24 (the latter two strains did not form resting cysts in any combination). The remaining two strains, DPR02 and DPR13 differed in their reproductive compatibility (distance = 1.7) and therefore formed separate mating types. The grouping of the two strains which did not form resting cysts in any combination (non-reproductive strains – strains DPR11 and DPR24), is likely to be artificial, as the true mating group affiliation of these strains could not be determined. Although these two strains did not form resting cysts in any combination in this group of strains, they may form resting cysts with other strains of this species and have quite different compatibility characteristics.

Table 3.4: Compatibility measures (see Section 3.2 for details of calculations) for ten clonal strains of *Protoceratium reticulatum* isolated from the Derwent Estuary, Tasmania. Means are $^{+}$/- standard error.

Strain	CI_s	AV_s	RC_s
DPR02	0.67	2.17	1.44
DPR13	0.33	1.33	0.44
DPR04	0.11	2.00	0.22
DPR05	0.11	1.00	0.11
DPR12	0.22	1.00	0.22
DPR20	0.22	2.00	0.44
DPR22	0.22	3.00	0.67
DPR11	0.00	0.00	0.00
DPR21	0.11	2.00	0.22
DPR24	0.00	0.00	0.00
Group mean	0.22 ± 0.06	1.61 ± 0.31	0.42 ± 0.14

	DERWENT *P. reticulatum* STRAINS									
	02	04	05	11	12	13	20	21	22	24
02	0	2	1	0	1	0	3	2	4	0
04	2	0	0	0	0	0	0	0	0	0
05	1	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0
12	1	0	0	0	0	1	0	0	0	0
13	0	0	0	0	1	0	1	0	2	0
20	3	0	0	0	0	1	0	0	0	0
21	2	0	0	0	0	0	0	0	0	0
22	4	0	0	0	0	2	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0

Figure 3.1: Matrix 1: Reproductive compatibility for ten strains of *Protoceratium reticulatum*. Scores of 1-4 indicate increasing numbers of cysts per cross (see Table 3.2). All strain designations have been abbreviated (e.g. 02 =DPR02).

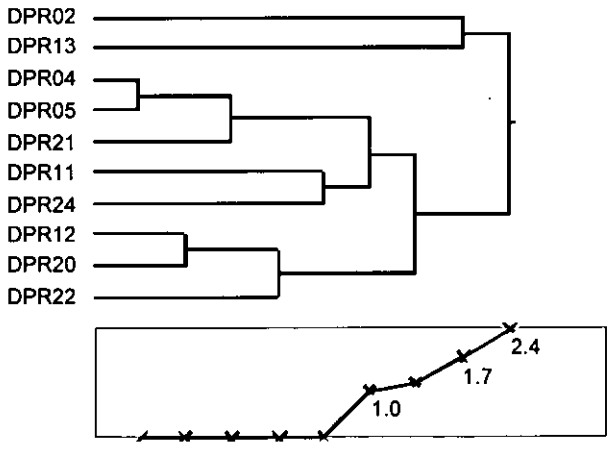

Figure 3.2: Cluster analysis of mating compatibility between strains of *Protoceratium reticulatum*. Lower plot indicates the distance between different clusters based on average linking. All distances between clusters are included in Appendix C.

3.5 *ALEXANDRIUM MINUTUM*

3.5.1 *ALEXANDRIUM MINUTUM* NON-CLONAL STRAINS

In this experiment only one of the four strains (AMAD25) produced hypnozygotes or resting cysts under nutrient limited conditions although planozygotes (some with detectable double longitudinal flagella) were observed in all strains during the course of the experiment. Compatibility measures and cluster analyses were not calculated for these strains, as only selfing ability was tested.

3.5.2 MATRIX 2: *ALEXANDRIUM MINUTUM* CLONAL STRAINS

The crossing matrix of *A. minutum* indicated that the mating system is complex (Fig. 3.3). *Alexandrium minutum* is primarily heterothallic (outcrossing) although there is some self-compatibility (homothallism) (Fig. 3.3). 37 out of a possible 78 pair combinations, in the 12x12 matrix of clonal strains produced resting cysts, (a high TCI of 47.4%). All strains were compatible with at least three other strains. Two of the twelve strains (AMAD02 and AMAD11) showed self-compatibility. Strain AMAD02 did not form resting cysts when combined with three of the other 11 strains, while strain AMAD11 readily produced resting cysts in clonal culture as well as in all other combinations. In four of the AMAD11 crosses, a greater concentration of resting cysts was formed than was seen in the clonal culture (Fig. 3.4) suggesting that both selfing and outcrossing may have been occurring. Strain AMAD08 produced resting cysts readily with all other strains but not in clonal culture. In several of the crosses (both self-crosses and out-crosses) there was evidence of 'partial compatibility' (*sensu* Destombe and Cembella, 1990) e.g. the cross AMAD02 x AMAD06 produced 'dancing gametes' (sensu von Stosch, 1973) but neither gamete fusion nor planozygote formation was observed. Also, in crosses of AMAD03 x AMAD12, AMAD13 x AMAD14, and self-crosses of AMAD08, planozygotes formed but no resting cysts were detected. In crosses of AMAD08 with AMAD02, AMAD03, AMAD06, and AMAD14, the resting cysts produced appeared to be non-viable. These resting cysts became blackened and were rapidly degraded by bacteria (Fig. 3.3).

Figure 3.3 shows the crossing matrix incorporating observations of partial compatibility. In Figure 3.4 the same matrix is converted to resting cyst production scores (1-4 based on increasing numbers of resting cysts per cross see Table 3.2). The compatibility measures for each individual strain are given in Table 3.5. Strain AMAD11 was the most reproductive strain with a RC_s of 3.17, and a CI_s of 1.00, (resting cysts were produced in all crosses with this strain as well as in self-crosses). The RC_s of strain AMAD08 was comparatively low, (2.33) given that it also formed resting cysts with all strains, however the number of resting cysts produced in each cross was lower, and AMAD08 was not selfing. The next most reproductive non-selfing strain was strain AMAD06, which had a RC_s of 2.18 and formed

	PORT RIVER *A. minutum* STRAINS											
	01	02	03	06	08	09	11	12	13	14	15	16
01	-	++	-	-	+++	-	+++	-	-	+?	-	-
02	++	+	-	-	+?	+++	+++	-	++	++	+	+
03	-	-	-	-	+?	++	++	PZ	PZ	PZ	-	-
06	-	-	-	-	+?	+++	+++	++	++	+	+++	++
08	+++	+?	+?	+?	PZ	++	+++	++	+++	+?	++	++
09	-	+++	++	+++	++	-	++	-	-	-	-	-
11	+++	+++	++	+++	+++	++	++	++	++	+	+	++
12	-	-	PZ	++	++	-	++	-	-	-	-	-
13	-	++	PZ	++	+++	-	++	-	-	-	-	-
14	+?	++	PZ	+	+?	-	+	-	-	-	-	-
15	-	+	-	+++	++	-	+	-	-	-	-	-
16	-	+	-	++	++	-	++	-	-	-	-	-

Figure 3.3: Matrix 2: Reproductive compatibility for twelve *Alexandrium minutum* strains isolated from the Port River, South Australia. PZ = planozygotes present; +? = resting cysts present but apparently non-viable; + = 10-100 resting cysts per cross; ++ = 101-1000 resting cysts per cross; +++ = > 1000 resting cysts per cross. All strain designations have been abbreviated (e.g. 01=AMAD01).

	PORT RIVER *A. minutum* STRAINS											
	01	02	03	06	08	09	11	12	13	14	15	16
01	0	3	0	0	4	0	4	0	0	1	0	0
02	3	2	0	0	1	4	4	0	3	3	2	2
03	0	0	0	0	1	3	3	0	0	0	0	0
06	0	0	0	0	1	4	4	3	3	2	4	3
08	4	1	1	1	0	3	4	3	4	1	3	3
09	0	4	3	4	3	0	3	0	0	0	0	0
11	4	4	3	4	4	3	3	3	3	2	2	3
12	0	0	0	3	3	0	3	0	0	0	0	0
13	0	3	0	3	4	0	3	0	0	0	0	0
14	1	3	0	2	1	0	2	0	0	0	0	0
15	0	2	0	4	3	0	2	0	0	0	0	0
16	0	2	0	3	3	0	3	0	0	0	0	0

Figure 3.4: Matrix 2: Reproductive compatibility for twelve *Alexandrium minutum* strains isolated from the Port River, South Australia. Symbols from Figure 3.3 have been converted here to scorings from 1 to 4 (see Table 3.2). All strain designations have been abbreviated (e.g. 01=AMAD01).

Table 3.5: Compatibility measures for twelve clonal strains of *Alexandrium minutum* isolated from the Port River, South Australia. Means are $^{+}$/- standard error.

Strain	CI_s	AV_s	RC_s
AMAD01	0.36	3.00	1.09
AMAD02	0.73	2.67	1.94
AMAD03	0.27	2.33	0.64
AMAD06	0.73	3.00	2.18
AMAD08	1.00	2.33	2.33
AMAD09	0.45	3.40	1.55
AMAD11	1.00	3.17	3.17
AMAD12	0.27	3.00	0.82
AMAD13	0.36	3.25	1.18
AMAD14	0.45	1.80	0.82
AMAD15	0.36	2.75	1.00
AMAD16	0.36	2.75	1.00
Group mean	0.53 ± 0.08	2.79 ± 0.13	1.48 ± 0.22

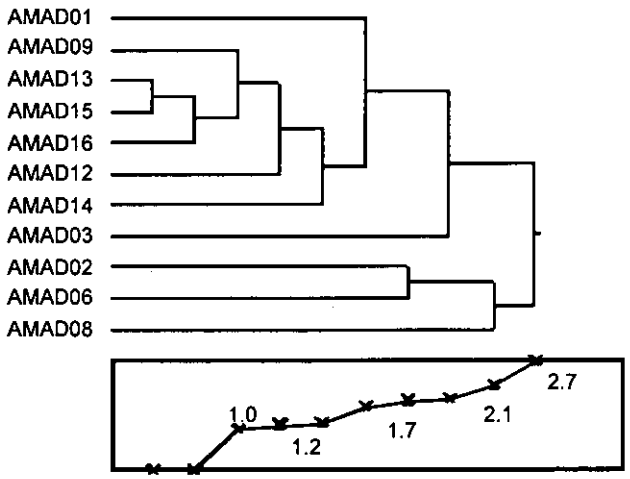

Figure 3.5: Cluster analysis of mating compatibility between strains of *Alexandrium minutum*. Lower plot indicates the distance between different clusters based on average linking. All distances between clusters are included in Appendix C.

resting cysts with eight of the eleven other strains. The least reproductive strain was strain AMAD03 (RC_s = 0.64), which formed resting cysts with only one strain other than the highly compatible strains AMAD08 and AMAD11.

Cluster analysis based on compatibility characteristics identified an overall distance between strains of 2.7 and a maximum of nine mating types (distance <1.0, Fig. 3.5), excluding the selfing strain AMAD11, the other selfing strain, AMAD02 was included in cluster analysis as it did not form resting cysts in all crosses. Only strains AMAD13, 15 and 16 had identical reproductive compatibility while strains AMAD09, 12 and 14 were quite closely linked to this group (distance = 1.2). All other strains were separated by distances of greater than 1.6.

3.6 ALEXANDRIUM CATENELLA

3.6.1 MATRIX 3: *ALEXANDRIUM CATENELLA* PORT RIVER STRAINS

The crossing matrix for the Port River population is shown in Figure 3.6. Nine out of a total of 21 strain combinations produced resting cysts, resulting in a relatively high TCI of 42.9%. Strain ACAD02, was the only self-compatible strain found for *A. catenella*, (although possibly not selfing in the strict sense as it is a non-clonal strain) and also had a high RC_s value of 1.33. The most reproductive outcrossing strain was ACAD01 (RC_s = 1.40), this strain was also of non-clonal origin but did not form resting cysts in self-crosses. The least reproductive strain was ACAD03 (RC_s= 0.2). ACAD03 only formed resting cysts in combination with the selfing strain ACAD02, therefore resting cysts from this combination may have been the result of selfing of ACAD02 rather than gamete fusion between ACAD02 and ACAD03. Compatibility measures for the other strains are presented in Table 3.6. Strains from the Port River were arbitrarily assigned mating types (mt(+) and mt(–) see Fig. 3.6), as four of the strains formed a binary heterothallic mating system; the first group containing only strain ACAD01 (designated mt(+)) and the second containing strains ACAD04, 05 and 06 (designated mt(–)). The two deviations from this mating system were the selfing strain ACAD02 and ACAD03 for which true compatibility affiliation could not be determined as it only crossed with the selfing strain. Cluster analysis (which excluded the selfing strain) based on reproductive compatibility identified an overall distance between strains of 1.9 and identified three clusters (mt(+) and mt(–) types, and non-compatible ACAD03) at a distance of 1.0 (Fig. 3.7).

3.6.2 MATRIX 4: *ALEXANDRIUM CATENELLA* SYDNEY HARBOUR STRAINS

The crossing matrix for *A. catenella* strains from the Sydney Harbour population is shown in Figure 3.8. The TCI for the Sydney Harbour population was a relatively high 42.9%, which was identical to the Port River strains; however, the Sydney Harbour crosses were

	PORT RIVER *A. catenella* STRAINS					
	+	-				
	01	04	05	06	02	03
01	0	2	2	1	2	0
04	2	0	0	0	2	0
05	2	0	0	0	1	0
06	1	0	0	0	1	0
02	2	2	1	1	1	1
03	0	0	0	0	1	0

Figure 3.6: Matrix 3: Reproductive compatibility for six strains of *Alexandrium catenella* isolated from the Port River, South Australia. Scores of 1-4 indicate increasing numbers of cysts per cross, see Table 3.2. Strain designations have been abbreviated (e.g. 01 = ACAD01).

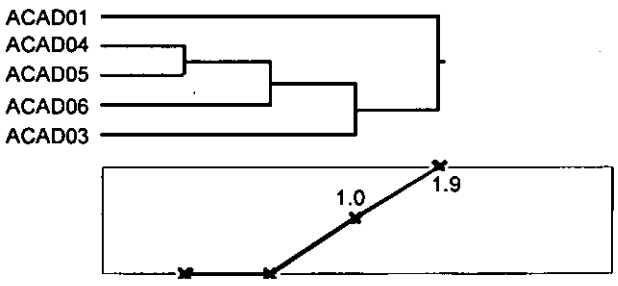

Figure 3.7: Cluster analysis of mating compatibility between strains of *Alexandrium catenella* from the Port River Estuary, South Australia. Lower plot indicates the distance between different clusters based on average linking. All distances between clusters are included in Appendix C.

Table 3.6: Compatibility measures for the three groups of *Alexandrium catenella* strains isolated from three geographic populations. Means are ± standard error. Indices with the same letter (in brackets) were not significantly different between populations ($p<0.05$, Mann Whitney Rank Sum Test).

Population	Strain	CI_s	AV_s	RC_s
Port River	ACAD01	0.80	1.75	1.40
	ACAD02	1.00	1.33	1.33
	ACAD03	0.20	1.00	0.20
	ACAD04	0.40	2.00	0.80
	ACAD05	0.40	1.50	0.60
	ACAD06	0.40	1.00	0.40
	Group mean	0.53 ± 0.12 (AB)	1.43 ± 0.16 (B)	0.79 ± 0.2 (B)
Sydney Harbour	ACSH02	0.60	2.67	1.60
	ACSH03	0.60	3.00	1.80
	ACSH05	0.60	2.33	1.40
	ACSH04	0.60	2.33	1.40
	ACSH06	0.60	3.67	2.20
	ACSH07	0.60	2.00	1.20
	Group mean	0.60 ± 0.0 (A)	2.67 ± 0.24 (A)	1.60 ± 0.15 (A)
Port Phillip Bay	ACPP01	0.43	3.00	1.29
	ACPP03	0.43	3.00	1.29
	ACPP04	0.43	3.00	1.29
	ACPP05	0.43	3.00	1.29
	ACPP02	0.57	2.75	1.57
	ACPP06	0.57	3.00	1.71
	ACPP10	0.57	3.00	1.71
	ACPP08	0.00	0.00	0.00
	Group mean	0.43 ± 0.07 (B)	2.59 ± 0.37 (A)	1.27 ± 0.19 (AB)
	Overall Mean	0.51 ± 0.05	2.27 ± 0.21	1.22 ± 0.13

SYDNEY HARBOUR *A. catenella* STRAINS						
	+			-		
	02	03	05	04	06	07
02	0	0	0	3	3	2
03	0	0	0	3	4	2
05	0	0	0	1	4	2
04	3	3	1	0	0	0
06	3	4	4	0	0	0
07	2	2	2	0	0	0

Figure 3.8: Matrix 4: Reproductive compatibility for six strains of *Alexandrium catenella* isolated from Sydney Harbour, New South Wales. Scores of 1-4 indicate increasing numbers of cysts per cross (Table 3.2). Strain designations have been abbreviated (e.g. 02 = ACSH02).

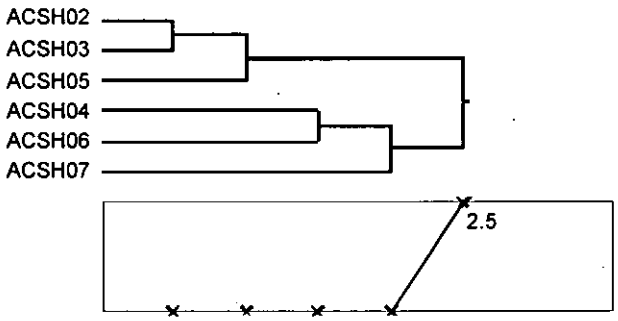

Figure 3.9: Cluster analysis of mating compatibility between strains of *Alexandrium catenella* from Sydney Harbour, New South Wales. Lower plot indicates the distance between different clusters based on average linking.

significantly more productive ($p<0.05$) with a mean RC_s of 1.6 compared with 0.79 (Table 3.6). The Sydney Harbour strains also formed a binary heterothallic mating system with all six strains fitting into two mating types and forming two mating incompatibility groups with exactly the same reproductive compatibility (Fig. 3.8). The first group (designated mt(+)) contained strains ACSH02, 03 and 05 and the second (designated mt(–)) contained strains ACSH04, 06 and 07. Cluster analysis clearly separated the mt(+) and mt(–) mating type strains with a distance between the two strain groups of 2.5 (Fig. 3.9).

3.6.3 MATRIX 5: *ALEXANDRIUM CATENELLA* PORT PHILLIP BAY STRAINS

The crossing matrix for *A. catenella* strains from the Port Phillip Bay population is presented in Figure 3.10. The TCI for this population was lower than for the other two populations at 33% while the mean RC_s of 1.27, was not significantly different ($p>0.05$) to the other two populations (Table 3.6). Therefore, although the total number of compatible crosses was lower than the other populations, the reproductive compatibility of most strains was quite high (Table 3.6). The exception was strain ACPP08, which did not form resting cysts in any combination and its reproductive compatibility must be considered to be low, but could not be quantified. All compatible strains produced medium to high numbers of resting cysts with all but one cross resulting in >100 resting cysts per cross (score of 3) (Fig. 3.10). Seven of the eight strains formed a binary heterothallic mating system (Fig. 3.10). The first mating group (designated mt(+)) contained strains ACPP01, 03, 04, and 05 and the second (designated mt(–)) contained strains ACPP02, 06, and 10, a third group contained only strain ACPP08. Cluster analysis of these strains clearly separated the mt(+) and mt(–) strains at a distance of 2.5, and ACPP08 was clustered with the mt(+) strains at a distance of 1.7 (Fig. 3.11).

3.6.4 *ALEXANDRIUM CATENELLA* POPULATION COMPARISON

These studies show that *A. catenella* strains from three distinct populations were largely heterothallic. The exception was a single strain from the Port River population, which formed resting cysts in self crosses, however, this strain (ACAD02) was non-clonal (see Table 3.1). Two mating types were identified from the six to eight strains from each population. The strains from the Sydney Harbour population had the highest compatibility measures although average vigour (AV) and reproductive compatibility (RC) were not significantly greater than the strains from the Port Phillip Bay population, and the compatibility index (CI) was not significantly greater than the strains from the Port River Estuary population (Table 3.6). The average vigour (AV) of the strains from the Port River Estuary population was significantly lower than strains from the Sydney Harbour and Port Phillip Bay populations (Table 3.6).

	PORT PHILLIP BAY *A. catenella* STRAINS							
	+				-			
	01	03	04	05	02	06	10	08
01	0	0	0	0	3	3	3	0
03	0	0	0	0	3	3	3	0
04	0	0	0	0	2	3	3	0
05	0	0	0	0	3	3	3	0
02	3	3	2	3	0	0	0	0
06	3	3	3	3	0	0	0	0
10	3	3	3	3	0	0	0	0
08	0	0	0	0	0	0	0	0

Figure 3.10: Matrix 5: Reproductive compatibility for six strains of *Alexandrium catenella* isolated from the Port Phillip Bay, Victoria population. Scores of 1-4 indicate increasing numbers of cysts per cross (Table 3.2). Strain designations have been abbreviated (e.g. 01 = ACPP01).

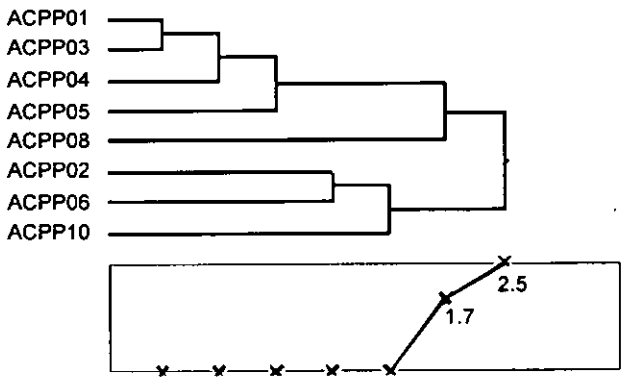

Figure 3.11: Cluster analysis of mating compatibility between strains of *Alexandrium catenella* from Port Phillip Bay, Victoria. Lower plot indicates the distance between different clusters based on average linking.

3.7 *GYMNODINIUM CATENATUM*

3.7.1 VARIABILITY IN DUPLICATE CROSSES

Crosses were replicated for a subset of the *G. catenatum* bloom strains. Results indicated that compatibility characteristics were generally consistent between duplicates. In no combination was there a shift in compatibility of more than one level (Figs. 3.14, 3.16 and 3.18). Of 56 duplicated crosses only 9 (16%) had different compatibility levels between duplicates, e.g. level 1 in one duplicate (1 – 9 resting cysts) and level 2 in the other (10 – 100 resting cysts). In four of these crosses, one duplicate was close to the border of two levels (e.g. 6 resting cysts produced in one duplicate (level 1) and 10 in the other (level 2). Only 3 crosses (5%) were reproductively compatible in one duplicate but not in the other, 2 crosses varied between level 1 and level 2, and 4 crosses varied between level 2 and level 3.

3.7.2 MATRIX 6: *GYMNODINIUM CATENATUM* LONG-TERM CULTURED STRAINS

Six of the seven long-term cultured *G. catenatum* strains investigated were found to be heterothallic and one, GCDE08, was homothallic or selfing (Fig. 3.12A). Strains GCHU02, GCHU09 and GCJP10 did not form resting cysts except in the presence of the selfing strain GCDE08. It was not possible to determine whether the resting cysts produced in these crosses resulted from selfing or outcrossing although fewer resting cysts in outcrosses may indicate an interactive effect. The three other strains formed resting cysts with at least one other strain. The TCI for all strains was 36%, which was relatively high, elevated mainly due to the presence of the selfing strain, the mean RC_s was low (0.45, Table 3.7). Cluster analysis indicated a maximum of four mating types (the selfing strain GCDE08 was included in this analysis for direct comparison with Blackburn et al. (2001) – analysis without GCDE08 can be found in Appendix C) and a high maximum distance between strains of 5.5. Strains GCDE02 and GCDE06 had identical reproductive compatibility, as did GCHU02, GCHU09 and GCJP10, although true reproductive compatibility of the latter group could not be determined as these strains only formed resting cysts with the selfing strain GCDE08 (Fig. 3.13).

Results from this study were compared with the results of Blackburn et al. (2001), from crosses of the same strains conducted in 1990, seven years prior to this study (1997) (Fig. 3.12B). Their compatibility measures were re-calculated to account for the fact that fewer strains were used in this study. Compatibility between strains differed between the two crossing experiments with the Blackburn et al. (2001) study having a TCI of 50% compared with 36% seven years later. All compatibility measures were significantly greater in the Blackburn et al. (2001) study than in this study (Table 3.7, $p<0.05$). Strain GCDE08 was not found to produce resting cysts in clonal culture in the original study and was known to be

A

LONG-TERM CULTURED *G.catenatum* STRAINS							
This Study							
	HU02	HU09	HU11	DE02	DE06	DE08	JP10
HU02	0	0	0*	0*	0	1	0
HU09	0	0	0	0	0*	1	0
HU11	0*	0	0	1	1	2	0
DE02	0*	0	1	0	0*	1	0
DE06	0	0*	1	0*	0	1	0
DE08	1	1	2	1	1	2	1
JP10	0	0	0	0	0	1	0

B

LONG-TERM CULTURED *G.catenatum* STRAINS							
From Blackburn et al., 2001							
	HU02	HU09	HU11	DE02	DE06	DE08	JP10
HU02	0	3	2	0	0	0	0*
HU09	3	0	2	3	3	3	3
HU11	2	2	0	3	3	1	0
DE02	0	3	3	0	2	2	1
DE06	0	3	3	2	0	0	0
DE08	0	3	1	2	0	0	1
JP10	0*	3	0	1	0	1	0

Figure 3.12: Matrix 6: Reproductive compatibility for seven long-term cultured strains of *Gymnodinium catenatum*. A: Results from crosses carried out on these strains in this study; B: results of crosses carried out by Blackburn et al. (2001). Scores of 1-4 indicate increasing numbers of cysts per cross (Table 3.3) 0* indicates less than 4 cysts per cross. Strain designations have been abbreviated (e.g. HU02 = GCHU02). HU, DE and JP prefixes designate strains isolated from the Huon Estuary, Derwent Estuary, and Japanese waters respectively.

Table 3.7: Compatibility measures for seven long-term cultured strains of *Gymnodinium catenatum*. Strains with the prefix GCHU and GCDE were isolated from the Huon and Derwent estuaries, Tasmania, respectively while the GCJP10 strain originated from Japanese waters. 1997 values are from this study and 1990 values from Blackburn et al. (2001). Means are ± standard error. Means of all compatibility measures for the 1990 crosses were significantly higher than the 1997 crosses ($p<0.05$. Mann Whitney Rank Sum Test).

Year	Strain	CI_s	AV_s	RC_s
1997	GCHU02	0.17	1.00	0.17
	GCHU09	0.17	1.00	0.17
	GCHU11	0.50	1.33	0.67
	GCDE02	0.33	1.00	0.33
	GCDE06	0.33	1.00	0.33
	GCDE08	1.00	1.29	1.29
	GCJP10	0.17	1.00	0.17
	Group mean	0.38 ± 0.11	1.09 ± 0.06	0.45 ± 0.16
1990	GCHU02	0.33	2.50	0.83
	GCHU09	1.00	2.83	2.83
	GCHU11	0.83	2.20	1.83
	GCDE02	0.83	2.20	1.83
	GCDE06	0.67	2.50	1.67
	GCDE08	0.67	1.75	1.17
	GCJP10	0.50	1.67	0.83
	Group mean	0.69 ± 0.08	2.24 ± 0.16	1.57 ± 0.27

clonal in both studies, therefore selfing in this strain represents a shift from heterothallic to homothallic reproductive compatibility over time. Aside from the selfing strain, all strains that produced resting cysts in this study also produced resting cysts in the original study. However, there was a greater degree of compatibility in the original study. For example, strain GCHU02 crossed with GCHU11 and GCHU09 in the first study but with neither strain in this study. Strain GCDE06 was originally found to be compatible with strains GCDE02, GCHU11 and GCHU09, while in the more recent study of these strains only GCHU11 successfully crossed with GCDE06.

Cluster analysis of the two crossing matrices (this study and that of Blackburn et al., 2001) gave very different results (Fig. 3.13A and B). The maximum distance between strains was greater in this study (5.5 compared with 4.4), while the maximum number of mating types was less, partly due to the three strains which only formed resting cysts with the selfing strain GCDE08. GCDE06 and GCDE02 had identical reproductive compatibility in this study but not in the 1990 study.

3.7.3 MATRIX 7: *GYMNODINIUM CATENATUM* SUMMER BLOOM

The compatibility matrix for the 15 strains isolated from the summer bloom is presented in Figure 3.14 and the compatibility measures in Table 3.8. All strains were reproductively compatible with at least one other strain. The most reproductive strain was GCBP13-97: this strain crossed successfully with 9/14 other strains and had a RC_s of 1.71 while the least reproductive strain, GCBP04-97, only produced a low number of resting cysts with strain GCW7-97 ($RC_s = 0.07$). Strains isolated from the same site (e.g. Wheatleys or Killala Bay), were reproductively compatible in some combinations for all sites (Fig. 3.14), with the exception of Brabazon Point, but only one strain from this site was included in the crossing matrix so intrasite compatibility was not tested in this instance.

Cluster analysis showed the summer bloom to be highly diverse with respect to reproductive compatibility, identifying 14 mating types with distances of 1 or greater. Only strains GCBP04-97 and GCBP05-97 had identical reproductive compatibility (Fig. 3.15). The maximum distance between strains was 3.3. There was some clustering of strains isolated from the Brabazon Park site, with strains GCBP04-97, GCBP05-97, GCBP10-97 and GCBP12-97 forming a cluster at a distance of 1.6 and strains GCBP06-97 and GCBP08-97 forming a cluster at a distance of 1.0. There was no clustering of strains from other sites.

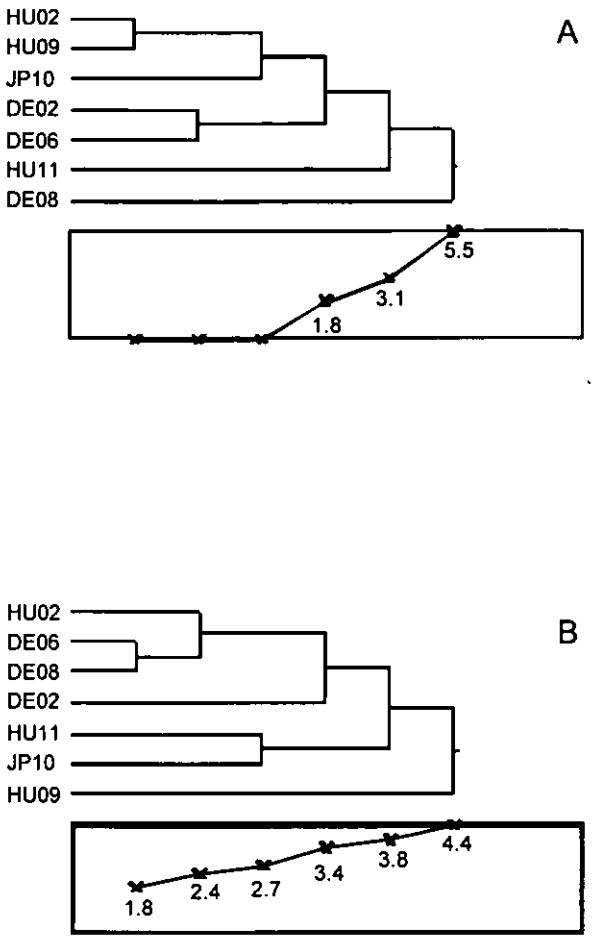

Figure 3.13: Cluster analysis of mating compatibility between long-term cultured strains of *Gymnodinium catenatum*. A: results from this study; B: results for the same strains from Blackburn et al. (2001). Lower plots indicate the distance between different clusters based on average linking. Strain designations have been abbreviated (e.g. HU02 = GCHU02). HU, DE and JP prefixes designate strains isolated from the Huon Estuary, Derwent Estuary, and Japanese waters respectively.

	WHEATLEYS				BRABAZON PARK								KILLALA		BPT.
	W1	W5	W7	HUN4	BPO1	BPO4	BPO5	BPO6	BPO8	BP10	BP12	BP13	HUN1	HUN2	HUN3
W1	0	3	2	1	0	0	0	0	0	0	1	3	3	0	0
W5	3	0	1	1	0	0	0	0	0	0	0	0	1	1	0
W7	2	1	0	2	1	1	2	2	3	1	2	0	3	2	2
HUN4	1	1	2	0	0	0	0	0	0	0	0	2	0	1	1
BPO1	0	0	1	0	0	0	0	2	0	0	2	0	2	2	1
BPO4	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
BPO5	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
BPO6	0	0	2	0	2	0	0	0	0	0	0	3	2	0	0
BPO8	0	0	3	0	0	0	0	0	0	0	0	3	1	0	0
BP10	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0
BP12	1	0	2	0	2	0	0	0	0	0	0	2	0	0	0
BP13	3	0	0	2	0	0	0	3	3	1	2	0	3	4	3
HUN1	3	1	3	0	2	0	0	2	1	0	0	3	0	3	2
HUN2	0	1	2	1	2	0	0	0	0	0	0	4	3	0	0
HUN3	0	0	2	1	1	0	0	0	0	0	0	3	2	0	0

Figure 3.14: Matrix 7: Reproductive compatibility for fifteen strains isolated from the summer 1997/98 bloom of *Gymnodinium catenatum* in the Huon Estuary, Tasmania. Outlined areas indicate strains isolated from the same site. Scores of 1-4 indicate increasing numbers of cysts per cross (Table 3.2). Strain designations have been abbreviated (e.g. W1 = GCW1-97).

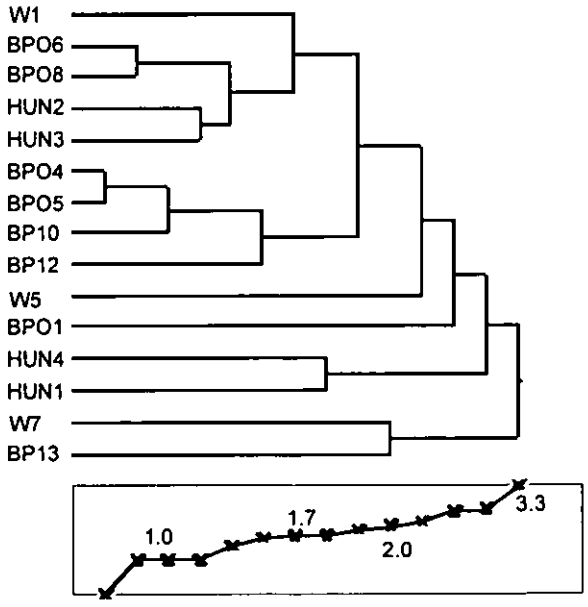

Figure 3.15: Cluster analysis of mating compatibility between strains of *Gymnodinium catenatum* isolated from the summer 1997/98 bloom in the Huon Estuary, Tasmania. Strain designations have been abbreviated (e.g. W1 = GCW1-97). Lower plot indicates the distance between different clusters based on average linking. BP=Brabazon Park, W =Wheatleys (also HUN4), HUN1,2 = Killala Bay), HUN3 = Brabazon Point. All distances between clusters are included in Appendix C.

Table 3.8: Compatibility measures for strains isolated from the summer 1997/98 and autumn 1998 blooms of *Gymnodinium catenatum* in the Huon Estuary, Tasmania. Means are ± standard error.

Population	Strain	CI_s	AV_s	RC_s
Summer bloom	GCW1-97	0.43	2.17	0.93
	GCW5-97	0.36	1.40	0.50
	GCW7-97	0.93	1.85	1.71
	GCHUN4-97	0.43	1.33	0.57
	GCBPO1-97	0.43	1.67	0.71
	GCBPO4-97	0.07	1.00	0.07
	GCBPO5-97	0.07	2.00	0.14
	GCBPO6-97	0.31	2.25	0.69
	GCBPO8-97	0.21	2.33	0.50
	GCBP10-97	0.14	1.00	0.14
	GCBP12-97	0.29	1.75	0.50
	GCBP13-97	0.64	2.67	1.71
	GCHUN1-97	0.64	2.22	1.43
	GCHUN2-97	0.43	2.17	0.93
	GCHUN3-97	0.36	1.80	0.64
	Group mean	0.38 ± 0.06	1.84 ± 0.13	0.75 ± 0.13
Autumn bloom	GCPC9802	0.57	2.13	1.21
	GCPC9803	0.71	1.80	1.29
	GCPC9806	0.07	2.00	0.14
	GCK9801	0.43	1.67	0.71
	GCK9804	0.07	3.00	0.21
	GCK9808	0.36	2.00	0.71
	GCK9811	0.14	1.50	0.21
	GCK9815	0.36	1.60	0.57
	GCBP9803	0.36	2.40	0.86
	GCBP9815	0.36	1.80	0.64
	GCBP9818	1.00	2.40	2.40
	GCHU9813	0.21	1.67	0.36
	GCHU9820	0.36	1.80	0.64
	GCHU9828	0.43	2.17	0.93
	GCHU9833	0.29	1.50	0.43
	Group mean	0.38 ± 0.06	1.96 ± 0.11	0.75 ± 0.15
	Overall mean	0.38 ± 0.04	1.90 ± 0.08	0.75 ± 0.1

3.7.4 MATRIX 8: *GYMNODINIUM CATENATUM* AUTUMN BLOOM

The compatibility matrix for the 15 strains isolated from the autumn bloom is presented in Figure 3.16 and the compatibility measures in Table 3.8. This matrix contained the only selfing strain, GCBP9818, of the 30 *G. catenatum* bloom strains investigated. The selfing strain GCBP9818 was clearly the most reproductive strain ($RC_s = 2.40$), while the most reproductive non-selfing strains were GCPC9803 ($RC_s = 1.29$) and GCPC9802 ($RC_s = 1.21$), which were sexually compatible with 10/14 and 8/14 other strains respectively. Strain GCPC9806 was the least reproductive strain ($RC_s = 0.14$) with resting cysts only being present when this strain was combined with the selfing strain GCBP9818. In several cases crosses of other strains with GCBP9818 yielded greater numbers of resting cysts than when it was self-crossed (a score of 3 (100-1000 resting cysts per cross) rather than a score of 2 (10-100 resting cysts per cross)) suggesting that outcrossing may be occurring, in addition to selfing. Interestingly, for the autumn bloom strains, there was no reproductive compatibility between strains isolated from the same site, if the selfing strain GCBP9818 is not considered (see Fig. 3.16).

Cluster analysis of the autumn bloom strains again indicated a high diversity of reproductive compatibility with a maximum of twelve mating types (excluding the selfing strain). Only strains GCPC9806 and GCK9804, and GCBP9815 and GCHU9820 had identical compatibility characteristics. However, the former group (GCPC9806 and GCK9804) consisted of strains that only combined with the selfing strain and compatibility measures are therefore somewhat ambiguous (Fig. 3.17). The maximum distance between strains was high at 6.8 and there was no close clustering of strains isolated from the same site.

3.7.5 MATRIX 9: *GYMNODINIUM CATENATUM* SUMMER X AUTUMN BLOOM

The compatibility matrix for the crosses between strains from the summer bloom and strains from the autumn bloom (summer x autumn) is presented in Figure 3.18. Strains within sites for the sixteen strains all showed some within-site compatibility (see Fig. 3.18). Compatibility measures are presented in Table 3.9.

When compared with the within-bloom crosses the results from the duplicates were generally consistent. In 9/56 crosses compatibility levels differed between duplicates. In only 3/56 crosses a score of 0 (no resting cysts produced) in one crossing matrix was replaced with a 1 (<10 resting cysts per cross) in the other. These crosses were GCHU9820 x GCK9804 (0 in the autumn matrix and 1 in the summer x autumn matrix), GCHUN4-97 x GCHUN2-97, and GCHUN1-97 x GCBP08-97 (1 in summer matrix and 0 in summer x autumn).

	PORT CYGNET			KILLALA BAY					BRABAZON PK.			WHEATLEYS			
	PC9802	PC9803	PC9806	K9801	K9804	K9808	K9811	K9815	BP9803	BP9815	BP9818	HU9813	HU9820	HU9828	HU9833
PC9802	0	0	0	1	0	0	0	0	2	2	3	2	3	3	1
PC9803	0	0	0	1	0	0	1	1	3	3	3	1	2	2	1
PC9806	0	0	0	0	0	0	0	0	0	0	2	0	0	0	0
K9801	1	1	0	0	0	0	0	0	2	0	3	0	0	1	2
K9804	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0
K9808	0	0	0	0	0	0	0	0	3	1	3	0	1	2	0
K9811	0	1	0	0	0	0	0	0	0	0	2	0	0	0	0
K9815	0	1	0	0	0	0	0	0	0	1	2	0	1	3	0
BP9803	2	3	0	2	0	3	0	0	0	0	2	0	0	0	0
BP9815	2	3	0	0	0	1	0	1	0	0	2	0	0	0	0
BP9818	3	3	2	3	3	3	2	2	2	2	3	2	2	2	2
HU9813	2	1	0	0	0	0	0	0	0	0	2	0	0	0	0
HU9820	3	2	0	0	0	1	0	1	0	0	2	0	0	0	0
HU9828	3	2	0	1	0	2	0	3	0	0	2	0	0	0	0
HU9833	1	1	0	2	0	0	0	0	0	0	2	0	0	0	0

Figure 3.16: Matrix 8: Reproductive compatibility for fifteen strains isolated from the autumn 1998 bloom of *Gymnodinium catenatum* in the Huon Estuary, Tasmania. Outlined areas indicate strains isolated from the same site. Scores of 1-4 indicate increasing numbers of cysts per cross (Table 3.2). Strain designations have been abbreviated (e.g. PC9802 = GCPC9802).

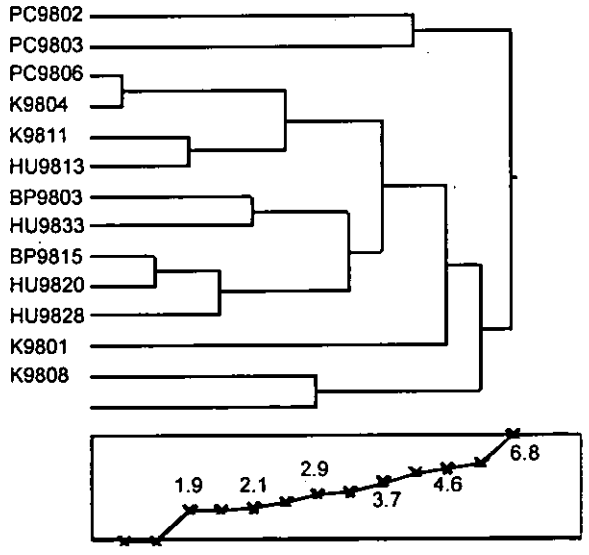

Figure 3.17: Cluster analysis of mating compatibility between strains of *Gymnodinium catenatum* isolated from the autumn 1998 bloom in the Huon Estuary, Tasmania. Strain designations have been abbreviated (e.g. PC9802 = GCPC9802). Lower plot indicates the distance between different clusters based on average linking. BP=Brabazon Park, K=Killala Bay, PC=Port Cygnet, HU=Wheatleys. All distances between clusters are included in Appendix C.

	PT. CYGNET		KILLALA BAY				BRABAZON PARK					WHEATLEYS				B PT.
	PC9802	PC9806	K9804	K9811	HUN1	HUN2	BP9803	BP9818	BP06	BP08	BP10	HU9820	HU9828	W1	HUN4	HUN3
PC9802	0	0	0	0	0	3	3	3	2	2	0	3	2	3	0	3
PC9806	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
K9804	0	0	0	0	0	1	0	3	1	1	0	1	0	2	0	0
K9811	0	0	0	0	0	1	0	3	0	0	0	0	0	0	0	0
HUN1	0	0	0	0	0	2	1	3	0	0	0	0	1	2	0	2
HUN2	3	0	1	1	2	0	2	3	0	0	0	0	0	0	1	0
BP9803	3	0	0	0	1	2	0	1	2	2	0	0	0	2	0	3
BP9818	3	2	3	3	3	3	1	0	2	3	2	2	2	3	2	3
BP06	2	0	1	0	0	0	2	2	0	0	0	0	0	0	0	0
BP08	2	0	1	0	0	0	2	3	0	0	0	1	0	0	0	0
BP10	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	0
HU9820	3	0	1	0	0	0	0	2	0	1	0	0	0	0	0	0
HU9828	2	0	0	0	1	0	0	2	0	0	0	0	0	1	0	0
W1	3	0	2	0	2	0	2	3	0	0	0	0	1	0	1	0
HUN4	0	0	0	0	0	1	0	2	0	0	0	0	0	1	0	0
HUN3	3	0	0	0	2	0	3	3	0	0	0	0	0	0	0	0

Figure 3.18: Matrix 9: Reproductive compatibility for sixteen strains isolated from both the summer 1997/98 and autumn 1998 blooms of *Gymnodinium catenatum* in the Huon Estuary, Tasmania (eight strains from each bloom). Outlined areas indicate strains isolated from the same site, autumn bloom strains are shaded and summer bloom strains are stippled. Scores of 1-4 indicate increasing numbers of cysts per cross (Table 3.2). Strain designations have been abbreviated (e.g. W1 = GCW1-97; PC9802 = GCPC9802).

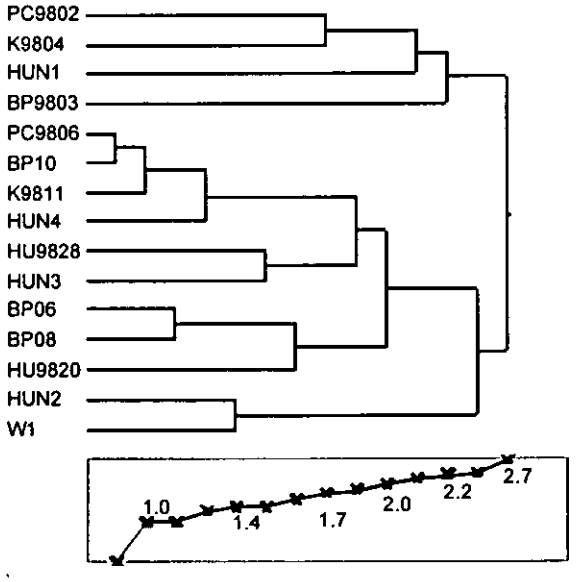

Figure 3.19: Cluster analysis of mating compatibility between strains of *Gymnodinium catenatum* isolated from the summer 1997/98 and autumn 1998 blooms in the Huon Estuary, Tasmania (eight strains from each). Strain designations have been abbreviated (e.g. W1 = GCW1-97; PC9802 = GCPC9802). Lower plot indicates the distance between different clusters based on average linking. BP=Brabazon Park, W, HU98 =Wheatleys (also HUN4), HUN1,2 = Killala Bay), HUN3 = Brabazon Point. All distances between clusters are included in Appendix C.

Cluster analysis of the strains again indicated high diversity of mating type in the summer x autumn strain crosses with a maximum number of mating types of fourteen (excluding the selfing strain GCBP9818). Only strains GCPC9806 and GCBP10-97 were identical in their mating characteristics. The maximum distance between groups was lower than that for either the autumn or summer strains alone at 2.7. There was no clustering of strains from either the same site (as was seen in the summer bloom strains), or strains isolated from the same bloom.

3.7.6 *GYMNODINIUM CATENATUM* BLOOM POPULATION COMPARISON

All of the thirty strains isolated from the summer and autumn bloom populations in 1997/98 were found to be heterothallic except the autumn bloom strain GCBP9818, which was homothallic as well as potentially outcrossing (it was not possible to determine whether resting cysts produced in out-crosses of selfing strains were due to selfing or outcrossing). The summer bloom strains had a low TCI of 27% and a low mean RC_s of 0.75 (Table 3.8). The autumn bloom strains had similar compatibility measures to the summer bloom strains with a slightly higher TCI of 33% and a mean RC_s of 0.75 (Table 3.8). When a subset of the strains from the two blooms were intercrossed (summer x autumn) the resulting mean compatibility measures were similar to those calculated for the crosses of strains from each bloom individually (TCI=29%, RC_s =0.69) (Table 3.9). There were no significant differences between the matrices in any of the compatibility measures ($p>0.05$, Mann Whitney Rank Sum Test).

Table 3.9: Compatibility measures for crosses between groups of strains from the summer 1997/98 and autumn 1998 blooms of *Gymnodinium catenatum* in the Huon Estuary, Tasmania. Means are $^{+}/_{-}$ standard error. S and A indicate strains isolated from summer and autumn blooms respectively.

Strain	Season	CI_s	AV_s	RC_s
GCPC9802	A	0.63	1.63	1.02
GCPC9806	A	0.00	0.00	0.00
GCK9804	A	0.50	1.25	0.63
GCK9811	A	0.13	1.00	0.13
GCHUN1-97	S	0.25	1.67	0.42
GCHUN2-97	S	0.50	2.00	1.00
GCBP9803	A	0.75	2.00	1.50
GCBP9818	A	1.00	2.63	2.63
GCBP06-97	S	0.38	1.75	0.66
GCBP08-97	S	0.50	1.80	0.90
GCBP10-97	S	0.00	2.00	0.00
GCHU9820	A	0.13	1.00	0.13
GCHU9828	A	0.25	1.00	0.25
GCW1-97	S	0.50	2.20	1.10
GCHUN4-97	S	0.00	2.00	0.00
GCHUN3-97	S	0.25	3.00	0.75
Group mean		0.36 ± 0.07	1.68 ± 0.18	0.69 ± 0.17

3.8 SPECIES COMPARISON

Means of compatibility measures for all the compatibility matrices are summarised in Table 3.10. *Protoceratium reticulatum* had significantly lower compatibility than all other species (except in the AV_s, which was not significantly lower than *G. catenatum*). The *G. catenatum* bloom strains also had relatively low reproductive compatibility, significantly lower than *A. catenella* and *A. minutum* in all but one of the measures (CI_s). The compatibility measures for *A. minutum* and *A. catenella* were not significantly different.

DISCUSSION

In many species of algae, fungi and protozoa there are multiple, genetically controlled mating types within a single species, identifiable by the specificity they express in sexual reproduction (Coleman, 1977). Although the primary reason for sexual reproduction in these organisms is undoubtedly genetic recombination (Sandgren, 1981), other outcomes can include the production of a resting stage, which aids both short and long-term survival (Pfiester and Anderson, 1987). This is the case in many dinoflagellates, including those studied here. Understanding sexual reproduction in dinoflagellates is of vital importance to an understanding of dinoflagellate autecology, and the mating system is a key component of this process. The four dinoflagellates studied here were found to have diverse mating systems, both in the number of potential mating types and in the reproductive compatibility of individual strains. All four species were heterothallic, (96% of all strains) however, at least one homothallic strain was found in all but *P. reticulatum*. The fact that all four species studied were primarily heterothallic suggests that out-crossed progeny may be more 'fit' or have increased viability over selfed progeny in these species. Progeny viability was not determined in this study (except for observations of apparently non-viable resting cysts of *A. minutum*) but Destombe and Cembella (1990) found that of seven selfing strains of *A. tamarense* only one produced viable progeny. Each of these heterothallic mating system must therefore be a trade-off between the need for reliable resting cyst production for genetic recombination and long term survival on the one hand (which would be optimised in a homothallic mating system), and the probable increased viability and vigour of out-crossed progeny on the other.

3.9 *ALEXANDRIUM CATENELLA*

The simplest mating system, a binary heterothallic one, was observed in *A. catenella* from all three locations. These results for Australian *A. catenella* strains agree with the work of Yoshimatsu (1981) on Japanese *A. catenella* strains, who previously identified a binary heterothallic mating system (just two mating types, mt(+) and mt(–)). Strains from all three populations studied here also expressed essentially two mating types, although there were

Table 3.10: Summary of mean compatibility indices for crossing matrices[1]. Means are ± standard error.
Indices with the same letter (in brackets) were not significantly different between matrices ($p<0.05$, Mann Whitney Rank Sum Test).

Species	Matrix	Population	TCI	CI_s	AV_s	RC_s	Max. distance between strains	Max. no. of mating types
Protoceratium reticulatum	1	Derwent Estuary	16.4	0.22 ± 0.06 (A)	1.61 ± 0.31 (A)	0.42 ± 0.14 (A)	2.4	5
Alexandrium minutum	2	Port River Estuary	47.4	0.53 ± 0.08 (BC)	2.79 ± 0.13 (B)	1.48 ± 0.22 (C)	2.8	10
Alexandrium catenella	3	Port River Estuary	42.9	0.53 ± 0.12	1.43 ± 0.16	0.79 ± 0.20	1.9	3
	4	Sydney Harbour	42.9	0.60 ± 0.00	2.67 ± 0.24	1.60 ± 0.15	2.4	2
	5	Port Phillip Bay	33.0	0.43 ± 0.07	2.59 ± 0.37	1.27 ± 0.19	2.5	3
	3,4,5	All *A. catenella* strains		0.51 ± 0.05 (C)	2.29 ± 0.21 (B)	1.22 ± 0.13 (C)		
Gymnodinium catenatum	7	Summer Bloom	27.0	0.38 ± 0.06	1.84 ± 0.13	0.75 ± 0.13	3.3	14
	8	Autumn Bloom	33.0	0.38 ± 0.06	1.96 ± 0.11	0.75 ± 0.15	6.8	12
	9	Summer x Autumn Bloom	29.0	0.36 ± 0.07	1.68 ± 0.18	0.69 ± 0.17	2.7	14
	7,8,9	All *G. catenatum* strains		0.38 ± 0.04 (B)	1.83 ± 0.08 (A)	0.73 ± 0.09 (B)		

[1] Matrix 6, the long-term cultured *G. catenatum* strain matrix is not included in this table as the compatibility indices were calculated using a different scoring method (Table 3.3).

some deviations from this simple mating system (minor deviations from the binary heterothallic mating system were also observed by Yoshimatsu (1981)). In the Port River strains, one selfing strain was observed and both the Port River and Port Phillip Bay strains contained one strain that did not show compatibility with any other strains. It is possible that in the latter case conditions required for sexual reproduction differ between strains and that these criteria were not met by the experimental conditions i.e. that these results were false negatives. Nevertheless, where it has been studied, the binary heterothallic mating system of *A. catenella* seems to be consistent within widely geographically separated populations. Further research should investigate whether the two mating types identified in the three Australian populations and in Japanese populations are reproductively compatible.

3.10 *PROTOCERATIUM RETICULATUM*

Protoceratium reticulatum had the next simplest mating system. The mating system of *P. reticulatum* based on the ten strains was heterothallic with no selfing observed. A maximum of five mating types were identified – one of these contained strains which did not form resting cysts in any combination and could therefore not be identified as a true mating type. *Protoceratium reticulatum* resting cysts are one of the most common and abundant resting cyst types found in the sediments of various regions (e.g. Tasmania Australia, Bolch and Hallegraeff, 1990; west coast of Sweden, Persson et al., 2000; southwest India, Godhe et al., 2000) although blooms are rarely seen in the water column (I. Jameson, personal communication). This suggests that when blooms do occur they may be brief in duration and that a relatively large proportion of the population undergoes sexual reproduction and/or that resting cysts remain dormant for a long period of time. Field observations indicate that both possibilities may be true. The bloom from which the strains for this study were isolated was sexually reproductive, as of 25 single cells isolated from the bloom one was found to be a planozygote, which successfully encysted. Although it is not possible to make any quantitative estimate of the proportion of the population that was encysting based on this observation, it is certainly interesting that of so few cells isolated one was a planozygote, and therefore encystment rates may have been high. After approximately 12 months, the *P. reticulatum* resting cyst, which had been stored under the normal growth conditions outlined in Section 3.1, in GSe medium, successfully excysted to produce a viable vegetative culture (N. Parker unpublished data). This observation suggests that *P. reticulatum* may have a long requisite dormancy period although further experimentation is required to adequately define the requisite dormancy period; this is the first estimate of dormancy period for this species. Counter-intuitively, very few of the *P. reticulatum* crosses in this study were reproductively compatible (TCI - 16.4%), although some crosses did produce large numbers of resting cysts (e.g. DPR02 x DPR22). The growth of *P. reticulatum* in culture frequently resulted in

somewhat malformed, angular or 'warty' cells (personal observation). This suggests that the growth conditions for this species were not optimal, and that sexual compatibility between strains may also have been affected. Complete documentation of the various life cycle stages and modified culture techniques are required to further elucidate the mating system, sexual life cycle and dormancy requirements of *P. reticulatum* and the implications of this mating system for the ecology and bloom dynamics of the species.

3.11 *ALEXANDRIUM MINUTUM*

Of the four species studied, the most detailed information on life history stages was obtained for *A. minutum*. The presence of gametes, planozygotes and apparently non-viable resting cysts were noted in detail and considered with respect to the mating system. The mating system of *A. minutum* based on the twelve strains studied was primarily heterothallic with only two homothallic or selfing strains (AMAD11 and AMAD02). Mating systems containing both homothallic and heterothallic strains have also been observed in other dinoflagellates (e.g. *Peridinium volzii*; Hayhome et al., 1987). The data in this study suggest that the selfing strain AMAD11 may also have shown heterothallic or outcrossing behaviour due to higher resting cyst numbers in outcrosses. The results for the other selfing strain, AMAD02 differ from AMAD11, as although this strain produced resting cysts by itself, resting cysts were not produced in combination with some other strains, suggesting some interactive effect. One possible explanation for this is that a mechanism similar to the ciliate *Euplotes raikovi* operates in *A. minutum*. In this ciliate the presence of pheromones from a cell of the same mating type promotes vegetative reproduction while the presence of the pheromone of a different mating type promotes sexual reproduction (Vallesi et al., 1995). A similar mechanism might prevent selfing in some combinations of AMAD02 with other strains. The presence of pheromone-like substances in *A. minutum* is explored in Chapter 5.

The mating system of *A. minutum* was more complex than the simple binary heterothallic mating system of *A. catenella* and was more similar to that of *A. tamarense* as described by Destombe and Cembella (1990) which also had multiple mating types. For *A. minutum*, gamete recognition appears to be independent of resting cyst or hypnozygote formation as gamete fusion does not necessarily lead to hypnozygote formation (this was also found for *A. tamarense*; Destombe and Cembella, 1990). In this study the formation of apparently non-viable resting cysts was observed in some crosses. These resting cysts blackened soon after formation with rapid bacterial degradation. Out of the five crosses in which these blackened resting cysts were observed, four contained strain AMAD08 which formed resting cysts in combination with all other strains and planozygotes in self crosses. The presence of these apparently non-viable resting cysts suggests that in these crosses, although resting cyst formation occurs, a reproductive barrier may be in place at a later stage in the life cycle

rather than at the point of gamete recognition or fusion. The rapid breakdown of these non-viable resting cysts suggests a compromised or incomplete cell wall that could act as an effective reproductive barrier.

The various levels of compatibility which were observed in the crosses of the twelve strains indicate that there may in fact be reproductive barriers in place at each step of the life cycle. Such multiple reproductive barriers have also been suggested to explain reproductive incompatibility in *A. tamarense* and *G. catenatum* with further barriers at the level of zygote viability (Destombe and Cembella, 1990; Blackburn et al., 2001) and in the desmid *Micrasterias thomasiana* (Blackburn and Tyler, 1987). In light of the complexity of reproductive compatibility observed in *A. minutum* it is suggested that mating type in this species is controlled by a gene complex rather than by a single pair of alleles, as has also been suggested for *G. catenatum* (Blackburn et al., 2001). The long-term stability of reproductive compatibility in *A. minutum* is discussed in Section 3.13.

3.12 *GYMNODINIUM CATENATUM*

Based on crosses of 13 Tasmanian strains Blackburn et al. (1989) identified *G. catenatum* as having a heterothallic mating system requiring compatible mating types (i.e. mt(+) and mt(–)) with no resting cysts forming in clonal cultures. However, they also found that although mt(+) / mt(+) or mt(–) / mt(–) combinations never formed resting cysts, neither did some mt(+) /mt (–) combinations under the conditions tested. A follow up study to assess compatibility of 21 *G. catenatum* strains from four global populations (Tasmania, Australia (15 strains), Japan (2 strains), Portugal (2 strains) and Spain (2 strains)) identified greater complexity in the mating system of *G. catenatum* (Blackburn et al., 2001). Based on this follow up study, Blackburn et al. (2000) suggested a multiple-group mating system of between three and seven mating types. They found that the geographically separated populations were able to intercross although Tasmania x Spain and Tasmania x Japan crosses had decreased post meiotic viability compared with Tasmania x Tasmania and Spain x Japan crosses. Within the 15 Tasmanian strains they found a high level of mating diversity which was also found in the present study. In this thesis the study of the *G. catenatum* mating system was extended to look at between bloom variation within the same estuary and long-term stability of reproductive compatibility; the latter is discussed in Section 3.13.

Based on the study of two groups of strains isolated from summer and autumn blooms, *G. catenatum* had the most complex mating system identified in this study. Maximums of fourteen and twelve out of a possible fifteen mating types were identified for the summer and autumn bloom strain groups respectively. The complexity of reproductive compatibility

identified for these 30 strains supports the hypothesis of Blackburn et al. (2001) that mating type in *G. catenatum* is determined by a gene complex, giving a greater variety of possible outcomes with respect to mating type from each recombination.

The mating systems for the strains from the two *G. catenatum* blooms had similar levels of compatibility. This was perhaps surprising given the different autecological characteristics of the two blooms (extensive sexual reproduction was observed in the summer bloom while sexual reproduction was undetected in the autumn bloom - see Chapter 6). The lack of sexual reproduction in the autumn bloom suggests the possibility that it was a less genetically diverse population with fewer compatible mating types, but the compatibility measures calculated for the three groups of strains (for each bloom and between the blooms) were quite similar (Tables 3.8 and 3.9). However, the distances between strains determined by cluster analysis were much higher for crosses of the autumn strains than the summer strains (6.8 compared with 3.3) i.e. there was greater diversity in reproductive compatibility between strains. Interestingly, there were no intrasite compatible strains in the autumn bloom (excluding the selfing strain) while compatible strains were found within all sites in the summer bloom. This result may indicate that at a smaller spatial scale (i.e. within site) populations were less genetically diverse in the autumn, resulting in a lack of compatible mating types for sexual reproduction. This hypothesised lower genetic diversity in the autumn bloom may have been due to a number of factors. One possibility is that there was a greater contribution of germinated resting cysts to the development of the summer bloom, while in contrast, the autumn bloom may have arisen from the limited number of cells carried over in the water column from the summer bloom (see Chapter 6). Bolch et al. (1999) hypothesised that a population arising largely from mass resting cyst germination is likely to have greater genetic diversity than one that arises from few vegetative cells. It follows that such a genetically diverse population is also more likely to contain compatible mating types, which should facilitate sexual reproduction and enable replenishment of resting cyst numbers in the sediments. Possible lower genetic diversity at a small spatial scale coupled with ecological factors such as temperature (see Chapters 5 and 6) may have contributed to the lack of sexual reproduction in the autumn bloom of 1998.

3.13 LONG TERM STABILITY OF MATING TYPE

The stability of mating type and reproductive compatibility was investigated for long term (seven years) cultured strains of both *A. minutum* and *G. catenatum*. Only one out of four of the *A. minutum* strains derived from wild resting cysts produced hypnozygotes (i.e. evidence for within culture reproductive compatibility) in this study. However, three of these four strains (AMAD25, 26, and 27) were found to be reproductively compatible within the culture when first isolated, seven years prior to the current study (S. Blackburn unpublished

data). Based on observations of *Alexandrium catenella*, Yoshimatsu (1984) hypothesised a segregation of mating type at meiosis in which the planomeiocyte divides into a 2 celled chain containing one mt(+) and one mt(–) cell. Although it is suggested that mating type determination in *A. minutum* is more complex than in *A. catenella*, the observation of sexual reproduction in strains derived from resting cysts suggests that, like *Ceratium cornutum* and *A. catenella* (Beam and Himes, 1984; Yoshimatsu, 1984), strains derived from resting cysts can contain compatible mating types. The apparent loss of reproductive compatibility within the *A. minutum* germinated resting cyst strains, after seven years in culture, may therefore suggest genetic selection acting within the culture to favour one mating type over another.

Several differences were found in the reproductive compatibility of seven long-term cultured strains of *G. catenatum* when they were re-crossed after seven years. There was a decrease in the TCI from 50% to 36% and the means of strain compatibility measures were all significantly lower in this study than in the first study. Seven of a total of 28 crosses, which were reproductively compatible in the original study, were not in this study. Unlike the *A. minutum* strains, all the *G. catenatum* strains studied were clonal isolates and therefore the observed change in reproductive compatibility suggests an actual shift in reproductive compatibility rather than selection for one mating type over another within the strain. Although slightly different conditions were used in the two studies, the magnitude of the differences in reproductive compatibility supports the conclusion that these are real changes in compatibility rather than due to some environmental effect.

Based on these observations of both *G. catenatum* and *A. minutum*, mating types in complex mating systems do not appear to be particularly stable in long term culture, either due to genetic changes, some artifact of culturing methods, environmental differences or a combination of these factors. It is possible that in *A. minutum* and *G. catenatum*, mating type mutations may result in a previously sexually viable strain losing the ability to undergo sexual reproduction. The loss of sexual viability has also been observed in the Chlorophyte *Gonium pectorale* (Coleman et al., 1994). Destombe and Cembella (1990), from studies of *A. tamarense*, have suggested that the opposite situation may also arise. They suggest that long term culture of clonal strains could result in mating type mutations, causing strains which were originally unable to form resting cysts without the presence of another strain to be able to do so. This appears to be the case for the *G. catenatum* strain GCDE08, which was found to be selfing in the second study but not in the original one.

3.14 METHODOLOGICAL CONSIDERATIONS

Cluster analysis highlighted the differing levels of affinity between strains. The use of cluster analysis proved to be a valuable way of defining potential mating types in this study.

By quantifying the similarity between strains, different numbers of mating groups could be identified. If a mating type is defined as comprising only strains with identical mating compatibility (distance <1.0) numbers of mating groups in the species studied varied from two for the *A. catenella* strains from Sydney Harbour and fifteen for crosses between strains isolated from the two *G. catenatum* blooms. A few strains in each species could be unambiguously assigned the same mating type (distances <1.0) while the majority of strains were combined into groups of decreasing similarity of reproductive compatibility. This is a useful way to look at reproductive affiliation within a gradient in which a strain can be seen to 'tend' toward a particular mating type rather than having a fixed mating type. Varying the acceptable linkage distance between groups for the definition of a mating type would give different numbers of mating types but determining where to put this cut-off needs to be given careful consideration and based on some evaluation of what defines a mating type within a complex mating system. The strength of this cluster analysis method is that it shows reproductive affiliations between strains and identifies the strength of the gradients between them. This cluster analysis method may prove useful for analysing the mating systems of other protists with multiple mating types such as the ciliate *Euplotes octocarinatus* (Kuhlmann et al., 1997).

One potential problem with the cluster analysis method was the interpretation of clusters containing selfing strains, which would result in strains being clustered more closely than they should be because all strains would have compatibility with the selfing strain in common. Removing all selfing strains from cluster analysis avoided this problem. There were also some other problems with interpreting the clusters. In this study the cluster analysis was based entirely on presence/absence data for resting cysts. In the *A. minutum*, *G. catenatum* and *P. reticulatum* matrices, few strains had identical compatibility characteristics and the degree of compatibility varied between combinations, which were scored here on a scale of 1 to 4. In all three species the number of combinations with low resting cyst yields under experimental conditions (i.e. a score of 1 or less than 10 resting cysts per cross) was high, particularly for *G. catenatum* and *P. reticulatum*. If such crosses are also low yielding in the natural environment, their importance in an ecological sense is difficult to determine. The development of techniques for quickly assessing sexual reproduction in field situations is required to investigate this. It may prove useful in future studies to include quantitative data on the number of resting cysts formed by a particular cross in the analysis so that higher yielding crosses are given more 'weight' in the analysis. For this to be effective, resting cyst production should be expressed quantitatively, as percentage encystment rather than the semi-quantitative 'resting cysts per cross' categories used in these experiments.

A second issue with regard to cluster analysis was that all crosses were performed under only one set of environmental conditions and most were not replicated. It is possible that some strains may be compatible under different conditions. Yoshimatsu (1981) found slightly different results when strains were crossed under different nutrient conditions and small differences in reproductive compatibility were also found between duplicates from the *G. catenatum* bloom strains in this study. Testing reproductive compatibility under different environmental conditions, with replication, should be considered for the species studied here to improve the rigour with which these mating systems are described, although this greatly increases the number of crosses required.

A broader methodological issue with the study was that progeny viability was not investigated aside from observations of apparently non-viable resting cysts in some *A. minutum* crosses. Destombe and Cembella (1990) found that various strain combinations and self crosses of *A. tamarense* produced non-viable resting cysts, as did Blackburn et al. (2001) with *G. catenatum*. Determining the viability of resting cysts from the various strain combinations would provide a much greater understanding of the mating systems of these species, give greater strength to the cluster analysis, and also provide further information relevant to their population dynamics. Determining resting cyst viability was outside the scope of this project where the primary aim was to provide an initial study of the mating systems of the four species from Australian waters and to investigate long term stability and inter-population variation in mating systems.

The large number of strains investigated here offers insight into the mating systems of these species, but care should be taken in extrapolating results from between 6 and 15 strains from a site or region to a widely distributed species . As Coleman (1962) states 'There is no reason to assume that all strains of any widely distributed species will exhibit the same breeding system, particularly since a number of species containing both heterothallic and homothallic strains are already known.' For species with relatively simple mating systems such as *A. catenella* it is possible to get a very good sense of the mating system from a limited number of strains. However, as the mating system becomes more complex the number of strains required becomes much greater. For example, based on probability theory, if equal frequencies of mating type are assumed, then to have a >99% chance of detecting all mating types in a random selection of strains in a two mating type system 8 strains would be required. If this were increased to a 3 mating type system then 15 strains would be required, and so on (Day and Parker, unpublished data). As the complexity of mating systems increases so does the number of strain crosses required to adequately describe the mating system, and the logistics of these studies becomes more difficult. A complete pairwise cross of 15 strains requires 120 different crosses (with crosses to test for selfing but without

replication). To increase that number of strains to 20 would require 210 crosses, 50 – 1275, 100 – 5050 and so on, with the added effort of maintaining that number of strains until they were ready for crossing. The increase in our knowledge of the genetic makeup of dinoflagellates may ultimately contribute to determining the genetic basis of mating type in dinoflagellates. A full understanding would require identifying the portion of the dinoflagellate genome that codes for sexuality and the development of quick methods for its determination. Until this is achieved, mating system studies using a limited number of strains provide important insight into the sexual reproduction of dinoflagellates.

3.15 ECOLOGICAL IMPLICATIONS OF MATING SYSTEMS

One of the key issues raised by this research is whether the mating system of a species has implications for its ecology. In other words, are there fundamental differences in the ecology of a species influenced by whether it has a binary heterothallic, complex heterothallic or homothallic mating system? In this section the following questions related to the ecological implications of mating systems are explored:

- What are the proportions or frequencies of mating types in natural populations or blooms and does this differ between different mating systems?
- Does mating system influence the frequency and success or 'efficiency' of sexual reproduction?
- How would the presence of pheromone-like substances influence mating systems and the efficiency of sexual reproduction?
- Are species with complex mating systems evolving towards forming a species complex of closely related species rather than being a true biological species?
- Does the nature of the mating system give us any information on the status of a species with respect to being 'native' or 'introduced' and how might mating systems be affected by a new introduction?

3.15.1 MATING TYPE FREQUENCY

In Section 3.4.6 an assumption was made that dinoflagellate mating types are found in equal frequencies or proportions in the natural environment, in order to explore the problem of the number of strains that may be required to define a mating system. Just how probable is it that this is true? Frequency-dependent sex-ratio theory predicts equal frequencies of fixed multiple mating types (Doerder et al., 1995) but this issue has not previously been considered with dinoflagellate populations. In a field study of the ciliate *Tetrahymena thermophila* Doerder et al. (1995) found that the seven mating types of this species did occur in approximately equal frequency throughout the study area. Of the species studied here,

only *A. catenella* has what could be considered as clearly fixed mating types. In this study and in previous work (S. Blackburn unpublished data) roughly equal mating type distribution has been observed in the limited number of strains isolated from Sydney Harbour and Port Phillip Bay which suggests that field populations may also have roughly equal mating type frequency. The strains from the Port River population deviated from the model of equal mating type frequency with only one mt(+) strain and three mt(–) strains, however all sample sizes were small. A detailed study of field populations with randomly selected sites is required to determine whether equal frequencies of mating type occur.

Alexandrium minutum, *G. catenatum* and *P. reticulatum* did not have clearly fixed mating types but have more complex mating systems, similar to that described by Destombe and Cembella (1990) for *A. tamarense* as a 'gradient of gametic affinity'. In these complex mating systems the frequencies of mating type are unknown and the degree to which these mating types are 'fixed' is also uncertain as it has been shown here that the expression of mating type in a strain can vary over time in culture. The way in which mating type is determined genetically has not been investigated for dinoflagellates with complex mating systems. If these mating types are determined through a gene complex rather than by a single pair of alleles as suggested by some authors for *G. catenatum* (Bolch et al., 1999; Blackburn et al., 2001) then recombination may result in a variety of phenotypic mating types reflecting the gradient of gametic affinity. The possibility that some products of meiosis may abort either at random or selectively (Pfiester and Skvarla, 1979) adds an additional level of complexity to the determination of mating type frequency in complex mating systems and the assumption of equal frequency seems unlikely but needs to be properly tested.

3.15.2 EFFICIENCY OF SEXUAL REPRODUCTION

Regardless of the mating system there is always a cost associated with sexual reproduction (Lewis, 1987) and the efficiency with which sexual reproduction occurs is therefore important. The amount of sexual reproduction that occurs in species with different mating systems may be closely linked to mating type frequency. For example, a simpler mating system, with equal mating type frequencies might be expected to yield a greater number of resting cysts based on probability of random encounters of opposite mating types. Alternatively, a species with a multiple mating type system might have lower probability of reproductively compatible gametes encountering one another, and may also have higher failure of sexual reproduction due to partial compatibility activating reproductive barriers. Variable compatibility levels due to multiple reproductive barriers have been suggested for *A. minutum* (this study), *A. tamarense* (Destombe and Cembella, 1990) and *G. catenatum* (Blackburn et al., 2001). This suggests that in some cases reproductive effort is being put

into sexual reproduction that is ultimately non-viable. Most research suggests that the various stages in dinoflagellate life cycles are non-reversible except for gametogenesis where gametes may revert to vegetative reproduction (von Stosch, 1973; Pfiester, 1975). Therefore, once committed to sexual reproduction sexual cells are lost from the water column population unless the life cycle is completed through hypnozygote germination and subsequent vegetative growth. Species with complex mating systems that may produce non-viable resting cysts may therefore be less efficient at sexual reproduction than species with either homothallic or simple heterothallic mating systems. The relationship between complexity of mating system and efficiency of mating system can only be determined through detailed field studies of species with known mating systems using sediment traps for measuring the frequency of sexual reproduction or rate of encystment in natural populations, and assessing resting cyst viability. In the absence of such detailed field data, if we consider the abundance of resting cysts of a species in the sediments as a rough estimate of the efficiency of sexual reproduction, there does not appear to be a relationship between the mating system of a species and its efficiency of sexual reproduction. For example, although *A. catenella* resting cysts (produced by a simple mating system) can be quite common in the sediments (Sonneman and Hill, 1997), *P. reticulatum* resting cysts, as mentioned previously, are often the most abundant resting cysts found in sediments and this species has a more complex mating system.

3.15.3 MATING SYSTEMS AND PHEROMONE-LIKE SUBSTANCES

One factor that may influence the association between mating system complexity and mating system efficiency is the possibility that pheromone-like substances play a role in sexual reproduction of dinoflagellates (Wyatt and Jenkinson, 1997; Chapter 5). If we consider that pheromone-like substances function in either gamete attraction or in gametogenesis then their specificity may both increase the encounter rate of compatible gametes and decrease the fusion of partially compatible gametes. Indeed, it is possible that the artificial nature of culture conditions disrupt these signals by limiting the available options for sexual reproduction, resulting in the fusion of partially compatible gametes being more abundant in culture than it would be in the natural environment. Complex mating systems may be just as efficient as simple mating systems with other environmental factors being more important than mating system in determining the amount or efficiency of sexual reproduction.

3.15.4 EVOLUTIONARY IMPLICATIONS OF DIFFERENT MATING SYSTEMS

The low degree of reproductive compatibility between strains identified in three of the species studied (*A. minutum, G. catenatum* and *P. reticulatum*), brings into question the validity of their classification as single species in a biological species sense (*sensu* Mayr,

1976). Wiese and Wiese (1977), based on their work on *Chlamydomonas* proposed a hypothesis of speciation by evolution of gametic incompatibility, which might occur through mutations in sexual recognition systems. If such a process is valid for dinoflagellates then the species recognised here as having complex mating systems may in fact be undergoing frequent mutations in sexual recognition systems. Therefore, although the various mating types may respond or bloom to the same environmental cues, they may be more accurately described as evolving towards a species complex.

3.15.5 MATING SYSTEMS AND INTRODUCED SPECIES

Three of the dinoflagellates studied here, *A. minutum*, *A. catenella* and *G. catenatum* are considered to have been introduced to Australian waters in recent times and are listed as 'introduced marine pests' by the Australian quarantine and inspection service (AQIS). This has not been substantiated for most populations although information from dated sediment cores from the Huon Estuary suggest that *G. catenatum* was not present in the region prior to the 1970s (McMinn et al., 1997). The most likely vectors of introduction of these species into Australian waters are through ballast water or shellfish translocation. Three different scenarios are possible with regard to the influence of new introduction on mating system if *G. catenatum* is an introduced species: 1) Some studies comparing the genetic variation of invading marine populations to native populations have found that invading populations are undifferentiated from core populations, implying a minimal role for founder effects or drift, presumably due to large colonizing populations or to maintained gene flow (Geller, 1996). Both of the possible vectors for translocation of dinoflagellate resting cysts are likely to involve large numbers of individuals, for example Hallegraeff and Bolch (1992) found 300 million viable dinoflagellate resting cysts in one ballast tank; 2) Other studies have found that introduced populations undergo significant losses of genetic diversity due to population bottlenecks during establishment (Ward and Andrew, 1995); 3) It is also possible that species introductions may lead to increased genetic diversity compared with the source population as the introduced species rapidly expands into new niches (Holland, 2000). Dinoflagellate mating system investigations have been carried out for very few dinoflagellate populations so it is not yet possible to make comparisons between different source and introduced populations.

This study found that variability in reproductive compatibility was extensive for *G. catenatum* strains isolated from the same or different blooms. Bolch et al. (1999) also found extensive genetic variation in *G. catenatum* strains isolated from the same or adjacent estuaries, based on RAPD (randomly amplified polymorphic DNA). They found that genetic variation (as determined by AMOVA; analysis of molecular variance) within a population (i.e. within a bloom) accounted for most of the total variation (87%) compared with variation

between populations within regions (6%) or between regions (8%). This extensive intrapopulation variation in genetic and reproductive compatibility measures may indeed be a reflection of the introduced status of *G. catenatum* as in scenario 3 above, but without analysis of the mating systems of other populations this can not be determined. It would be instructive to explore the genetic variation of the strains used in this study using RAPD analysis to compare genetic variation between sites and blooms directly with strain reproductive compatibility and determine if there is any correlation between the two measures.

3.16 CONCLUSION

Because the continuity of their existence has been protected by asexual reproduction, unicellular algae have been able to explore their full evolutionary potential with respect to sexual differentiation and reproduction, leading to a great variety in their sex phenomena (Wiese, 1984). The dinoflagellates are no exception to this, with homothallic and both simple and complex heterothallic mating systems being identified (Pfiester and Anderson, 1987; Destombe and Cembella, 1990; Blackburn et al., 2001). In this chapter, mating systems of four ecologically important Australian dinoflagellates were examined. The mating systems of *A. minutum* and *P. reticulatum* were described for the first time and that of *A. catenella* for the first time from Australian populations. For *G. catenatum* long term stability of reproductive compatibility and the mating systems of two blooms in the same region were compared. In these four species a high degree of variability was found in mating systems and in reproductive compatibility between strains both within species and within populations. With this increased knowledge of mating systems it is important to remember that these mating systems do not act in isolation in the field but are influenced by physical, chemical and biological factors. In the natural environment the extent of sexual reproduction that occurs must be a combination of both genetic composition and environmental cues regulating gene expression, either at the transcriptional, translational or post-translational phase. In order for dinoflagellate autecology to be understood, it is important that the mating systems of other dinoflagellates are also investigated. This study represents a significant contribution to the small body of literature on dinoflagellate mating systems and addresses the important issues associated with the role of mating systems in dinoflagellate ecology.

REFERENCES

Allen JR, Roberts TM, Loeblich AR III, Klotz LC (1975) Characterization of the DNA from the dinoflagellate *Crypthecodinium cohnii* and implications for nuclear organization. Cell 6: 161–169.

Beam CA, Himes M (1974) Evidence for sexual fusion and recombination in the dinoflagellate *Crypthecodinium (Gyrodinium) cohnii.* Nature 250: 435–436.

Beam CA, Himes M (1984) Dinoflagellate Genetics. In Spector DL (ed), Dinoflagellates. Academic Press Inc., Orlando: 263–298.

Blackburn SI, Tyler PA, (1987) On the nature of eclectic species – a tiered approach to genetic compatibility in the desmid *Micrasterias thomasiana.* British Phycological Journal 22: 277–298.

Blackburn SI, Hallegraeff GM, Bolch CJ (1989) Vegetative reproduction and sexual life cycle of the toxic dinoflagellate *Gymnodinium catenatum* from Tasmania, Australia. Journal of Phycology 25: 577–590.

Blackburn SI, Bolch CJ, Haskard KA, Hallegraeff GM (2001) Reproductive compatibility among four global populations of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). Phycologia 40: 78–87.

Bolch CJ, Hallegraeff GM (1990) Dinoflagellate cysts in recent marine sediments from Tasmania, Australia. Botanica Marina 33: 173–192.

Bolch CJS, Blackburn SI, Hallegraeff GM, Vaillancourt RE (1999) Genetic variation among strains of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). Journal of Phycology 35: 356–367.

Coleman AW (1962) Sexuality. In Lewin RA (ed), Physiology and Biochemistry of Algae. Academic Press, New York: 711–729.

Coleman AW (1977) Sexual and genetic isolation in the cosmopolitan algal species *Pandorina morum.* American Journal of Botany 64: 316–368.

Coleman AW, Suarez A, Goff LJ (1994) Molecular delineation of species and syngens in volvocacean green algae (Chlorophyta). Journal of Phycology 30: 80–90.

Destombe C, and Cembella A (1990) Mating type determination, gametic recognition and reproductive success in *Alexandrium excavatum* (Gonyaulacales, Dinophyta), a toxic red-tide dinoflagellate. Phycologia 29: 316–325.

Doerder FP, Gates MA, Eberhardt FP, Arslanyolu M (1995) High frequency of sex and equal frequencies of mating types in natural populations of the ciliate *Tetrahymena thermophila.* Proceedings of the National Academy of Science USA 92: 8715–8718.

Doerder FP, Arslanyolu M, Saad Y, Kaczmarek M, Mendoza M, Mita B (1996) Ecological genetics of *Tetrahymena thermophila*: mating types, i-antigens, multiple alleles and epistasis. Journal of Eukaryotic Microbiology 43: 95–100.

Ferris PJ, Pavlovic C, Fabry S, Goodenough UW (1997) Rapid evolution of sex-related genes in *Chlamydomonas.* Proceedings of the National Academy of Sciences USA 94: 8634–8639.

Geller J (1996) Molecular approaches to the study of marine biological invasions. In Ferraris JD, Palumbi SR (eds), Molecular zoology: advances, strategies, and protocols. Wiley-Liss Inc: 123–132.

Godhe A, Karunasagar I, Karunasagar I, Karlson B (2000) Dinoflagellate cysts in recent marine sediments from SW India. Botanica Marina 43: 39–48.

Hallegraeff GM, Bolch CJ (1992) Transport of diatom and dinoflagellate resting spores in ships' ballast water: Implications for plankton biogeography and aquaculture. Journal of Plankton Research 14: 1067–1084.

Hayhome BA, Whitten DJ, Harkins KR, Pfiester LA (1987) Intraspecific variation in the dinoflagellate *Peridinium volzii*. Journal of Phycology 23: 573–580.

Holland BS (2000) Genetics of marine bioinvasions. Hydrobiologia 420: 63–71.

Ichimura T, Kasai F (1989) Life cycles of homothallic and heterothallic clones of *Triploceras gracile* Bailey (Desmidiaceae, Chlorophyta). Phycologia 28: 212–221.

Ichimura T, Kasai F (1990) Mating systems and speciation in haplontic unicellular algae, Desmids. In Kawano S (ed), Biological Approaches and Evolutionary Trends in Plants. Academic Press Ltd., London: 309–332.

Ichimura T, Kasai F (1995) Morphological variation and zygotic defective mating-type plus in a population of *Closterium ehrenbergii* (Desmidiales, Chlorophyta) from Boest Brook, Denmark. Phycologia 34: 45–50.

Kremp A, Heiskanen A-S (1999) Sexuality and cyst formation of the spring-bloom dinoflagellate *Scrippsiella hangoei* in the coastal northern Baltic Sea. Marine Biology 134: 771–777.

Kuhlmann H-W, Brunen-Nieweler C, Heckmann K (1997) Pheromones of the ciliate *Euplotes octocarinatus* not only induce conjugation but also function as chemoattractants. Journal of Experimental Zoology 277: 38–48.

Lewis WM Jr (1987) The cost of sex. In Stearns SC (ed), The evolution of sex and its consequences. Birkhauser Verlag Basel: 33–57.

Mayr E (1976) Evolution and diversity of life. Harvard University Press, Cambridge: 721 pp.

McMinn A, Hallegraeff GM, Thomson P, Short S, Heijnis H (1997) Microfossil evidence for the recent introduction of the toxic dinoflagellate *Gymnodinium catenatum* into Tasmanian waters. Marine Ecology Progress Series 161: 165–172.

Oshima Y, Itakura H, Lee K-C, Yasumoto T, Blackburn SI, Hallegraeff G (1993) Toxin production by the dinoflagellate *Gymnodinium catenatum*. In Smayda TJ, Shimizu Y (eds) Toxic phytoplankton blooms in the sea, Elsevier Science Publishers, B.V., Amsterdam: 907–912.

Persson A, Godhe A, Karlson B (2000) Dinoflagellate cysts in recent marine sediments from the west coast of Sweden. Botanica Marina 43: 69–79.

Pfiester LA (1975) Sexual reproduction of *Peridinium cinctum* f. ovoplanum (Dinophyceae) Journal of Phycology 11: 259–265.

Pfiester LA, Anderson DM (1987) Dinoflagellate reproduction. In Taylor FJR (ed) The biology of dinoflagellates. Blackwell Scientific Publications, Oxford: 611–648.

Pfiester LA, Skvarla JJ (1979) Heterothallism and thecal development in the sexual life history of *Peridinium volzii* (Dinophyceae). Phycologia 18: 13–18.

Rizzo PJ (1987) Biochemistry of the dinoflagellate nucleus. In Taylor FJR (ed) The biology of dinoflagellates. Blackwell Scientific Publications, Oxford: 143–173.

Sandgren CD (1981) Characteristics of sexual and asexual resting cyst (statospore) formation in *Dinobryon cylindricum* Imhof (Chrysophyta). Journal of Phycology 17: 199–210.

Sandgren CD, Flanagin J (1986) Heterothallic sexuality and density dependent encystment in the Chrysophycean alga *Synura petersenii* Korsh. Journal of Phycology 22: 206–212.

Satake M, MacKenzie L, Yasumoto T (1997) Identification of *Protoceratium reticulatum* as the biogenetic origin of yessotoxin. Natural Toxins 5: 164–167.

Satake M, Ichimura T, Sekiguchi K, Yoshimatsu S, Oshima Y (1999) Confirmation of yessotoxin and 45, 46, 47 – Trinoryessotoxin production by *Protoceratium reticulatum* collected in Japan. Natural Toxins 7: 147–150.

Sonneborn TM (1957) Breeding systems, reproductive methods, and species problems in protozoa. In Mayr E (ed), The Species Problem. American Association for the Advancement of Science, Washington DC: 155–324.

Sonneman JA and Hill DRA (1997) A taxonomic survey of cyst-producing dinoflagellates from recent sediments of Victorian coastal waters, Australia. Botanica Marina 40: 149–177.

Spector DL (1984) Dinoflagellate nuclei. In Spector DL (ed), Dinoflagellates. Academic Press Inc., Orlando: 107–147.

Steidinger KA, Tangen K (1997) Dinoflagellates. In Tomas CR (ed), Identifying Marine Phytoplankton. Academic Press, San Diego: 387–584.

Vallesi A, Giuli G, Bradshaw RA, Luporini P (1995) Autocrine mitogenic activity of pheromones produced by the protozoan ciliate *Euplotes raikovi*. Nature 376: 522–524.

van den Ende H, van Winkle-Swift KP (1994) Mating-type differentiation and mate selection in the homothallic *Chlamydomonas monoica*. Current Genetics 25: 209–216.

van den Hoek C, Mann DG, Jahns HM (1995) Algae: an introduction to phycology. Cambridge University Press, Cambridge: 627 pp.

von Stosch HA (1973) Observation on vegetative reproduction and sexual life cycles of two freshwater dinoflagellates, *Gymnodinium pseudopalustre* Schiller and *Woloszynskia apiculata* sp. nov. British Phycological Journal 8: 105–134.

Ward RD, Andrew J (1995) Population genetics of the northern Pacific seastar *Asterias amurensis* (Echinodermata: Asteriidae): Allozyme differentiation among Japanese, Russian, and recently introduced Tasmanian populations. Marine Biology 124: 99–109.

Wiese L (1984) Mating systems in unicellular algae. In Encyclopedia of Plant Physiology Volume 17. Springer-Verlag, Berlin: 238–260

Wiese L, Wiese W (1977) On speciation by evolution of gametic incompatibility: a model case in *Chlamydomonas*. American Naturalist 111: 733–742.

Wyatt T, Jenkinson IR (1997) Notes on *Alexandrium* dynamics. Journal of Plankton Research 19: 551–575.

Yoshimatsu S (1981) Sexual reproduction of *Protogonyaulax catenella* in culture. I. Heterothallism, Bulletin of the Plankton Society of Japan 28: 131–139.

Yoshimatsu S (1984) Sexual reproduction of *Protogonyaulax catenella* in culture. II. Determination of mating type. Bulletin of the Plankton Society of Japan 31: 107–111.

CHAPTER 4

GROWTH OF THE TOXIC DINOFLAGELLATE *ALEXANDRIUM MINUTUM* (DINOPHYCEAE) USING HIGH BIOMASS CULTURE SYSTEMS

INTRODUCTION

Toxic dinoflagellates are important microalgae in a global context. The combination of the production of toxins along with their capacity to form blooms or 'red tides' is significant both ecologically and economically. While their environmental effects are often serious and negative, the toxins and other secondary metabolites that they produce have great potential as useful bioactive molecules for biotechnology. Dinoflagellates are a particularly diverse group of organisms, exhibiting biological phenomena such as bioluminescence, symbiosis with invertebrates, parasitism, complex life cycles and varied nutritional strategies (Taylor, 1987). Many different bioactive molecules have been discovered from both free-living and symbiotic dinoflagellates (Faulkner, 2001). Marine dinoflagellates contain metabolites shown to be nutritionally valuable, such as DHA (docosahexaenoic acid) which is produced commercially in heterotrophic systems from *Crypthecodinium cohnii* (Martek Biosciences Corporation). Metabolites from dinoflagellates have also been found to be valuable laboratory tools and show promise for drugs (Pietra, 1997). Of the bioactive compounds produced by dinoflagellates, the suite of toxins is particularly significant. Okadaic acid, a diarrhoeic shellfish toxin and tumour promoter found in many dinoflagellates of the genera *Dinophysis* and *Prorocentrum,* is used for studies of cellular regulation (Cohen et al., 1990). Saxitoxin and its analogues; the gonyautoxins, neosaxitoxin and C toxins (the paralytic shellfish toxins (PSTs) produced by *Gymnodinium catenatum* and many species of *Alexandrium* including *A. minutum*), along with the brevetoxins (neurotoxic shellfish toxins produced by *Gymnodinium breve* and other red tide dinoflagellates) are used to investigate the structure and function of ion channels in cell membranes (Pietra, 1997; Yasumoto and Satake, 1998). These compounds are therefore important tools in the study of the effect of various agents on neuromuscular systems (Shimizu, 1993). The production of toxins for toxin standards is also essential for seafood and water monitoring programs.

Alexandrium minutum (Halim) Balech is a PST producing 'red tide' dinoflagellate. First described by Halim (1960) from Alexandria Harbour in Egypt it was redescribed by Balech (1989). Since the original record, *A. minutum* has been reported from South Australia (Hallegraeff et al., 1988), Spain and Portugal (Balech, 1985; as *A. ibericum*), Italy (Montresor et al., 1990), Turkey (Koray and Buyukisik, 1988), and France (LeDoux et al., 1989). Australian blooms of *A. minutum* commonly reach cell densities of 10^6 cells L^{-1} and can be as high as 10^7 cells L^{-1} (Cannon, 1993a). *Alexandrium minutum*, like some other toxic dinoflagellates, can reproduce sexually, forming a resting cyst or hypnozygote (Bolch et al., 1991). Complete description of the sexual life cycle and elucidation of the mating system of *A. minutum* has recently been achieved (see Chapter 2 and Chapter 3). The toxin profile of *A. minutum* has been determined for Australian, Spanish and New Zealand populations (Oshima et al., 1989; Flynn et al.,

1994) and is usually dominated by gonyautoxins 1 and 4 with lower concentrations of gonyautoxins 2 and 3. New Zealand strains also produce high concentrations of neosaxitoxin (Hoe Chang et al., 1997). Due to its relatively simple toxin profile, *A. minutum* is a good candidate for toxin production applications.

In order to utilise the research, pharmacological and therapeutic potential of toxins and other bioactive molecules produced by *A. minutum* and other dinoflagellates it is essential to be able to cultivate these organisms and study their growth and physiology in relatively high cell density, high productivity culture systems. Commercial viability can be assessed only when this is realised. The use of photobioreactor technology for microalgal production has advanced over the last 40 years with the development of many closed photobioreactors (reviewed by Tredici, 1999). An important advantage of closed photobioreactors over open pond systems is enhanced quality control, permitting uni-algal production with a lower risk of contamination by heterotrophs (fungi, bacteria, protozoa) and by other autotrophs which may out-compete the cultivated species (Tredici, 1999). At present only a few of the more than 50,000 known species of microalgae are commercially exploited, all in open pond systems. The use of photobioreactors offers the potential to extend this to many other species which are potential sources of high value products (Tredici, 1999).

Dinoflagellates are more fragile than other microalgae currently cultivated in photobioreactors, with considerable evidence of negative effects from turbulent conditions (Estrada and Berdalet, 1998) and as such offer a challenge to microalgal biomass production. Successful cultivation of dinoflagellates for bioactive molecule production requires the refining and customising of photobioreactor design, which is likely to prove useful for relatively fragile microalgae in general.

In this chapter, the results of an exploration into the feasibility of high density autotrophic cultivation of *A. minutum* and the resulting production of paralytic shellfish toxins are presented. Growth and toxin production of *A. minutum* in Erlenmeyer flasks, with and without aeration as well as in two laboratory systems designed for high cell density cultivation (aerated tubes and an alveolar panel photobioreactor) were investigated. Two clonal strains and a combination of these two strains were compared in order to examine cellular interactions, including sexual reproduction, in these culture systems.

METHODS

4.1 ORGANISMS AND CULTURE CONDITIONS

Alexandrium minutum strains AMAD06 (CS-323, isolated by S. Blackburn 1987) and AMAD16 (CS-324, isolated by S. Blackburn and J. Cannon 1988) were obtained from the CSIRO Collection of Living Microalgae (http://www.marine.csiro.au/microalgae). Both strains are clonal isolates of vegetative cells from the Port River Estuary in South Australia and have been maintained in non-axenic culture. These two strains were found to be sexually compatible through crossing experiments (see Chapter 3). Both strains were maintained in GSe medium (modified GP medium, 28 psu with added selenium; Blackburn et al., 2001 and Appendix A) in 500 mL Erlenmeyer flasks at a temperature of 18°C ± 1°C with cool white fluorescent illumination (80 µmol photon m^{-2} s^{-1}) and a 12:12 light:dark cycle. Experimental inocula were grown in 500 mL Erlenmeyer flasks to a density ranging from 5 x 10^4 to 1.2 x 10^5 cells mL^{-1}. All inocula were grown in static cultures (non-aerated), except the inoculum for the vertical alveolar panel culture which was aerated.

4.2 EXPERIMENTAL DESIGNS

4.2.1 GROWTH IN 2-L ERLENMEYER FLASKS WITH AND WITHOUT AERATION (TRIAL 1)

This initial trial investigated growth of AMAD06 and AMAD16 in 2-L Erlenmeyer flasks with and without aeration. Two experimental 1.5-L cultures were set up for each strain at an initial density of approximately 8 x 10^3 cells mL^{-1}. For each strain, one culture was aerated at a rate of 27 mL L^{-1} min^{-1} with air / CO_2 (100:1, v/v) through a 2 mm internal diameter glass tube, while the other was a static culture. Culture temperature was 18°C ± 1°C; light intensity was 90 µmol photons m^{-2} s^{-1} on a 12:12 light:dark cycle. Cell density was monitored every 2 days until day 27 when the experiment was terminated as the two aerated cultures had reached stationary phase. At day 23, samples were taken for toxin analyses.

4.2.2 INTERACTIONS BETWEEN STRAINS: CLONAL/NON-CLONAL COMPARISON (TRIAL 2)

Experimental conditions for the investigation of interactions between strains were the same as Trial 1 except that the initial cell concentration was approximately doubled and all six cultures were aerated at a rate of 67 mL L^{-1} min^{-1} with air / CO_2 (100:1, v/v). These changes were made following the increased productivity of aerated cultures in Trial 1 and to better simulate the conditions experienced by cells in bubbled photobioreactors. Duplicate 1.5-L cultures in 2-L flasks were set up by inoculating with exponential phase pre-experimental cultures of AMAD06, AMAD16 and a combination of the two strains to an initial density of 1.7 x 10^4 cells mL^{-1}. Every 2 days, for 38 days,

subsamples were taken for the measurement of cell density and for microscopic observations (general cell condition and determining whether sexual stages were present). The cultures were harvested in early stationary phase on day 38 and filtered for analysis of toxins and dry weight.

4.2.3 GROWTH IN AERATED TUBES (TRIAL 3)

This trial was carried out to test growth in 500 mL glass aerated tubes (40 cm long, 5 cm diameter, containing 450 mL of culture), designed to simulate the conditions of a bubble column photobioreactor (Tredici, 1999). Exponential phase inocula were transferred to the culture tubes to obtain a cell density of 7.5 x 10^4 cells mL^{-1}. The culture conditions and use of strains were the same as in Trial 2 except that all six cultures were aerated at a rate of approximately 200 mL L^{-1} min^{-1} with air / CO_2 (100:1, v/v). Samples were taken every 2 days for microscopic observations, dry weight and monitoring of pH, and every 4 days for cell counts. Cultures were harvested on day 26 in stationary phase and toxin content determined.

4.2.4 GROWTH IN A 4-L ALVEOLAR PANEL PHOTOBIOREACTOR (TRIAL 4)

This trial tested growth in a laboratory scale alveolar panel photobioreactor (Tredici et al., 1991). Dimensions of the panel were 535 x 630 mm with an illuminated surface area of 0.31 m^2 and a light path length of 12 mm. Light intensity incident on the panel was 100 µmol photons m^{-2} s^{-1}. The culture was aerated at a rate of 200 mL L^{-1} min^{-1} with air / CO_2 (100:1, v/v) through only 5 of the 17 available alveoli so as to avoid excessive shear stress on the cells. Two litre exponential phase cultures of AMAD06 and AMAD16 were combined in the photobioreactor with an initial cell density of 5 x 10^4 cells mL^{-1}. Samples were taken every 2-3 days for dry weight, cell density, monitoring of pH, and for checking the condition of the cells microscopically. Toxin content was measured on day 24 in early stationary phase. Growth of *A. minutum* was continued in the photobioreactor for several months, maintaining the culture in linear phase. A weekly harvest of 1-L of culture was replaced by 2GSe medium (twice the nutrient and soil extract concentration of GSe). Cell densities and dry weights were determined at every harvest.

4.3 ANALYTICAL METHODS

4.3.1 CELL DENSITY

For the determination of cell densities as a measure of biomass, sub-samples (3 mL) were taken and fixed with Lugol's iodine solution (Sherr and Sherr, 1993). A 1 mL aliquot was then counted in a Sedgewick-Rafter chamber.

4.3.2 DRY WEIGHT

For determination of dry weight, 10 mL of culture was filtered onto a pre-weighed 47 mm diameter Whatman GF/C filter and rinsed twice with 5 mL of 0.65M ammonium formate. The filters were then dried overnight in an oven at 70°C and left to stand in a dessicator for 30 minutes before re-weighing.

4.3.3 MICROSCOPIC OBSERVATIONS

Sub-samples (2 mL) were taken every 2 – 3 days to observe cell 'health' and life history stages using a Leitz Labovert FS inverted microscope at 320x magnification with phase optics. These observations included cell motility, shape (which can change, particularly under nutrient stress, making cells more angular or 'warty') and presence of sexual stages including gametes, planozygotes, and hypnozygotes.

4.3.4 TOXIN ANALYSIS

Samples of 40-100 mL were filtered on pre-weighed 47 mm diameter Whatman GF/C filters, rinsed twice with 5 mL of 0.65M ammonium formate and then freeze dried. Dried filters were re-weighed and transferred to a 10 mL centrifuge tube. Acetic acid (4 mL, 0.05M) was added to the tube and the sample sonicated 5 times for 3 seconds, with a probe sonicator (B.Braun Labsonic 1510) on high power. Samples were stored at –20 °C prior to analysis. HPLC analyses of the three classes of paralytic shellfish toxins (C-toxins, gonyautoxins (GTX) and saxitoxins (STX)) were made using a method adapted from Oshima et al. (1993). Separations were performed on a Waters 600 HPLC, combined with a Pickering PCX 5100 Post-Column Reactor using a 5 µm, 250 x 4.6 mm Alltima ODS column (Alltech, IL) at a flow rate of 0.8 mL min^{-1} (Negri and Llewellyn, 1998). Fluorescent toxin derivatives were detected using a Linear (LC305) spectrofluorometric detector with excitation at 330 nm and emission at 390 nm. Toxins were identified by comparison of retention times and fluorescence emission maxima with toxin standards, the disappearance of peaks by eliminating post-column oxidation and spiking experiments. Toxin standards were kindly donated by Prof. Y. Oshima of Tohoku University, Japan.

4.3.5 CALCULATION OF GROWTH RATE.

Growth rate was calculated based on cell density using the equation:

$$\mu = \frac{\ln(Pt_1) - \ln(Pt_0)}{t_1 - t_0} \qquad \text{(Equation 1)}$$

where µ is specific growth rate in d^{-1}, P is the measured parameter (in this case cell density) and t is time (Guillard, 1973). Growth rates reported are the maximum values calculated during early linear phase, since exponential growth phase was not observed.

RESULTS

Alexandrium minutum was successfully cultivated at high cell densities (ranging between 9.9 x 10^4 in aerated Erlenmeyer flasks to 4.9 x 10^5 in aerated tubes) in all culture systems investigated in the four trials.

4.4 GROWTH IN 2 L ERLENMEYER FLASKS WITH AND WITHOUT AERATION - (TRIAL 1)

Aerated cultures grew better than non-aerated cultures, with no apparent damage to cells and flagella from aeration. There was no lag phase in the cultures except strain AMAD16 without aeration, which had a short (1 day) lag phase (Fig. 4.1). An exponential growth phase was not observed in the cultures although the aerated AMAD06 culture approached exponential growth between days 1 and 5. By day 17-19 the two aerated cultures entered stationary phase, while the non-aerated cultures continued slower and linear growth (Tredici, 1999) (Fig. 4.1). Maximum growth rates during early linear phase were greater in aerated than non-aerated cultures (0.36 and 0.27 d^{-1}). The mean productivity and the maximum cell concentration of the aerated cultures were also greater than in the non-aerated cultures (Table 4.1). Failure of aeration for 12 hours in AMAD16 at day 22 resulted in sinking and temporary loss of cell motility. Cells quickly recovered motility after swirling the flask and resumption of aeration.

For both strains, the total toxin content per cell (measured on day 23) was greater in non-aerated cultures than in aerated cultures (Table 4.1). However, the volumetric toxin concentrations of all cultures were similar due to the higher cell density in aerated cultures. Toxin composition for all cultures was dominated by gonyautoxins 1 and 4 with small amounts of gonyautoxins 2 and 3 and trace amounts of C toxins. The exception was the non-aerated AMAD16, which had unusually high amounts of gonyautoxins 2 and 3 relative to all other samples (Table 4.2).

Table 4.1: Summary of results for Trial 1 – cultures in 2-L flasks with [+AIR] and without [-AIR] aeration.

Strain Details	Mean Productivity[a] (cells x10^3 $mL^{-1}d^{-1}$)	Maximum Growth Rate[b] (d^{-1})	Cell Concentration[b] (cells x10^4 mL^{-1})	Toxin Content (fg $cell^{-1}$)	Toxin Concentration (µg mL^{-1})
AMAD06 [- AIR]	2.4	0.26	7.2	372	21.2
AMAD16 [- AIR]	1.8	0.28	5.6	474	23.6
AMAD06 [+ AIR]	4.6	0.35	9.4	318	28.4
AMAD16 [+ AIR]	4.8	0.37	9.9	264	21.8

[a] Calculated during early linear phase.
[b] Calculated at the end of the linear growth phase [+ AIR] or at the end of the experiment [- AIR].

Table 4.2: Toxin proportion and content for all trials.

Strain Details	n	Toxin proportion (mol %) GTX4	GTX1	GTX3	GTX2	TOTAL (fg cell^{-1})
TRIAL 1						
AMAD06 [- AIR]	1	78.7	20.9	0.29	0.14	372
AMAD16 [- AIR]	1	49.2	18.4	27.4	5.10	474
AMAD06 [+ AIR]	1	70.3	29.3	0.24	0.20	318
AMAD16 [+ AIR]	1	46.7	49.6	2.21	1.50	264
TRIAL 2						
AMAD06	2	58.5	41.3	0.10	0.12	409
AMAD16	2	57.4	42.3	0.13	0.21	402
AMAD06xAMAD16	2	58.1	41.7	0.09	0.13	460
TRIAL 3						
AMAD06	1	53.3	46.4	0.12	0.18	405[a]
AMAD16	2	52.4	47.2	0.17	0.31	377
AMAD06xAMAD16	2	53.3	46.3	0.12	0.21	339
TRIAL 4						
AMAD06xAMAD16	1	63.2	32.3	0.23	0.34	417
mean		58.3	38.1	2.82	0.77	426
SE		9.40	10.8	8.16	1.49	154

[a] The unusually high value of the AMAD06 replicate which lost aeration (1308.36 fg cell^{-1}) is not included in this number.

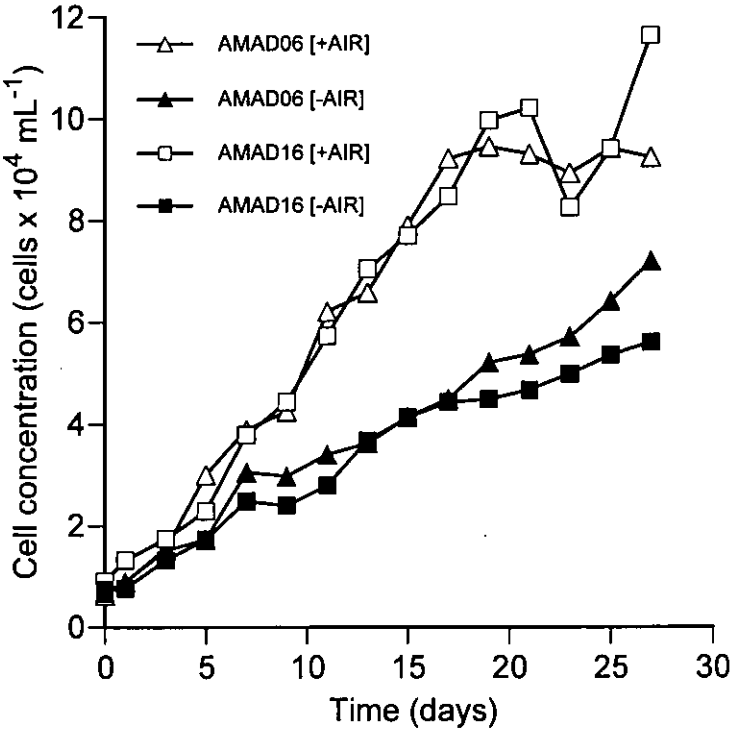

Figure 4.1: Trial 1 -Cell density over time of strains AMAD06 and AMAD16 grown in 2-L flasks with [+AIR] and without [-AIR] aeration.

4.5 INTERACTIONS BETWEEN STRAINS: CLONAL/NON-CLONAL COMPARISON - (TRIAL 2)

Both the clonal strains and the combined strains (non-clonal and sexually compatible) grew well under aerated conditions. Growth curves in the aerated flasks were similar for the different strains (Fig. 4.2). In all cultures a short lag phase of approximately 3 days was followed by linear growth until day 29-30 when cultures entered stationary phase. Two distinct linear phases were observed: from day 3 to day 17, with a mean productivity of 6.5 x 10^3 cells mL^{-1} d^{-1}, and from day 17 to day 30, with a mean productivity of 21.4 x 10^3 cells mL^{-1} d^{-1}. Exponential growth was not observed. No differences were evident in growth rate or final cell densities between the two strains and the combination of the two strains. Maximum growth rate for all cultures was in the range 0.25 – 0.27 d^{-1} and cell density at day 38 ranged between 3.9 x 10^5 and 4.8 x 10^5 cells mL^{-1} for all the cultures (Fig. 4.2). The increased air flow rate (compared with Trial 1) resulted in increased productivity and final cell densities, rather than having any adverse effect on growth. Cells appeared healthy throughout the experiment, and in the combined strain cultures there was no evidence of sexual reproduction. Toxin composition for all cultures was similar to that seen in Trial 1, with gonyautoxins 1 and 4 dominating and lesser amounts of gonyautoxins 2 and 3. Trace amounts of neosaxitoxin and saxitoxin were also detected. Toxin content per cell was similar to the non-aerated cultures in the first trial at 402-460 fg $cell^{-1}$ (Table 4.2).

4.6 GROWTH IN AERATED TUBES - (TRIAL 3)

All experimental cultures in aerated tubes had similar growth characteristics, with linear growth until day 16 when cultures entered stationary phase (Fig. 4.3). An exception was one replicate of AMAD06 in which interruption of aeration for approximately 16 hours on day 10 resulted in cell lysis and death, a dramatic increase in bacteria levels, and a decrease in cell density of approximately 50%. This treatment has been excluded from Figure 4.3 and the calculation of means. Mean productivity during linear phase was 2.6 x 10^4, 1.9 x 10^4 and 2.1 x 10^4 cells mL^{-1} d^{-1} for AMAD06, AMAD16 and AMAD06xAMAD16, respectively. The greatest cell density at the end of the linear phase (4.9 x 10^5 cells mL^{-1}) was reached by AMAD06. Maximum growth rates in the cultures ranged from 0.22 to 0.25 d^{-1}. No sexual stages were detected. Cells did not appear as healthy as those in the Erlenmeyer flasks, with flagella damage and loss of motility observed. Toxin composition was generally similar to Trials 1 and 2, but toxin per cell was generally lower than in Trial 2 for all but the AMAD06 replicate (which lost aeration). This culture had the highest toxin concentration value recorded (1308 fg $cell^{-1}$) (Table 4.2)

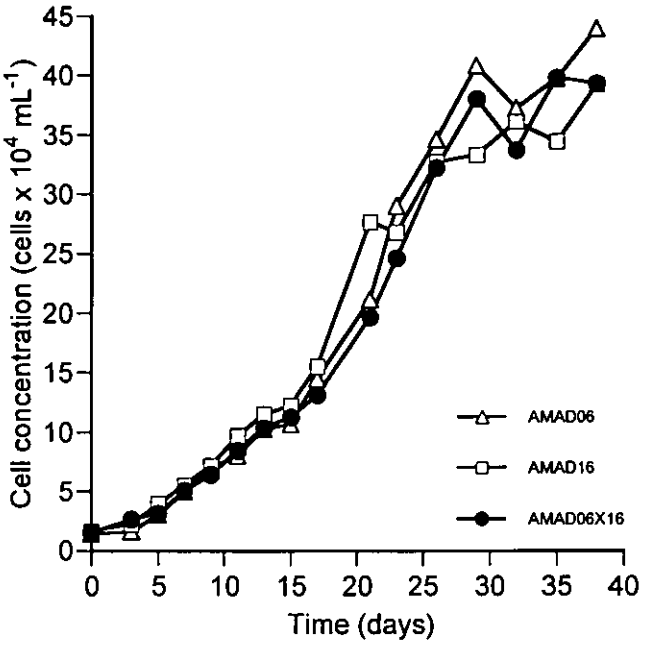

Figure 4.2: Trial 2 - Cell density over time of *A. minutum* strains AMAD06, AMAD16 and AMAD06xAMAD16 grown in 2-L flasks. The mean of two replicates is reported.

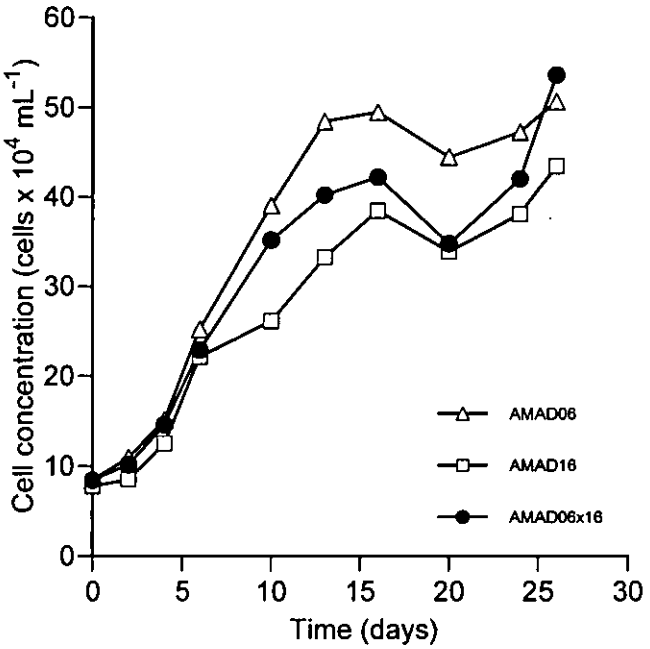

Figure 4.3: Trial 2 - Cell density over time of *A. minutum* strains AMAD06, AMAD16 and AMAD06xAMAD16 grown in 2-L aerated tubes. The mean of two replicates is reported. Only one of the duplicates for AMAD06 is shown.

4.7 GROWTH IN A 4 L VERTICAL ALVEOLAR PANEL PHOTOBIOREACTOR - (TRIAL 4)

Alexandrium minutum grew well in the 4-L alveolar panel photobioreactor. The culture showed linear growth until day 26 when it entered stationary phase; no lag or exponential phases were observed (Fig. 4.4). Maximum growth rate during early linear phase was 0.22 d^{-1} and mean productivity was 1.4 x 10^4 cells $mL^{-1}d^{-1}$. A maximum cell density of 3.3 x 10^5 cells mL^{-1} (corresponding to a dry weight of 0.9 g L^{-1}) was reached at the beginning of stationary phase (Fig. 4.4). The cells appeared healthier in the panel than in the culture tubes, exhibiting no flagella damage. There was evidence of sexual reproduction by day 9 of the experiment, with many gametes, fusing gametes and planozygotes being observed. The toxin composition was similar to all other trials and mean cellular toxin content was 417 fg $cell^{-1}$ (Table 4.2). *Alexandrium minutum* was successfully cultured semi-continuously in the alveolar panel photobioreactor for five months (Fig. 4.5 gives an example of growth over this period). During this time dilutions were made at 5-9 day intervals with a mean biomass concentration of 1.27 g L^{-1}, attaining a mean productivity of 1.3 x 10^4 cells mL^{-1} d^{-1} (0.062 g L^{-1} d^{-1}).

4.8 TOXIN COMPOSITION

With the exception of the culture which lost aeration (Trial 3) toxin composition and concentration for all strains in all treatments were similar, with an average toxin concentration over all trials of 426 fg $cell^{-1}$. Gonyautoxins (GTX) 1 and 4 were the most abundant toxins in each of the cultures (Table 4.2, Fig. 4.6). The only other toxins consistently detected in each trial were GTX2 and GTX3. Minor components probably included C-toxins C1, C2, and C4, neosaxitoxin (NEO) and saxitoxin (STX), but the concentrations of these minor components were too low for the identities of their peaks to be confirmed by standard techniques (e.g. see Fig. 4.6). These minor components were therefore not included when quantifying the cellular toxin concentrations. The only culture that showed some deviation from the usual composition was the non-aerated AMAD16, which had unusually high levels of GTX2 and GTX3. The highest toxin concentration per cell of 1308 fg $cell^{-1}$ was reached in a culture of AMAD06 as part of Trial 3 (aerated tubes). This culture crashed after a loss of aeration, but whether this was related to the higher toxin levels could not be determined. *Alexandrium minutum* grown in batch culture in the panel photobioreactor produced a similar toxin concentration to the average of all the combined trials (Table 4.2).

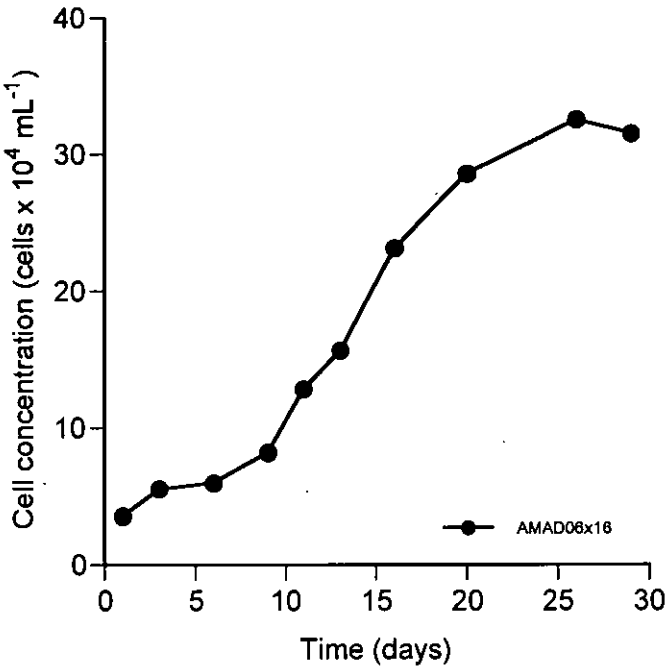

Figure 4.4: Trial 2 - Cell density over time of *A. minutum* strains AMAD06xAMAD16 grown in an alveolar panel photobioreactor.

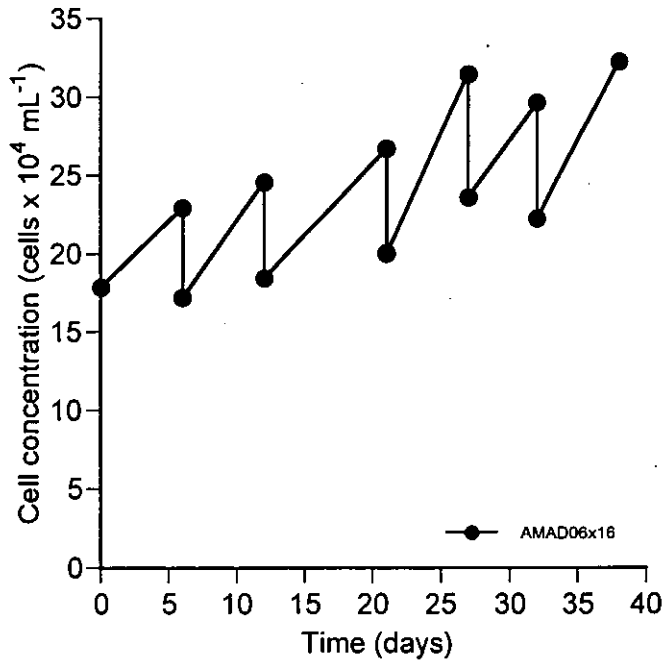

Figure 4.5: Trial 2 - Cell density over time of *A. minutum* strains AMAD06xAMAD16 grown in an alveolar panel photobioreactor under semicontinuous conditions.

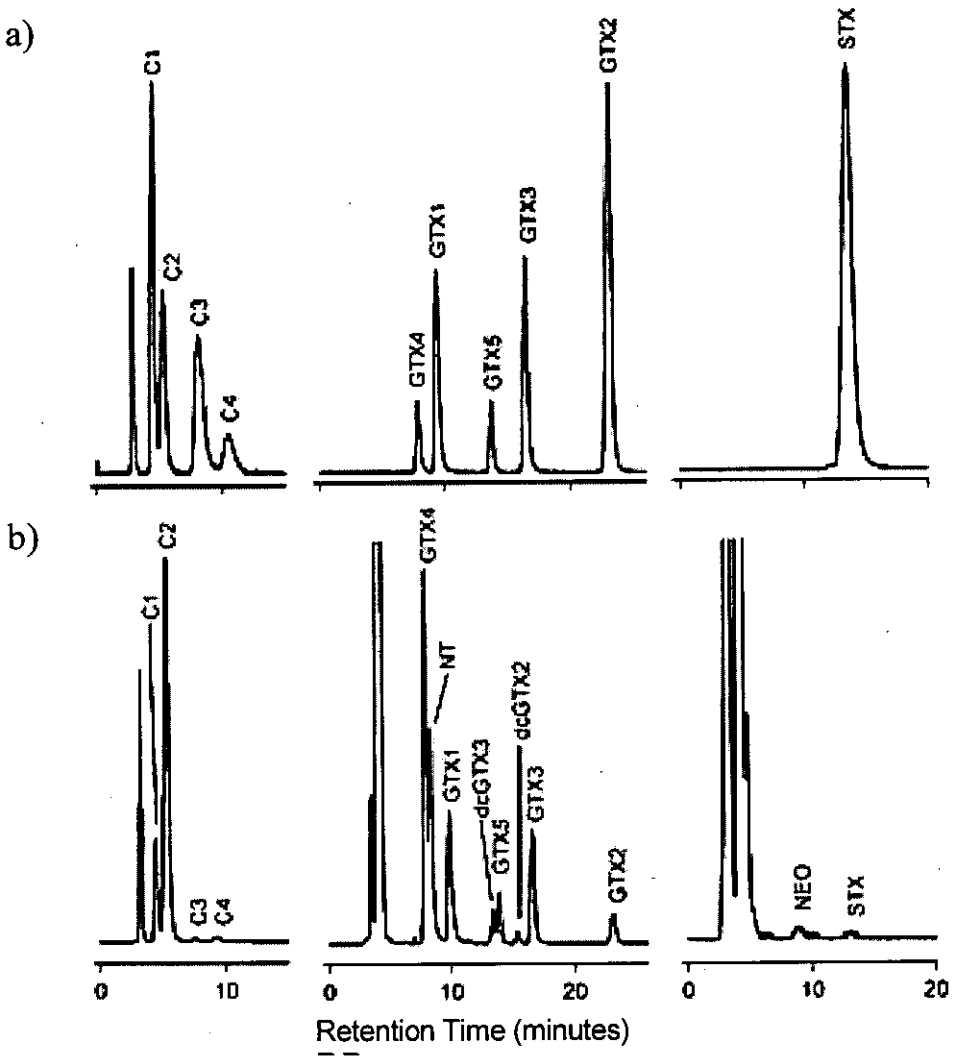

Figure 4.6 Chromatograms of a) PST standards and b) an extract of *A. minutum* (AMAD16 grown in an aerated tube - Trial 3). Note that fluorescence scales are not equivalent between chromatograms.

DISCUSSION

In assessing the use of microalgae as sources of bioactive compounds for biotechnology, there are three issues that need to be considered. The first is whether the organism can be cultured, the second is whether the organism can produce the desired compound in culture (Shimizu, 1993), and the third is whether the organism can be cultured at the scales required for commercial production. It has been shown that it is feasible to grow and maintain *A. minutum* at high cell densities, in three different laboratory scale culture systems, and that cell density in aerated cultures is greater than in non-aerated culture, with no decrease in production of the target toxin compounds. Previous studies of the culture of *A. minutum* reported cell densities of up to 1.5 x 10^5 cells mL^{-1} after 20 days in culture (Hwang and Lu, 2000) and 6.5 x 10^4 cells mL^{-1} in 5 weeks (La Barre et al., 1999). In this study, higher cell densities (over 5.0 x 10^5 cells mL^{-1} - corresponding to a dry weight biomass of approximately 0.8 g L^{-1}) have been achieved during an equivalent or even shorter time period.

Maximum growth rates measured during early linear phase were less than 0.4 d^{-1} and varied little across the trials. These low growth rates are not unusual for dinoflagellates, which only rarely have growth rates exceeding one doubling per day (Taylor and Pollingher, 1987). The maximum growth rate reported for *A. minutum* is 0.6 d^{-1} (for a Spanish strain; Flynn et al., 1994). Most other microalgal species of economic importance have growth rates much higher than 1.0 d^{-1} e.g. *Dunaliella tertiolecta* 1.4 d^{-1}, *Thalassiosira pseudonana* 1.8 d^{-1} and *Chaetoceros calcitrans* 2.0 d^{-1} (Thompson et al., 1990). Modification of environmental conditions of *A. minutum* cultures, to increase growth rate and achieve higher rates of production, requires investigation. The highest cell density was achieved in the aerated tubes (Trial 3), with a maximum of 5.5 x 10^5 cells mL^{-1}.

Exponential growth was only observed in one of the cultures studied. Due to the low doubling time, the sampling regime should have been able to detect exponential growth. This suggests that even at the beginning of the experiments there was some growth limiting factor. Although light saturation intensities are highly variable for dinoflagellates, it is likely that light was the limiting factor. Cannon (1993b) found that maximum growth of Australian *A. minutum* occurred at 100 µmol photon m^{-2} s^{-1}, which was the highest light intensity tested and similar to the intensities used in these studies. Spanish strains of *A. minutum* tested from 83 to 366 µmol photon m^{-2} s^{-1} also showed increased growth up to the highest intensity (Davidson et al., 1999). The related species *A. tamarense* has also been found to have a high light saturation intensity of 250 µmol photon m^{-2} s^{-1} (Langdon, 1987, 1988). Therefore, the light levels used in these

experiments could result in all the cultures in this study being photolimited even if initial cell density was low enough to avoid substantial self-shading.

Sexual reproduction was only observed in the alveolar panel photobioreactor (Trial 4) where fusing gametes and planozygotes were seen, although no resting cysts were observed. Resting cysts of *A. minutum* have a mucoid sheath and therefore may have escaped detection by adhering to the surfaces of the photobioreactor. The capacity of cells to sexually reproduce indicates that they were 'fully functional' despite exposure to the turbulent environment. This result also suggests that the conditions in the alveolar panel photobioreactor were more conducive to the induction of sexual reproduction than the other systems, although the cause of this is not clear. Importantly, the presence of a potentially sexually reproductive population did not appear to have any negative effects on growth and productivity in these systems, although a clonal strain was not tested in the alveolar panel photobioreactor.

The increase in productivity in response to aeration is worth emphasizing given the many examples in the literature of the damage suffered by dinoflagellates in turbulent environments (e.g. see Estrada and Berdalet, 1998). Some flagella damage did occur, particularly in the aerated tubes, but this was not extensive and in general cells were healthy in aerated cultures. They may have been responding favourably to increased CO_2 levels and/or increased irradiance brought about by mixing. It has been found that aeration also improves growth and biomass production of other dinoflagellates including other *Alexandrium* species, *Scrippsiella* sp. and *Gymnodinium catenatum* (Frampton and Blackburn, unpublished data). Increased growth rates of *G. catenatum* in turbulent conditions was also noted by Sullivan et al. (1997). These findings suggest that carefully regulated aeration in appropriate systems may result in increased growth rates and biomass concentration for dinoflagellates, as for most microalgae.

The paralytic shellfish toxin (PST) composition detected in these trials was similar to other reports of Australian *A. minutum*, although the trace levels of presumed C-toxins, neosaxitoxin and saxitoxin observed in this study have not previously been documented (Oshima et al., 1989). The most likely explanation is that *A. minutum* may produce a relatively wide range of PSTs at very low concentrations, but those reported are dependent on the detection limits of HPLC. The only culture that deviated from the usual composition was the non-aerated AMAD16 in Trial 1 which, as mentioned previously, had higher levels of GTX2 and GTX3. The cause of this compositional difference is unclear. It is possible that the lack of mixing from aeration may have resulted in zonation of O_2, CO_2 or nutrients, leading to preferential production of non-hydroxy toxins. Alternatively, bacterial strains have been shown to convert GTX1 and GTX4 to their non-hydroxy analogues GTX2 and GTX3 (Smith et al., 2001). An

infection of a single culture of AMAD16 by similar bacteria may have resulted in the toxin profiles observed in this study.

Alexandrium minutum harvested from the 4-L panel photobioreactor contained similar concentrations of PSTs to the other culture systems tested in this study. This demonstrates that use of the panel photobioreactor is an effective method of producing these metabolites. The panel photobioreactor culture was maintained at a cell concentration of more than 3.1 x 10^5 cells mL^{-1} with weekly harvesting. Assuming an average toxin concentration of 420 fg $cell^{-1}$, toxin production in this system was approximately 20 µg $L^{-1}d^{-1}$. The cellular concentration of PSTs in these experiments was much lower than for New Zealand strains which contain between 1800 and 11000 fg $cell^{-1}$ and are dominated by neosaxitoxin (MacKenzie and Berkett, 1997; Hoe Chang et al., 1997). Careful selection of *A. minutum* strains could therefore significantly enhance PST yields from photobioreactors.

In these trials the experimental parameters were based on the knowledge of dinoflagellate growth in small scale culture and may be conservative in terms of environmental factors promoting growth. In this study, relatively low light was used on a 12:12 light:dark cycle, which as discussed above may be well below the saturation light intensity for *A. minutum*. Temperature and nutrient concentrations were also not increased above the usual small scale culture conditions. Several investigators have found that phosphorous stress increases toxin per cell (Boyer et al., 1987; Anderson et al., 1990; Flynn et al., 1994; Béchemin, 1999). Maintaining low levels of phosphorus in cultures may therefore increase toxin production, and by careful monitoring of nutrients and growth it should be possible to harvest cells during the period of maximum toxin content. Refinement of these parameters and optimisation of aeration rate should lead to increased growth, biomass and toxin production of *A. minutum*.

Cultures used in this study were non-axenic. For commercial production purposes axenic culture may be required to produce a pure product. Successful axenic cultivation of *A. minutum* and various other *Alexandrium* species has been achieved (Maas, 1997; Hold et al., 2001). However, the interaction between dinoflagellates, bacteria and toxin production is complex and some caution needs to be taken in choosing axenic culture (Gallacher and Smith, 1999). In some cases, axenic conditions result in reduced dinoflagellate growth rates and reduced toxin production (Alavi et al., 2001; Hold et al., 2001). A defined and well- characterized bacterial population may be a better compromise.

The success of *A. minutum* in the alveolar panel photobioreactor demonstrated that this culturing system is suitable for growth of *A. minutum*, a relatively fragile species compared with the microalga for which this system was originally designed i.e.

Nannochloropsis (Chini Zittelli et al., 2000). It was not determined in this study whether *A. minutum* would respond well to scale-up in a larger panel photobioreactor (e.g. Chini Zittelli et al., 2000) or whether other photobioreactor designs may improve growth. These issues should be explored. Testing the growth of *A. minutum* in large scale flat plate photobioreactors (Richmond and Cheng-Wu, 2001) would be particularly interesting given the success of this species in aerated laboratory cultures. These reactors are open throughout, without the alveoli of the panels, therefore reducing the internal surface area to volume ratio. As such, a reactor of this type may be gentler on the cells and decrease flagella damage due to a reduction in shear stress during mixing.

Another issue that requires further attention for the effective high concentration culture of *A. minutum* is the cause of cells sinking when aeration stops. Cells appear healthy, are motile, and recover once aeration is resumed. However, it is possible that aeration is having a physiological effect on the cells' motility and ability to maintain their position in the water column that was not obvious from microscopic observations. The possibility that aeration may compromise long-term health of *A. minutum* cultures should therefore be investigated.

In nature, different microalgal species have their own ecological niches, thriving under particular combinations of physical and chemical conditions. Some species, including *A. minutum*, have the ability to form dense, almost monospecific, blooms. Where such species produce important bioactive molecules, the fact that they occur at high densities in the natural environment suggests that they may prove to be excellent candidates for high biomass production and subsequent commercial exploitation under the right growth conditions. The specific conditions required for individual species may require further customisation of photobioreactor design.

The work presented here indicates that the dinoflagellate *A. minutum* can be grown successfully in high density systems. With further refinement of strain selection, culture conditions and photobioreactor design, phototrophic high biomass culture of *A. minutum* and other dinoflagellates for bioactive molecule production is a viable proposition for biotechnology applications.

REFERENCES

Alavi M, Miller T, Erlandson K, Schneider R, Belas R (2001) Bacterial community associated with *Pfiesteria*-like dinoflagellate cultures. Environmental Microbiology 3: 380–396.

Anderson DM, Kulis DM, Sullivan JJ, Hall S, Lee C (1990) Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Marine Biology 104: 511–524.

Balech E (1985) The genus *Alexandrium* or *Gonyaulax* of the tamarense group. In Anderson DM, White AW, Baden DG (eds), Toxic Dinoflagellates. Elsevier/North Holland, Amsterdam: 33–38.

Balech E (1989) Redescription of *Alexandrium minutum* Halim (Dinophyceae) type species of the genus *Alexandrium*. Phycologia 28: 206–211.

Béchemin C, Grzebyk D, Hachame F, Hummert C, Maestrini SY (1999) Effect of different nitrogen/phosphorus nutrient ratios on the toxin content in *Alexandrium minutum*. Aquatic Microbial Ecology 20: 157–165.

Blackburn SI, Bolch CJ, Haskard KA, Hallegraeff GM (2001) Reproductive compatibility among four global populations of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). Phycologia 40: 78–87.

Bolch CJ, Blackburn SI, Cannon JA, Hallegraeff GM (1991) The resting cyst of the red-tide dinoflagellate *Alexandrium minutum* (Dinophyceae). Phycologia 30: 215–219.

Boyer GL, Sullivan JJ, Andersen RJ, Harrison PJ, Taylor FJR (1987) Effects of nutrient limitation on toxin production and composition in the marine dinoflagellate *Protogonyaulax tamarensis*. Marine Biology 96: 123–128.

Cannon JA (1993a) Germination of the toxic dinoflagellate *Alexandrium minutum*, from sediments in the Port River, South Australia. In Smayda TJ, Shimizu Y (eds), Toxic Phytoplankton Blooms in the Sea. Elsevier, New York: 103–107.

Cannon JA (1993b) Growth in culture of the toxic dinoflagellate *Alexandrium minutum* from the Port River, South Australia. In Smayda TJ, Shimizu Y (eds), Toxic Phytoplankton Blooms in the Sea. Elsevier, New York: 741–745.

Chini Zittelli G, Rastorelli R, Tredici MR (2000) A Modular Flat Panel Photobioreactor (MFPP) for indoor mass cultivation of *Nannochloropsis* sp. under artificial illumination. Journal of Applied Phycology 12: 521–526.

Cohen P, Holmes CFB, and Tsukitani Y (1990) Okadaic acid: a new probe for the study of cellular regulation. Trends in Biochemical Science 15: 98–102.

Davidson K, Wood G, John EH, Flynn KJ (1999) An investigation of non-steady-state algal growth. I. An experimental model system. Journal of Plankton Research 21: 811–837.

Estrada M, Berdalet E (1998) Effects of turbulence on phytoplankton. In Anderson DM, Cembella AD, Hallegraeff GM (eds), Physiological ecology of harmful algal blooms. Springer-Verlag, Berlin: 601–618.

Faulkner DJ (2001) Marine Natural Products. Natural Products Report 18: 1–49.

Flynn K, Franco JM, Fernandez P, Reguera B, Zapata M, Wood G, Flynn KJ (1994) Changes in toxin content, biomass and pigments of the dinoflagellate *Alexandrium minutum* during nitrogen refeeding and growth into nitrogen or phosphorus stress. Marine Ecology Progress Series 111: 99–109.

Gallacher S, Smith EA (1999) Bacteria and paralytic shellfish toxins. Protist 150: 245–255.

Guillard RRL (1973) Division rates. In Stein JR (ed) Handbook of Phycological Methods. Cambridge Univ. Press, Cambridge: 289–312.

Halim Y (1960) *Alexandrium minutum* n. gen. s. sp., dinoflagelle provocant "des eaux rouges". Vie et Milieu 11: 102–105.

Hallegraeff GM, Steffenson DA, Wetherbee R (1988) Three estuarine Australian dinoflagellates that can produce paralytic shellfish toxins. Journal of Plankton Research 10: 533–541.

Hoe Chang F, Anderson DM, Kulis DM, Till DG (1997) Toxin production of *Alexandrium minutum* (Dinophyceae) from the Bay of Plenty, New Zealand. Toxicon 35: 393–409.

Hold GL, Smith EA, Birkbeck TH, Gallacher S (2001) Comparison of paralytic shellfish toxin (PST) production by the dinoflagellates *Alexandrium lusitanicum* NEPCC 253 and *Alexandrium tamarense* NEPCC 407 in the presence and absence of bacteria. FEMS Microbiology Ecology 36: 223–234.

Hwang DF, Lu YH (2000) Influence of environmental nutritional factors on growth, toxicity, and toxin profile of dinoflagellate *Alexandrium minutum*. Toxicon 38: 1491–1503.

Koray T, Buyukisik B (1988) Toxic dinoflagellate blooms in the harbour region of Izmir Bay (Aegean Sea). Revue Internationale d'Oceanographie Medicale 91–92, 25–42.

LaBarre S, Singer S, Erard-Le Denn E, Jozefowicz M, (1999) Controlled cultivation of *Alexandrium minutum* and [33P] orthophosphate cell labelling towards surface adhesion tests. Journal of Biotechnology 70: 207–212.

Langdon C (1987) On the causes of interspecific differences in the growth-irradiance relationship for phytoplankton. Part I. A comparative study of the growth-irradiance relationship of three marine phytoplankton species: *Skeletonema costatum*, *Olisthodiscus luteus* and *Gonyaulax tamarensis*. Journal of Plankton Research 9: 459–482.

Langdon C (1988) On the causes of interspecific differences in the growth-irradiance relationship for phytoplankton. II A general review. Journal of Plankton Research 10: 1291–1312.

LeDoux M, Fremy JM, Nezan E, Erard E (1989) Recent occurrence of paralytic shellfish poisoning (PSP) toxins from the northwestern coasts of France. Journal of Shellfish Research 8: 486.

Maas EW (1997) The causative agent of toxic shellfish poisoning. Thesis submitted for the degree of Doctor of Philosophy of the University of Otago, Dunedin, New Zealand. University of Otago, Dunedin, 260 pp.

MacKenzie LM and Berkett N (1997) Cell morphology and PSP-toxin profiles of *Alexandrium minutum* in the Marlborough Sounds, New Zealand. New Zealand Journal of Marine and Freshwater Research 31: 403–409.

Montresor M, Marino D, Zingone A, Dafnis G (1990) Three *Alexandrium* species from coastal Tyrrhenian waters (Mediterranean Sea). In Graneli E, Sundstroem B, Edler L, Anderson DM (eds), Toxic Marine Phytoplankton. Elsevier, New York: 82–87.

Negri AP, Llewellyn LE (1998) Comparative analyses by HPLC and the sodium channel and saxiphilin ^{3}H-Saxitoxin receptor assays for paralytic shellfish toxins in crustaceans and molluscs from tropical North-West Australia. Toxicon 36: 283–298.

Oshima Y, Hirota M, Yasumoto T, Hallegraeff GM, Blackburn SI, Steffensen DA (1989) Production of paralytic shellfish toxins by the dinoflagellate *Alexandrium minutum* Halim from Australia. Nippon Suisan Gakkaishi 55: 925.

Oshima Y, Itakura H, Lee KC, Yasumoto T, Blackburn S, Hallegraeff G (1993) Toxin production by the dinoflagellate *Gymnodinium catenatum*. In Smayda TJ, Shimizu Y (eds), Toxic Phytoplankton Blooms in the Sea. Elsevier, New York: 907–912.

Pietra F (1997) Secondary metabolites from marine microorganisms: bacteria, protozoa, algae and fungi. Achievements and prospects. Natural Products Report 1997: 453–464.

Richmond A, Cheng-Wu Z (2001) Optimization of flat plate reactor for mass production of *Nannochloropsis* sp. outdoors. Journal of Biotechnology 85: 259–269.

Sherr EB, Sherr BF (1993) Preservation and storage of samples for enumeration of heterotrophic protists. In Kemp PF, Sherr BF, Sherr EB, Cole JJ (eds), Handbook of methods in aquatic microbial ecology. Lewis publishers, Florida: 207–212.

Shimizu Y (1993) Dinoflagellates as sources of bioactive molecules. In Attaway DH, Zaborsky OR (eds), Marine Biotechnology Volume 1: Pharmaceutical and bioactive natural products. Plenum Press, New York: 391–410.

Smith EA, Grant F, Ferguson CMJ, Gallacher S (2001) Biotransformations of paralytic shellfish toxins by bacteria isolated from bivalve molluscs. Applied and Environmental Microbiology 67: 2345–2353.

Sullivan JM, Rines J, Swift J, Donaghay PL (1997) The effects of small-scale turbulence on marine dinoflagellates. In ICES council meeting papers, ICES, Copenhagen: 1–7.

Taylor FJR (1987) The biology of dinoflagellates. Blackwell Scientific Publications, London, 785pp.

Taylor FJR, Pollingher U (1987) Ecology of dinoflagellates. In Taylor FJR (ed), The Biology of Dinoflagellates. Blackwell Scientific Publications, London: 398–529.

Thompson PA, Harrison PJ, Whyte JNC (1990) Influence of irradiance on the fatty acid composition of phytoplankton. Journal of Phycology 23: 278–288.

Tredici MR (1999) Bioreactors, Photo. In Flickinger MC, Drew SW (eds), Encyclopedia of Bioprocess Technology, fermentation, biocatalysis, and bioseparation Volume 1. John Wiley and Sons, Inc., New York: 395–419.

Tredici MR, Carlozzi P, Chini Zittelli G, Materassi R (1991) A vertical alveolar panel (VAP) for outdoor mass cultivation of microalgae and cyanobacteria. Bioresource Technology 38: 153–159.

Yasumoto T, Satake M (1998) Bioactive compounds from marine microalgae. Chimia 52: 63–68.

CHAPTER 5

FACTORS INFLUENCING ENCYSTMENT AND EXCYSTMENT OF THE DINOFLAGELLATES *ALEXANDRIUM MINUTUM* AND *GYMNODINIUM CATENATUM* (DINOPHYCEAE)

INTRODUCTION

For many years the importance of sexual reproduction in bloom dynamics has been a key question in the study of toxic dinoflagellates (Anderson et al., 1983). Of particular relevance was the discovery that many dinoflagellates formed resting cysts as part of their sexual life cycles (Huber and Nipkow, 1922, 1923; Braarud, 1945; Nordli, 1951). This discovery led to questions of the importance of resting cysts in the autecology and bloom dynamics of dinoflagellate populations. More recently resting cysts have also been recognised as possible vectors of the global expansion of toxic and other species by translocation in ballast tanks (Hallegraeff and Bolch, 1991). An understanding of the factors which trigger both encystment (resting cyst formation) and excystment (resting cyst germination) is crucial if the role of sexual reproduction in bloom dynamics is to be understood.

Although the study of excystment has received more attention to date (e.g. Anderson and Wall, 1978; Bravo and Anderson, 1994), encystment or resting cyst formation is an equally important component of dinoflagellate autecology and bloom dynamics. It has been suggested that encystment may play a major role in bloom decline (Anderson and Wall. 1978; Ishikawa and Taniguchi, 1996) as well as enabling populations to survive unfavourable environmental conditions. Nutrient depletion (particularly nitrogen) has been suggested to be the cue for encystment in many dinoflagellate species (Chapter 1, Table 1.1), with the inference that encystment occurs once conditions become sub-optimal for vegetative growth. However, this focus on nutrients may be at least partially due to the fact that the primary aim of early studies was to document sexuality, rather than to investigate encystment cues, and success with inducing sexual reproduction by nutrient starvation precluded the testing of other factors (Pfiester and Anderson, 1987). Both laboratory and field observations indicate that resting cysts can form under apparently optimal nutrient conditions (Anderson et al., 1983; Pfiester et al., 1984, Chapter 6). Over the last thirty years, many other potential encystment factors, apart from macronutrient concentrations, have been suggested and investigated. These include external factors such as temperature, with temperature windows for encystment being identified in several species (Ellegaard, et al., 1998; Sgrosso et al., 2001). Micronutrients such as selenium (Doblin, 1998) and other trace metals including iron (Blanco, 1995) have also been studied and found to enhance encystment in some species. Photoperiod was found to have a significant impact on encystment in some calcareous resting cyst forming dinoflagellates with shorter day lengths promoting encystment (Sgrosso et al., 2001) and oceanographic fronts have also been suggested as particularly active regions of encystment (Tyler et al., 1982). Endogenous or biological factors such as pheromone-like substances or chemical cues that facilitate sexual reproduction have also been suggested to be present in dinoflagellates (Destombe and

Cembella, 1990; Wyatt and Jenkinson, 1997). Internal nutrient pools have been suggested to trigger encystment (Probert et al., 1998) and natural bacterial assemblages associated with an *Alexandrium tamarense* bloom were implicated in enhancing encystment of *A. catenella* in culture (Adachi et al., 1999).

Many studies of sexual reproduction in dinoflagellates have focussed on the process of excystment. Excystment has been suggested as a means by which bloom initiation may occur, with 'cyst beds' inoculating overlying waters (Anderson and Wall 1978; Kremp, 2000a, 2000b). Therefore, understanding excystment cues may lead to greater predictability of harmful algal blooms and more informed management decisions. Various factors have been investigated for their potential to influence excystment. One crucial factor is the length of the requisite dormancy period or internal maturation period. The requisite dormancy period is the interval after encystment during which a resting cyst is unable to germinate, regardless of physical or chemical conditions (Pfiester and Anderson, 1987). This period varies greatly from species to species. For example, some dinoflagellates, such as *Alexandrium minutum* and *Gymnodinium catenatum* (Chapter 2 and Blackburn et al., 1989 respectively), have short requisite dormancy periods of approximately 2 to 4 weeks. Other dinoflagellates have much longer requisite dormancy periods e.g. 4.5 months for *Ceratium hirundinella* (Rengefors and Anderson, 1998) and 6 months for *Scrippsiella hangoei* (Kremp and Anderson, 2000). Another factor, which has received much attention as a cue for excystment, is temperature (Bravo and Anderson, 1994; Hallegraeff et al., 1998; Kremp and Anderson, 2000). Of the few dinoflagellates studied, most have a 'temperature window' outside of which they are unable to germinate. The effect of light on excystment has also been investigated and most dinoflagellate resting cysts are either unable to germinate or have very low excystment in the dark (Blackburn et al., 1989; Bravo and Anderson, 1994; Nuzzo and Montresor, 1999). Anoxic conditions have also been found to inhibit excystment in several dinoflagellates (Bravo and Anderson, 1994; Kremp and Anderson, 2000). In various studies the macronutrient concentration in the external medium has been shown to have no effect on excystment (e.g. Bravo and Anderson, 1994; Rengefors and Anderson, 1998). Other factors which have been investigated include the identification of optimal salinity conditions (Cannon, 1993), and the addition of growth factors (platelet-derived growth factor and fetal bovine serum) which significantly enhanced excystment (Costas et al., 1993).

This chapter explores the effect of several factors, which have not previously been studied in culture, on the encystment and excystment of the two toxic dinoflagellates *Gymnodinium catenatum* and *Alexandrium minutum* which bloom in Australian waters (Hallegraeff et al., 1988). Strains used in the study were isolated from Australian populations in the Huon Estuary in southeast Tasmania (*G. catenatum*) and the Port River Estuary in South Australia

(*A. minutum*). The role of sexual reproduction in the ecology of these populations is largely unknown. The factors studied were nutrients, temperature, light, algicidal bacterial exudates, pheromone-like substances and anaerobic conditions. The importance of each of these factors in the encystment and excystment of these key dinoflagellate species is discussed.

MATERIALS AND METHODS

A series of experiments were conducted to determine the effect of various factors on encystment and excystment in *A. minutum* and *G. catenatum*. The factors were selected for one of two reasons: 1) that they have been found to be important in other species (temperature, nutrients, anaerobic conditions and light) or 2) because they have not previously been investigated (algicidal bacterial exudates and pheromone-like substances). The experiments are summarised in Table 5.2.

Table 5.2: Summary of factors tested for effects on encystment and excystment of *G. catenatum* and *A. minutum* in this study.

Species	Encystment	Excystment
Gymnodinium catenatum	Temperature	Algicidal bacterial exudates
	Nutrient concentrations	Anaerobic conditions (and nutrients)
	Pheromone-like substances	
	Algicidal bacterial exudates	
Alexandrium minutum	Temperature	Algicidal bacterial exudates
	Nutrient concentrations (and light)	Temperature (and light)
	Pheromone-like substances	

5.1 STRAIN DETAILS

Eight strains were used in these experimental investigations of encystment and excystment cues. Six of these strains, the *A. minutum* strains AMAD06 and AMAD16, AMAD11 and AMAD12 and *G. catenatum* strains GCDE08 and GCHU11, were obtained from the CSIRO Collection of Living Microalgae (http://www.marine.csiro.au/microalgae). A further two *G. catenatum* strains GCHUN1-97 and GCHUN2-97 were isolated as part of this project. Full isolation details for all strains are given in Table 5.3. Strains were maintained at 18 °C ± 0.5 °C at 80 µmol photons PAR m^{-2} s^{-1} on a 12:12 light:dark cycle in 50 mL Erlenmeyer flasks in 40 mL of 28 psu (practical salinity units) GSe medium (Blackburn et al., 2001 and Appendix A). Cool white fluorescent lights were used for maintaining strains, and also in all experiments. The two pairs of strains for each species were selected as they were previously found to be sexually compatible (i.e. capable of producing resting cysts or hypnozygotes when combined, see Chapter 3).

Table 5.3: Isolation details for strains used in encystment and excystment experiments. Brackets indicate sexually compatible strains.

Strain Number	Isolation Date	Isolator	Location	Toxic	Clonal
AMAD06	27 Oct 1987	S. Blackburn	Port River, South Australia	yes	yes
AMAD16	27 Oct 1987	S. Blackburn	Port River, South Australia	yes	yes
AMAD11	27 Oct 1987	S. Blackburn	Port River, South Australia	yes	yes
AMAD12	4 June 1989	S. Blackburn, J. Cannon	Port River, South Australia	yes	yes
GCDE08	15 June 1987	S. Blackburn	Derwent Estuary, Tasmania	yes	yes
GCHU11	20 June 1988	S. Blackburn	Huon Estuary, Tasmania	yes	yes
GCHUN1-97	5 Dec 1997	N. Parker	Huon Estuary, Tasmania	na*	yes
GCHUN2-97	5 Dec 1997	N. Parker	Huon Estuary, Tasmania	na*	yes

*na = culture not analysed for toxicity

5.2 CROSSING OF STRAINS

Strain crosses were made using a modification of the resting cyst formation methods of Oshima et al. (1993). For all experiments, culture volumes were first increased from 40 to 70 or from 40 to 400 mL under the same environmental conditions as indicated above (Section 5.1). Cultures were then transferred into $GSe/_{20}$ (i.e. GSe with $^{1}/_{20}$ nutrients and soil extract, see Appendix A) for 1-2 weeks before the start of an experiment. Compatible strains were then combined with various treatment media, according to the experimental designs outlined below, in pre-sterilised polystyrene petri dishes (Falcon™), multi-well plates (Falcon™) or sterilised polycarbonate culture jars (70 mL Technoplas™). Encystment experiments generally continued until resting cyst formation slowed, i.e. stationary phase of encystment.

5.3 STATISTICAL ANALYSIS

Significance of differences between treatments were determined by one-way ANOVA using SigmaStat (version 2.0 SPSS Inc.), assumptions were tested and where necessary log transformations were performed which resolved the failed assumptions. If differences were found to be significant, one-way ANOVA analysis was followed by post hoc pair-wise multiple comparison analysis using the Tukey Test to determine which treatments were significantly different ($p<0.05$).

5.4 ENCYSTMENT EXPERIMENTS

5.4.1 *GYMNODINIUM CATENATUM* - EFFECT OF TEMPERATURE ON ENCYSTMENT

The effect of temperature on encystment in *G. catenatum* was tested over a temperature range of 5 to 34 °C (at 5, 8, 11, 14, 17, 19, 22, 24, 27, 29, 31, and 34 °C). Strains GCHUN1-97 and GCHUN2-97 were pre-conditioned for crossing in $GSe/_{20}$ for 8 days (at 18 °C).

Strains were then crossed in sterilised polycarbonate culture jars (70 mL Technoplas™) with 2 mL of each strain being added to 25 mL of fresh $GSe/_{20}$ medium. An initial sample of 4 mL was taken from each jar and fixed with Lugol's iodine solution to determine the initial cell concentration (48 cells mL^{-1}; n=10, s.e.=9). Jars were then placed in an insulated solid-cast aluminium block temperature gradient table (6 rows x 12 wells). The table was illuminated from below at approximately 70 µmol photons PAR m^{-2} s^{-1}. The temperature gradient was established by a heating water bath circulating through one end and a cooling bath circulating through the other (Jameson et al. in prep.). Cultures were initially placed towards the centre (14 – 27 °C) of the table to acclimate over 24 hours before being placed at the final temperatures. Each temperature had three replicate crosses. After 41 days resting cysts per jar were counted using a Nikon Diaphot inverted microscope. A 5 mL subsample of each cross was then fixed in Lugol's iodine solution and vegetative cells enumerated using a Sedgewick-Rafter Cell. Percentage encystment was calculated as:

$$\%\ Encystment = \left(\frac{2 \times a\ (resting\ cysts)}{b\ (vegetative\ cells) + 2 \times a\ (resting\ cysts)} \right) \times 100 \qquad \text{(Equation 1)}$$

where '*a* (resting cysts)' is the resting cyst concentration per mL and 'b (vegetative cells)' is the vegetative cell concentration per mL. This formula is based on the assumption that resting cysts are the result of the fusion of two vegetative cells (functional gametes) (Sgrosso et al., 2001).

5.4.2 *ALEXANDRIUM MINUTUM* - EFFECT OF TEMPERATURE ON ENCYSTMENT

The effect of temperature on encystment in *A. minutum* was tested at temperatures of 11, 14, 16, 20, 25 and 28 °C. Strains AMAD06 and AMAD16 were pre-conditioned for crossing in $GSe/_{20}$ for 8 days (at 18°C). Strains were then crossed in sterilised polycarbonate culture jars (70 mL Technoplas™) with 0.5 mL of each strain being added to 25 mL of fresh $GSe/_{20}$ medium. An initial sample of 3 mL was taken from each jar and fixed with Lugol's iodine solution. Two of these were enumerated to determine an approximate initial cell concentration (2062 cells mL^{-1}; n=2, s.e.=32). Jars were then placed in an aluminium temperature gradient table (described above). Cultures were initially placed towards the centre of the table (14 – 22 °C) to acclimate over 24 hours before being placed at their final temperatures. Each temperature had three replicate crosses. After 18 days resting cysts per jar were counted using a Nikon Diaphot inverted microscope. A 5 mL subsample of each cross was then fixed in Lugol's iodine solution and vegetative cells enumerated using a Sedgewick-Rafter Cell. Percentage encystment was calculated as above (Equation 1).

5.4.3 *GYMNODINIUM CATENATUM* - EFFECT OF NUTRIENT CONCENTRATION ON ENCYSTMENT

The effect of initial nutrient concentration on encystment of *G. catenatum* was tested over a range of concentrations of GSe medium. After pre-conditioning of *G. catenatum* strains in $GSe/_{20}$, 2 mL each of GCHU11 and GCDE08 were combined in pre-sterilised 55mm diameter polystyrene petri dishes (Falcon™) with 8 mL of one of 6 treatment media (GSe, $GSe/_{10}$, $GSe/_{20}$, $GSe/_{50}$, $GSe/_{100}$, $GSe/_{1000}$, see Appendix A). All treatments were carried out in triplicate. Temperature, irradiance and photoperiod were as described in Section 5.1. A sub-sample of 2 mL was removed and fixed for initial vegetative cell numbers (246 cells mL^{-1}; n=18, s.e.=39). Resting cysts per dish were enumerated every 4 days until day 19, with a final count on day 34. At the end of the experiment all crosses were fixed for a final count of vegetative cells and resting cysts so that percentage encystment could be calculated (Equation 1).

5.4.4 *ALEXANDRIUM MINUTUM* - EFFECT OF NUTRIENT CONCENTRATION ON ENCYSTMENT

Two experiments were conducted simultaneously to assess the effect of nutrients on *A. minutum* encystment. The first experiment was designed similarly to the *G. catenatum* experiment above (Section 5.4.3). After pre-conditioning of the *A. minutum* strains, 0.5 mL of each of the compatible strains was added to 30 mm diameter polystyrene petri dishes (Falcon™), along with 3.5 mL of 6 different treatment media ($GSe/_{10}$, $GSe/_{20}$, $GSe/_{50}$, $GSe/_{100}$, $GSe/_{1000}$ and GSe^{-N-P} (i.e. without nitrate and phosphate)). All treatments were carried out in triplicate and all petri dishes were placed at approximately 75 µmol photons PAR m^{-2} s^{-1} on a 12:12 light:dark cycle. Resting cysts per dish were enumerated every 4-6 days for 40 days.

The second experiment used to assess the effect of nutrients on encystment monitored both resting cysts and *in situ* fluorescence (as an indicator of vegetative growth). There were 3 duplicated treatments, $GSe/_{20}$, $GSe/_{100}$ and $GSe/_{1000}$. One mL each of the compatible strains was added to polycarbonate jars (70 mL Technoplas™), modified to accommodate a fluorometer tube (Fig. 5.1, Jameson et al., in prep.) along with 30 mL treatment media. Jars were placed at 18 °C with approximately 25µmol photons PAR m^{-2} s^{-1} on a 12:12 light:dark cycle. Resting cysts per jar and fluorescence were monitored every 4-6 days for a period of 40 days. Fluorescence was measured using a Turner Designs Fluorometer (model number 10-005R) by aseptically attaching the fluorometer tube, gently mixing the culture and inverting the culture into the tube. Resting cyst counts were normalised to fluorescence (ie cyst count/fluorescence units). However, changes in fluorescence with physiological changes in cells limited the usefulness of this measure. Non-normalised counts are therefore presented in the results and cell counts were used in subsequent experiments.

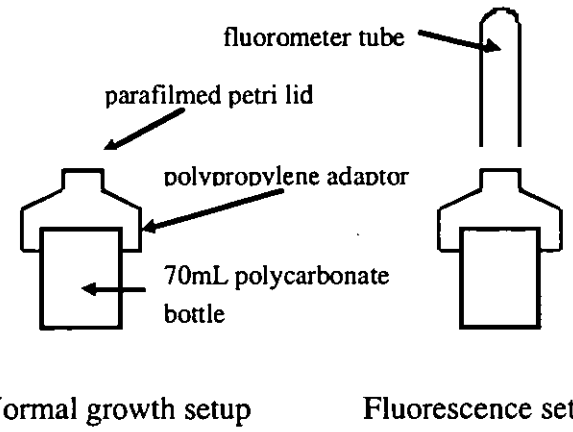

Figure 5.1: Modified jar for fluorescence measurements (reproduced from Jameson et al., in prep.).

5.4.5 *GYMNODINIUM CATENATUM* - EFFECT OF CELL:CELL INTERACTIONS ON ENCYSTMENT

A series of experiments were carried out to determine whether cell:cell interactions play a role in encystment i.e. that compounds produced by *G. catenatum* vegetative cells, (e.g. pheromone-like substances) act as cues or triggers for encystment. Each experiment involved crossing cultured strains in different treatment media made from filtrates of either field populations or stationary phase cultured strains. All filtrates were filtered through a 0.22 µm filter (sterile Millipore Sterivex™ GP 0.22µm filter unit cat. No. SVGP01050) to remove algal and bacterial cells.

Bloom filtrate experiment

The first investigation of whether compounds produced by *G. catenatum* influence encystment used a filtrate from a naturally occurring bloom of *G. catenatum* (integrated water column sample, *G. catenatum* cell concentration of 3.2×10^5 cells L^{-1}), which was actively encysting, as one treatment. The field sample was collected from the Huon Estuary at Brabazon Park – site H3 – on 9/12/1997 (see Chapter 6, Fig. 6.3 for site location). The second treatment in this experiment was a medium made up of filtered seawater and filtered Huon River water (from humic rich fresh waters above Huonville – see Fig. 6.3), to test for any effect of humic addition. The control medium was filtered seawater plus milliQ™ water. All treatments were the same salinity as the field filtrate (20 psu). Three mL of each of strains GCDE08 and GCHU11 were combined with 6 mL of each treatment media in 55mm diameter polystyrene petri dishes (Falcon™) with four replicates of each treatment. Crosses were monitored, and resting cysts enumerated until day 44 when the experiment was terminated as resting cyst excystment began to be observed.

Non-bloom filtrate experiment

The second experiment to investigate cell:cell communication also used a filtrate from a natural population. However, the water sample for the natural filtrate treatment for this experiment was taken from a non-bloom population (integrated water column sample, *G. catenatum* cell density = 1.9 x 10^3 cells L^{-1}; taken from the Huon Estuary at Brabazon Park – site H3 – 6/8/1998, see Chapter 6, Fig. 6.3 for site location). *Gymnodinium catenatum* encystment was undetectable in this population from observations of net samples and a 1-L fixed integrated water column sample. The other treatments were the same as for the previous bloom filtrate experiment, except that they were adjusted to a salinity of 33 psu to emulate the salinity of the non-bloom filtrate. As in the bloom filtrate experiment, 3 mL each of strains GCDE08 and GCHU11 were combined with 6 mL of treatment medium in 55mm diameter polystyrene petri dishes (Falcon™) with four replicates of each treatment. Crosses were monitored, and resting cysts enumerated until day 35 when the experiment was terminated as resting cyst excystment began to be observed.

Culture filtrate experiment 1

To investigate cell:cell interactions in a more controlled manner, filtrates derived from cultured strains were used. In this first experiment the treatments were 1) filtrate of GCDE08, 2) filtrate of GCHU11, 3) filtrate of GCDE08 x GCHU11 (strains were combined for 1 week and sexual reproduction was occurring), 4) $GSe/_{20}$, 5) filtered seawater + milliQ™ water + soil extract and 6) a milliQ™ water + filtered seawater control. All cultures that were used to make filtrates were in stationary phase. For each cross (all treatments were carried out in triplicate) 2 mL of strain GCDE08 and strain GCHU11, which had been pre-conditioned in $GSe/_{20}$ for 8 days, were combined in 55 mm diameter pre-sterilised polystyrene petri dishes (Falcon™) with 2 mL of $GSe/_{20}$ and 5 mL of treatment media. Light conditions were 70 µmol photons PAR m^{-2} s^{-1} on a 12:12 light:dark cycle. Crosses were monitored and resting cysts enumerated until day 21 when resting cyst excystment began to be observed.

Culture filtrate experiment 2

The experimental design for the second culture filtrate experiment was similar to that of the first except that different strains were used for the crosses – GCHUN1-97 and GCHUN2-97. The treatments tested were 1) filtrate of GCHUN1-97, 2) filtrate of GCHUN2-97, 3) filtrate of GCHUN1-97 x GCHUN2-97 (strains were combined for 1 week and sexual reproduction was occurring) and 4) a milliQ + filtered seawater control. Sixteen crosses (4 x 4 replicates) were carried out in 70 mL sterilised polycarbonate jars (Technoplas™) with 2 mL of each strain, 10 mL of $GSe/_{20}$ and 10 mL of treatment medium (4 replicates). An initial 4 mL subsample was taken from each cross and fixed in Lugol's iodine solution. Eight of these

sixteen subsamples (2 from each treatment) were enumerated using a Sedgewick-Rafter Cell to determine an approximate initial cell concentration (38 cells mL^{-1}; n=8, s.e.=4). Crosses were monitored until day 34. After day 34 resting cysts per jar were enumerated using a Nikon Diaphot inverted microscope. A 5 mL subsample of each cross was then fixed in Lugol's iodine solution and vegetative cells enumerated using a Sedgewick-Rafter Cell. Percentage encystment was calculated as in temperature experiments (Equation 1).

5.4.6 *ALEXANDRIUM MINUTUM* - EFFECT OF CELL:CELL INTERACTIONS ON ENCYSTMENT

A culture filtrate experiment similar to the two outlined for *G. catenatum* (Section 5.4.5) was carried out for *A. minutum* to determine whether cell:cell interactions play a role in encystment of this species. Strains AMAD06 and AMAD16 were used. Treatments were 1) filtrate of AMAD06, 2) filtrate of AMAD16, 3) filtrate of AMAD06 x AMAD16 (strains were combined for 1 week and sexual reproduction was occurring), 4) $GSe/_{20}$, 5) Filtered seawater + milliQ™ + soil extract and 6) a milliQ™ + filtered seawater control. All cultures that were used to make filtrates were in stationary phase. For each cross (all treatments were carried out in triplicate) 0.5 mL each of strains AMAD06 and AMAD16, which had been pre-conditioned in $GSe/_{20}$ for 8 days were combined in 30 mm diameter pre-sterilised polystyrene petri dishes (Falcon™) with 4 mL of treatment media. Crosses were monitored and resting cysts enumerated every few weeks until day 94.

5.4.7 *GYMNODINIUM CATENATUM* - EFFECT OF ALGICIDAL BACTERIAL EXUDATES ON ENCYSTMENT

Exudates from three bacterial strains, which were known to have algicidal effects on *G. catenatum* and *A. minutum* vegetative cells (Skerratt, 2001), were tested to see if they had any effect on encystment when added at low concentrations. Bacterial species and strain details are given in Table 5.4. All species were isolated from the Huon Estuary and maintained in marine liquid agar (strains are housed in the Australian Collection of Estuarine Microorganisms - ACEM - University of Tasmania). ACEM1 is known to be highly algicidal (Lovejoy et al., 1998), ACEM21 has rapid swarming capability and is presumed to be associated with algal bloom decline (Skerratt, 2001). ACEM22 was not producing algicidal compounds at the time of the experiment (based on activity against *G. catenatum* vegetative cells; J. Skerratt personal communication) and was chosen to determine whether other non-algicidal bacterial products may affect encystment.

Bacterial cultures were centrifuged and the supernatant filtered through 0.8 and then 0.2 µm filters (Gelman™). Treatments consisted of different volumes of supernatants from the three bacterial cultures (10, 100, 500, and 1000 µL in $1/10^{th}$ marine agar (Difco™)). The number of bacterial cells prior to centrifugation and filtration for a treatment volume of 100µL are

given in Table 5.4 for each bacterial strain. The effect of bacterial cells (strain ACEM1) on encystment was also tested at a volume of 10 µL with both full strength marine agar and $1/10^{th}$ marine agar (both strengths were tested to determine if the high nutrient marine agar was affecting the dinoflagellates). All strain and concentration combinations were duplicated. Controls included sterile bacterial liquid media ($1/10^{th}$ strength) added at volumes of 10, 100, 500, and 1000 µL and a blank in which no media was added.

Strains GCDE08 and GCHU11 were pre-conditioned in $GSe/_{20}$ for 7 days. 2 mL of each strain was then combined with 5 mL of GSe and the different treatments (different concentrations of supernatants, bacterial cells and marine agar) in sterilised 55 mm diameter pre-sterilised petri dishes (Falcon™) (approx. 330 algal cells mL^{-1} initial concentration). Crosses were placed at 18 °C, and approximately 70 µmol photons PAR m^{-2} s^{-1} on a 12:12 light:dark cycle, and monitored for 25 days. On day 25 a sub-sample was taken and fixed in Lugol's iodine solution. Resting cysts and vegetative cells were enumerated and percentage encystment calculated (see Section 5.4.1).

Table 5.4: Equivalent algicidal bacterial concentrations added for encystment and excystment experiments (Skerratt, 2001).

Bacterial Species	Bacterial strain no.	No. of bacterial cells per 100µL (added as either intact cells or as effective number of cells in supernatant)
Pseudoalteromonas sp.	ACEM1	2.6×10^1
Cytophaga lytica	ACEM21	1.5×10^3
Planococcus sp.	ACEM22	6.2×10^4

5.5 EXCYSTMENT EXPERIMENTS

5.5.1 *GYMNODINIUM CATENATUM* - EFFECT OF ALGICIDAL BACTERIAL EXUDATES ON EXCYSTMENT

The influence of three algicidal bacterial strains on excystment of *G. catenatum* resting cysts was tested. *Gymnodinium catenatum* resting cysts were produced by crossing strains GCDE08 and GCHU11. Resting cysts were isolated into a sterile 24 well polystyrene tissue culture plate (Falcon™) using a micropipette, and 5 mL of fresh GSe medium added. Each well had a minimum of 11 viable resting cysts (average = 19 viable resting cysts per well). Supernatants of ACEM1, ACEM21, and ACEM22 (0.2 µm filtered) were added to triplicate wells at two different volumes (100 and 200 µL – see Table 5.4 for corresponding effective number of cells i.e. cell density prior to centrifugation and filtration). ACEM1 was also added as an unfiltered bacterial culture (100µL). Controls of liquid marine agar only (100 and 200 µL) and no treatment, were also tested. The number of viable resting cysts, non-viable resting cysts (cyst wall clearly compromised and contents blackened), and empty

resting cysts (i.e. successfully germinated) were enumerated at the beginning of the experiment and after 8 and 25 days. The proportion of resting cysts which either appeared to be viable or had germinated was calculated as a percentage i.e. (viable + empty) / (non-viable + viable + empty) x 100. This percentage was corrected for any resting cysts that were non-viable or empty at the start of the experiment.

5.5.2 *ALEXANDRIUM MINUTUM* - EFFECT OF ALGICIDAL BACTERIAL EXUDATES ON EXCYSTMENT

The influence of three algicidal bacterial strains on excystment of *A. minutum* resting cysts was tested. *Alexandrium minutum* resting cysts were produced by crossing strains AMAD06 and AMAD16 in sterilised polycarbonate culture jars (70 mL Technoplas™). Once resting cysts were produced, all vegetative cells were removed by gently washing with sterile seawater (28 psu) three times. As resting cysts are surrounded by a mucilaginous sheath, they stick to the surface of the jar, and vegetative cells can be removed without disrupting the resting cysts. 10 mL of fresh GSe medium was then added to each jar. Aliquots of 5 mL $1/10^{th}$ strength marine agar (Difco™) containing bacterial cells of either ACEM1, ACEM21 or ACEM22 (see Table 5.4 for corresponding number of cells) were added to duplicate jars as well as a single marine agar control containing no bacterial cells. Resting cysts were checked for successful excystment after 27 days. One jar from each treatment was then washed three times with sterile seawater (28 psu) and observed for excystment after a further 2 and 5 days to determine whether resting cysts would germinate once the treatment was removed or at least diluted.

5.5.3 *GYMNODINIUM CATENATUM* - EFFECT OF ANAEROBIC CONDITIONS ON EXCYSTMENT

This experiment investigated the influence of anaerobic conditions on the viability and excystment rate of *G. catenatum* resting cysts. *Gymnodinium catenatum* resting cysts were produced by crossing strains GCDE08 and GCHU11 in $GSe/_{20}$ in a 2 L conical flask. Once resting cysts were produced, they were siphoned from the bottom of the flask and placed in 55 mm diameter pre-sterilised polystyrene petri dishes (Falcon™); 4 dishes with 10 mL of the resting cyst preparation (–GSe) and 4 dishes with 8 mL of the resting cyst preparation combined with 4 mL of full strength GSe medium (+GSe). Each dish contained several thousand resting cysts. The initial number of germinated resting cysts (empty resting cysts) was enumerated in all dishes and six dishes placed in an anaerobic chamber (BBL GasPak™ System anaerobic chamber with GasPak™ Plus anaerobic system envelopes with Palladium catalyst (H_2 and CO_2)). The remaining 2 dishes were placed under normal growth conditions (Section 5.1). After 7 days, 2 of the dishes (one +GSe and one –GSe dish) were removed from the chamber and placed under normal growth conditions, another 2 after 14 days and the last 2 after 28 days in the anaerobic chamber. All transfers were performed under

anaerobic conditions in a N_2 filled glove bag. Once they had been removed from the chamber the number of germinated resting cysts was enumerated weekly until day 51 when the experiment was terminated.

5.5.4 *ALEXANDRIUM MINUTUM* - EFFECT OF LIGHT AND TEMPERATURE ON EXCYSTMENT

Two experiments were carried out to test the effect of temperature on excystment in *A. minutum*. The first experiment also tested some effects of light. *Alexandrium minutum* resting cysts were produced by crossing strains AMAD11 and AMAD12 in 60 mm diameter sterilised glass petri dishes (Schott™). After 2 weeks, resting cysts were isolated using a micropipette and Teflon scraper into 12 wells (approximately 30 resting cysts per well) of sterile 24-well polystyrene tissue culture plates (Falcon™) and 5 mL of GSe added. Plates were then placed under various combinations of light and temperature; 2 plates were placed at 4°C, one in the dark and one under very low light (2 µmol photons PAR m^{-2} s^{-1}); 4 plates were placed at 18 °C, one in the dark and three at 80 µmol photons PAR m^{-2} s^{-1} on a 12:12 light:dark cycle; 1 plate was placed at 25°C under dim blue light at approximately 20 µmol photons PAR m^{-2} s^{-1}. All plates were left for 3 months and then checked for any evidence of resting cyst excystment. All plates were then moved to normal growth conditions (see Section 5.1) to determine whether those resting cysts that had not germinated were still viable. All plates were checked for excystment and motile cells after 1, 2 and 4 weeks in normal growth conditions.

In the second experiment to test the effect of temperature on excystment of *A. minutum*, a temperature gradient table was used (as described in Section 5.4.1). *Alexandrium minutum* cysts were produced by crossing strains AMAD06 and AMAD16 in 29 sterilised polycarbonate culture jars (70 mL Technoplas™). Once resting cysts were produced, all vegetative cells were removed by gently washing three times with sterile seawater (GSe salinity of 28 psu), leaving the resting cysts, which are surrounded by a mucilaginous sheath, adhered to the bottom of the jar. Fresh GSe (25 mL) was then added to all jars. Initial resting cyst densities were counted and averaged 8700 resting cysts per jar. Jars were then placed in the aluminium temperature gradient table. Cultures were initially placed towards the centre of the table (14 – 27 °C) to acclimate over 24 hours before being placed at their final temperatures (5, 8, 15, 18, 20, 23, 26, 28, 31, and 36 °C). Each temperature had at least 2 replicate jars. Due to the high number of resting cysts and the patchiness of distribution in the jars, the error in counting resting cysts was too high to assess percentage resting cyst excystment. Jars were observed every 1 to 2 days for 3 weeks for evidence of excystment and then checked once more after 2 months. After 2 months in the temperature gradient

table any jars in which no resting cyst excystment had been observed were transferred to normal growth conditions (Section 5.1) to see if resting cysts were still viable.

RESULTS

5.6 ENCYSTMENT EXPERIMENTS

5.6.1 *GYMNODINIUM CATENATUM* - EFFECT OF TEMPERATURE ON ENCYSTMENT

Increased temperature resulted in increased encystment of *G. catenatum* up to 27 °C (Fig. 5.2). Above 27 °C vegetative cells died and no resting cysts were produced, while at 11 °C no resting cysts were formed although vegetative growth occurred. At 5 and 8 °C, living vegetative cells were found but in very low numbers and no resting cysts were present. Temperature was found to have a significant effect on vegetative cell yield ($F_{11,24}$=103.3, p<0.001), resting cyst yield ($F_{11,24}$=5.1, p<0.001) and percentage encystment ($F_{11,24}$=6.8, p<0.001) after 41 days. The greatest encystment was observed at 27 °C, although percentage encystment was not significantly different between 20 and 27 °C. In contrast, the optimum temperature for vegetative biomass was approximately 20 °C.

5.6.2 *ALEXANDRIUM MINUTUM* - EFFECT OF TEMPERATURE ON ENCYSTMENT

An optimal encystment temperature of 20 °C was identified for *A. minutum* with both higher and lower temperatures yielding fewer resting cysts (Fig. 5.3). Maximum percentage encystment was relatively high (8%) compared with *G. catenatum* (0.6 %). Temperature was found to have a significant effect on the vegetative cell yield, ($F_{5,12}$=7.7, p=0.002), resting cyst yield ($F_{5,12}$=34.6, p=<0.001) and percentage encystment ($F_{5,12}$=38.4, p=<0.001) after 18 days. The optimum temperature range for vegetative growth was relatively broad with no significant difference in vegetative cell yield between 14 and 20 °C. The greatest encystment was observed at 20 °C and this was high enough (~700 resting cysts mL^{-1}) to be reflected in a dip in the number of vegetative cells mL^{-1}.

5.6.3 *GYMNODINIUM CATENATUM* - EFFECT OF NUTRIENTS ON ENCYSTMENT

Nutrient concentration was found to have a significant effect on vegetative cell yield of *G. catenatum* with the highest nutrient concentration (full strength GSe) having the greatest cell yield ($F_{5,12}$=50.4, p=<0.001) (Fig. 5.4). Resting cysts were produced in all treatments and there was no significant difference in resting cysts mL^{-1} between treatments (Fig. 5.4, $F_{5,12}$=1.7, p=0.218) on day 34. The only significant difference in percentage encystment was found between GSe and $GSe/_{100}$, which had the lowest and highest percentage encystment respectively ($F_{5,12}$=5.3, p=0.008) (Fig. 5.4), although there was a slight trend towards higher encystment at lower nutrient concentrations.

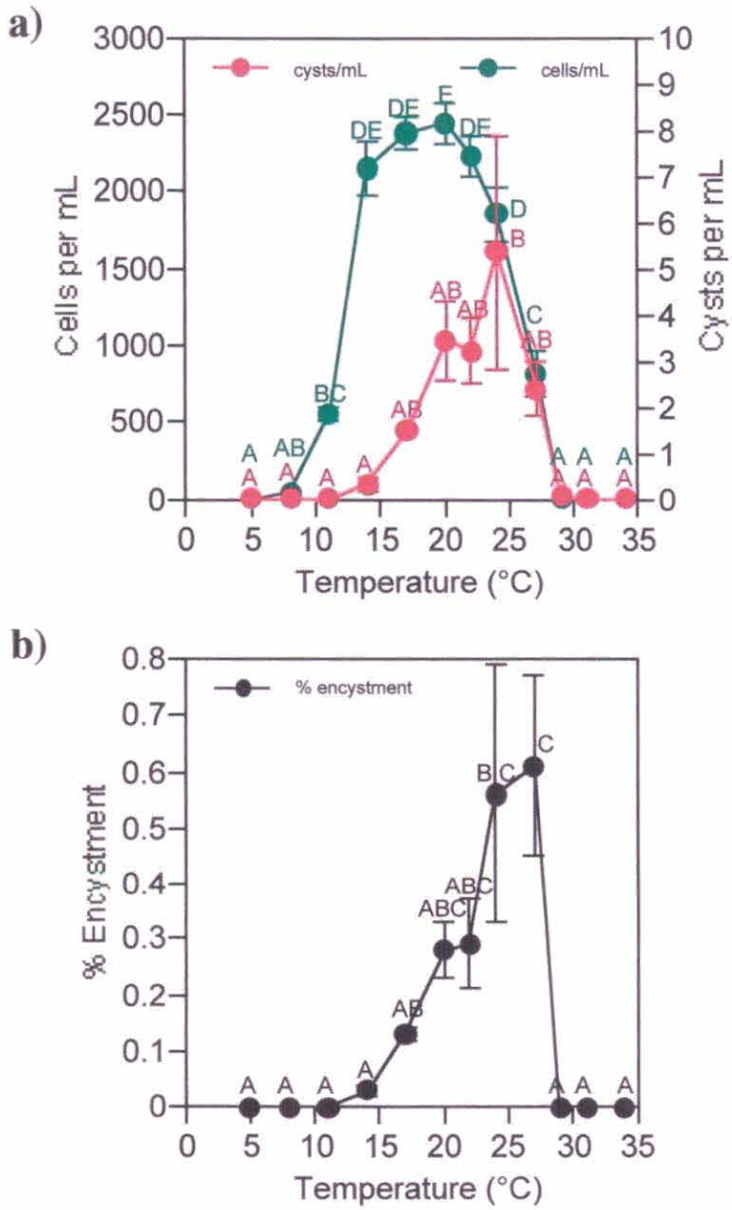

Figure 5.2: *Gymnodinium catenatum* - After 41 days of incubation at different temperatures a) the production of vegetative cells and resting cysts mL^{-1} and b) % encystment (mean ± standard error, n=3). Treatments marked with the same letter were not significantly different from one another ($p>0.05$, Tukey Test).

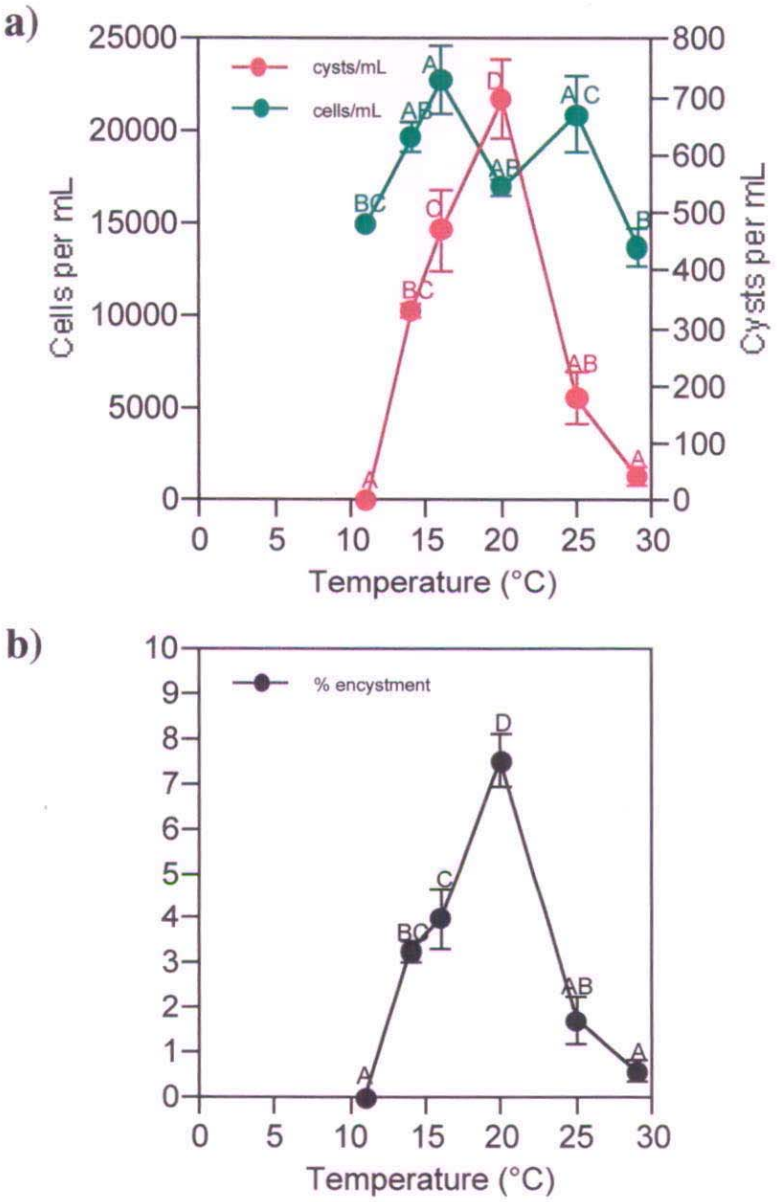

Figure 5.3: *Alexandrium minutum* - After 18 days of incubation at different temperatures a) the production of vegetative cells and resting cysts mL^{-1} and b) % encystment (mean ± standard error, n=3). Treatments marked with the same letter were found to be not significantly different from one another
($p>0.05$, Tukey Test).

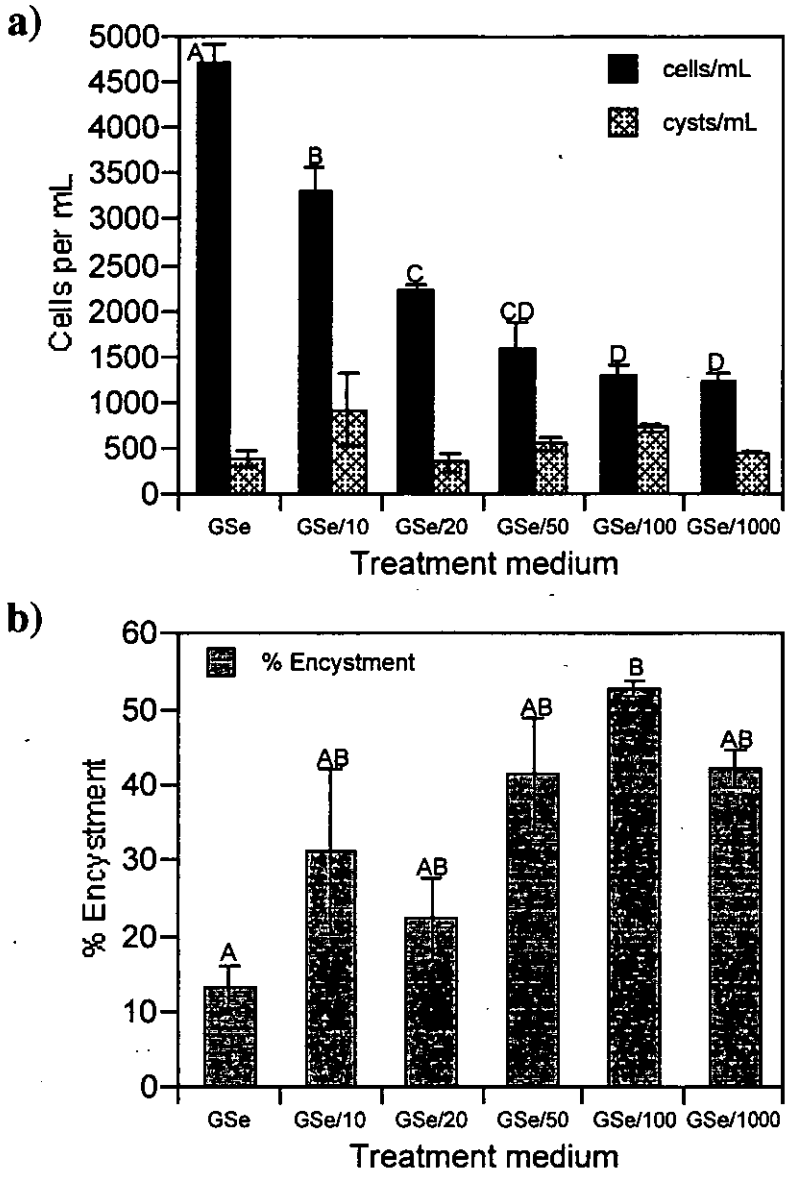

Figure 5.4: *Gymnodinium catenatum* - The effect of nutrient concentration after 34 days incubation on a) production of vegetative cells and resting cysts mL^{-1} and b) % encystment (mean ± standard error, n=3). Treatments marked with the same letter were found to be not significantly different from one another
($p>0.05$, Tukey Test).

5.6.4 *ALEXANDRIUM MINUTUM* - EFFECT OF NUTRIENTS ON ENCYSTMENT

In the nutrient experiment conducted under higher light conditions (75 µmol photons PAR m^{-2} s^{-1}), the greater nutrient concentrations ($GSe/_{10}$ and $GSe/_{20}$) produced very few resting cysts, significantly fewer than the lower nutrient concentrations, which produced large numbers of resting cysts ($F_{5,12}$=11.7, p=<0.001) (Fig. 5.5). This indicates that under these conditions encystment was enhanced by nutrient depletion although the $GSe/_{1000}$ treatment produced significantly fewer resting cysts than the $GSe/_{50}$ treatment, potentially due to lower cell densities although cells mL^{-1} were not enumerated in this experiment.

In the second experiment, under lower light conditions (25 µmol photons PAR m^{-2} s^{-1}) there were significantly greater numbers of resting cysts produced at the greater nutrient concentrations than at the lower nutrient concentrations ($F_{2,3}$=11.075, p=0.041) (Fig. 5.6). That is, in this experiment the number of resting cysts produced reflected the difference in biomass of the treatments (determined by fluorescence) and encystment was enhanced by greater nutrient concentrations.

5.6.5 *GYMNODINIUM CATENATUM* - EFFECT OF CELL:CELL INTERACTIONS ON ENCYSTMENT

***Gymnodinium catenatum* Bloom and Non–bloom Filtrate Experiments**

Encystment of *G. catenatum* (GCDE08 x GCHU11) was significantly greater in the presence of the *G. catenatum* bloom filtrate than in both of the other treatments ($F_{2,9}$=49.9, p=<0.001) (Fig. 5.7). The second most abundant resting cyst producer was the filtered seawater + milliQ treatment while the filtered seawater + filtered river water treatment produced the fewest numbers of resting cysts. This result indicates that some unidentified component of the bloom filtrate, potentially produced by *G. catenatum* cells, enhanced or activated encystment.

In contrast, when the non-bloom filtrate (few *G. catenatum* cells in the water and no detectable encystment) was added to the same cross (GCDE08 x GCHU11), the filtrate did not enhance encystment and had the lowest resting cyst production of the three treatments investigated (Fig. 5.8) although differences between treatments were not significant ($F_{2,9}$=0.523, p=0.609). Coupled with the previous experiment these results support the suggestion that the factor enhancing encystment in the first experiment was produced by *G. catenatum* under bloom conditions.

There was an order of magnitude difference between the number of resting cysts produced in the first experiment and those produced in the second experiment, this was probably due to a similar difference in the initial cell density of the two experiments.

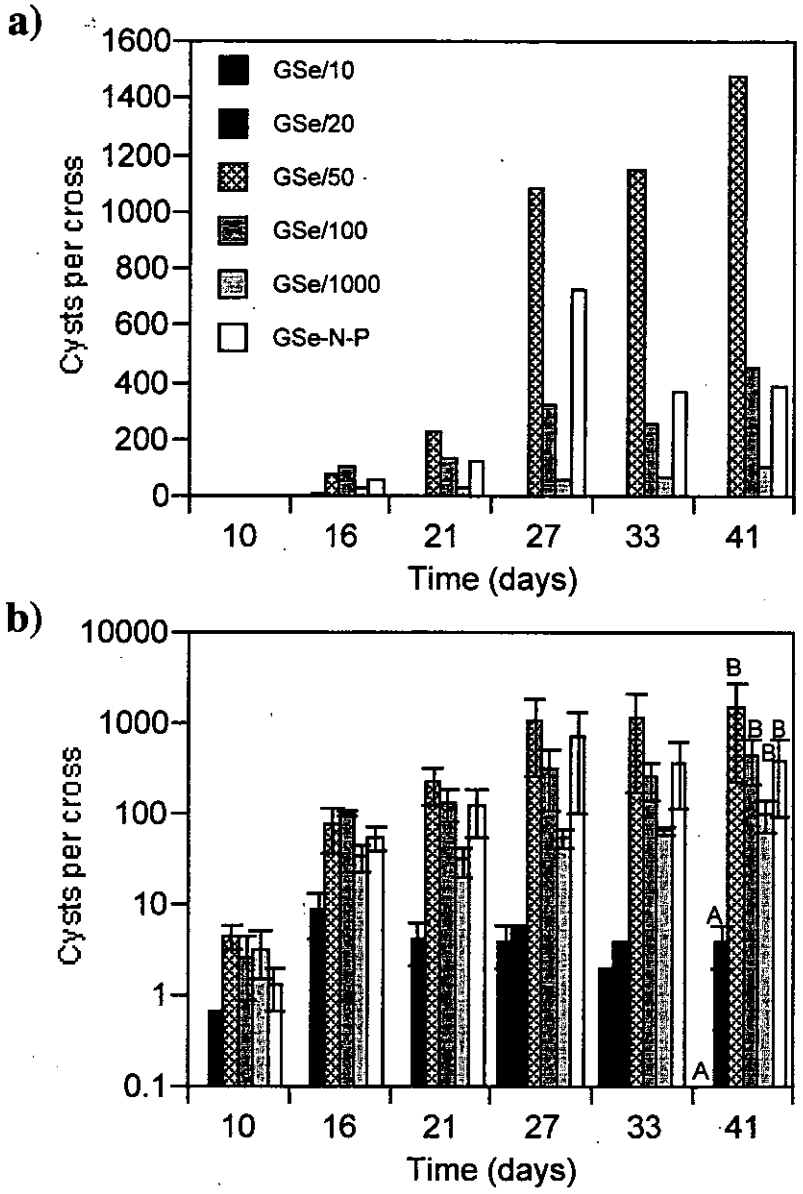

Figure 5.5: *Alexandrium minutum* - The effect of nutrient concentration on encystment under higher light (75 µmol photons PAR m^{-2} s^{-1}). a) normal scale b) log plot (mean ± standard error, n=3). Treatments marked with the same letter were found to be not significantly different from one another ($p>0.05$, Tukey Test).

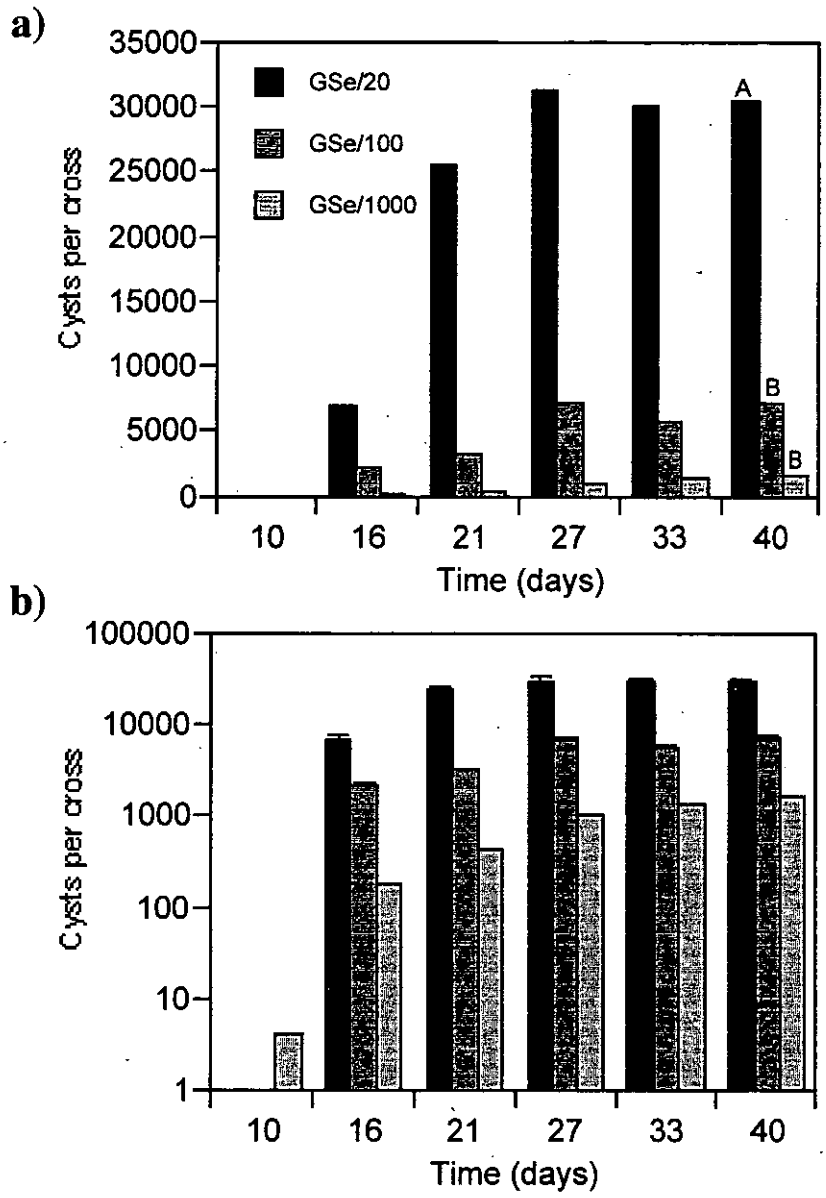

Figure 5.6: *Alexandrium minutum* - The effect of nutrient concentration on encystment of *Alexandrium minutum* under lower light (25 µmol photons PAR m^{-2} s^{-1}). a) normal and b) log scale. (mean ± standard error, n=2). Treatments marked with the same letter were found to be not significantly different from one another ($p>0.05$, Tukey Test).

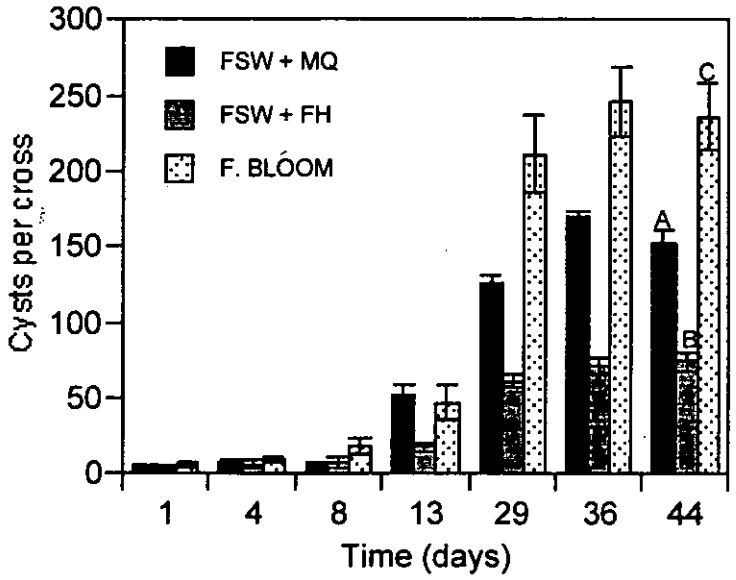

Figure 5.7: The effect of different filtrates on encystment of *Gymnodinium catenatum* (mean ± standard error, n=4). FSW+MQ = a 20 psu seawater control, FSW+FH = filtered seawater with added humic rich river water, and F. BLOOM = filtered water from an actively encysting bloom population of *Gymnodinium catenatum*. Treatments marked with the same letter were found to be not significantly different from one another ($p>0.05$, Tukey Test).

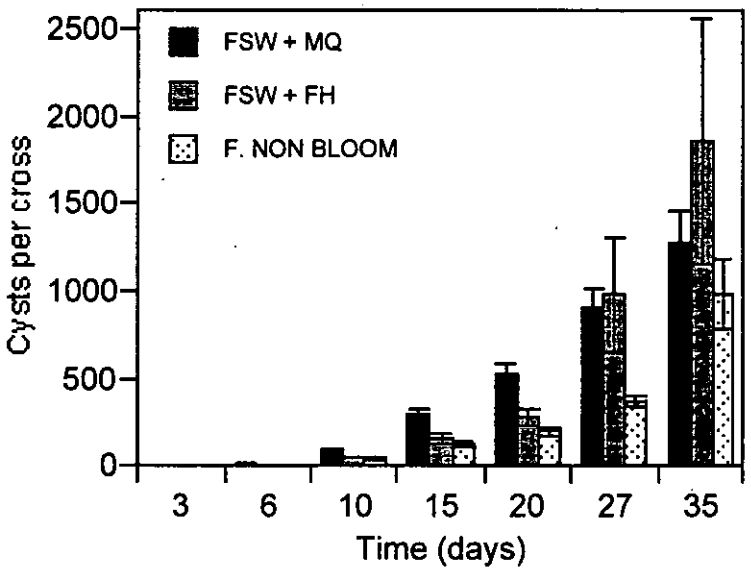

Figure 5.8: The effect of different filtrates on encystment of *Gymnodinium catenatum* (mean ± standard error, n=4). FSW+MQ = a 33 psu seawater control, FSW+FH = filtered seawater with added humic rich river water, and F. NON BLOOM = filtered water from a low biomass field population containing some *Gymnodinium catenatum* cells in which encystment was not occurring. Differences between treatments were not significant ($p>0.05$, Tukey Test).

***Gymnodinium catenatum* culture filtrate experiments**

The addition of filtrates from old cultures of either of the *G. catenatum* strains GCDE08 and GCHU11 or from a cross of those strains did not have a significant effect on encystment of GCDE08 x GCHU11 crosses ($F_{5,12}$=0.7, p=0.652) (Fig. 5.9). There were also no significant differences in encystment between any of the other treatments.

In contrast to the first culture filtrate experiment, a significant effect of the filtrate on percentage encystment was identified when the experiment was repeated using strains GCHUN1-97 and GCHUN2-97 ($F_{3,12}$=5.4, p=0.014) (Fig. 5.10). The filtrate of GCHUN1-97 (F.HUN1), while not significantly different to the milliQ+seawater control, produced a greater number of resting cysts than either the GCHUN2-97 filtrate (F.HUN2) or the cross filtrate (F.HUN1xHUN2).

5.6.6 *ALEXANDRIUM MINUTUM* - EFFECT OF CELL:CELL INTERACTIONS ON ENCYSTMENT

There was a significant effect by one of the culture filtrates on encystment of *A. minutum* (Fig. 5.11). The filtrate of strain AMAD06 had a significant positive effect on encystment relative to the other treatments ($F_{5,12}$=17.2, p<0.001). All other treatments were not significantly different from one another.

5.6.7 *GYMNODINIUM CATENATUM* - EFFECT OF ALGICIDAL BACTERIA ON ENCYSTMENT

The presence of supernatants of the algicidal bacteria ACEM1, ACEM21 and ACEM22 did not have a significant effect on encystment of *G. catenatum* after 25 days incubation (Fig. 5.12). There was also no significant effect on encystment due to the presence of bacterial cells of ACEM1. Supernatant volumes of 500 and 1000 µL of all strains as well as the marine agar controls resulted in vegetative cell lysis. Vegetative cells also lysed when ACEM1 cells in $1/10^{th}$ marine agar were added. Vegetative growth was significantly lower in the presence of ACEM1 supernatant at the 10 and 100µL concentrations compared with ACEM22 (p<0.05) but not significantly different to ACEM21. Resting cysts were produced in all crosses with either 10 or 100 µL of supernatant or marine agar added, and percentage encystment was not significantly different between the bacterial strains or between the concentrations of supernatant. There was also no apparent difference between the treatments and the marine agar controls or the controls without addition of supernatant or marine agar.

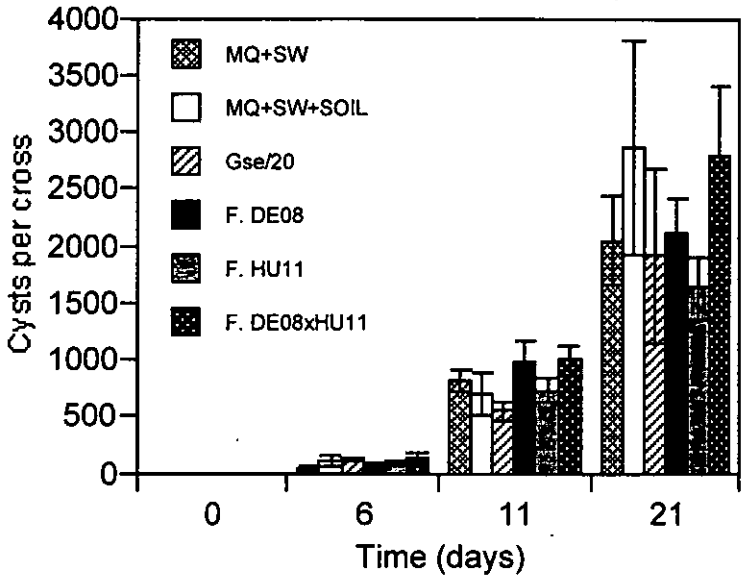

Figure 5.9: *Gymnodinium catenatum* - The effect of different culture derived filtrates on encystment (mean ± standard error). FSW+MQ = a 28 psu seawater control, FSW+MQ+soil = filtered seawater with added soil extract, F. DE08 and F. HU11 are filtrates from cultures of these two strains and F. DE08xHU11 is a filtrate from a cross of the two strains. Differences between treatments were not significant ($p>0.05$, Tukey test).

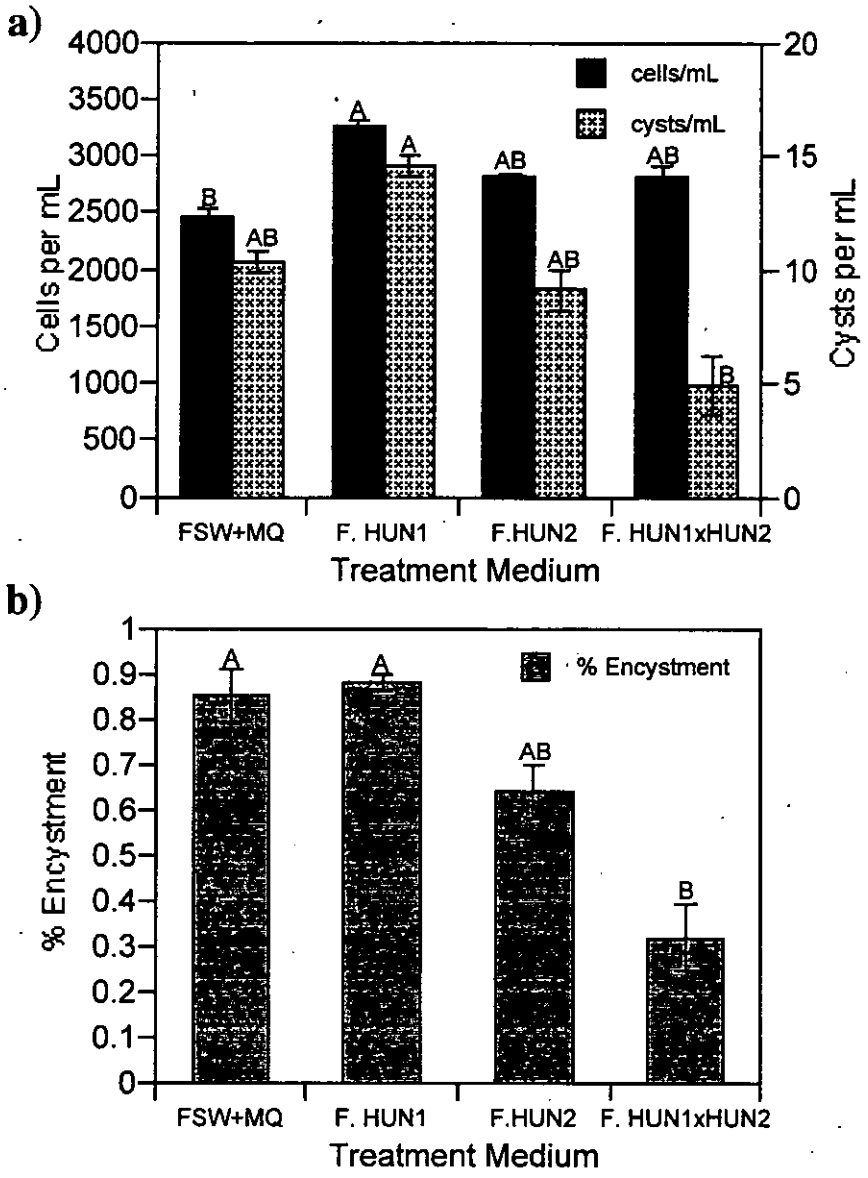

Figure 5.10: *Gymnodinium catenatum* - The effect of different culture derived filtrates after 34 days incubation on a) production of vegetative cells and resting cysts mL^{-1} and b) % encystment of: FSW+MQ = a 28 psu seawater control, F. HUN1 and F. HUN2 are filtrates from cultures of the strains GCHUN1-97 and GCHUN2-97 respectively and F. HUN1xHUN2 is the filtrate from a cross of the two strains (mean ± standard error). Treatments with the same letter were found to be not significantly different from one another ($p>0.05$, Tukey Test).

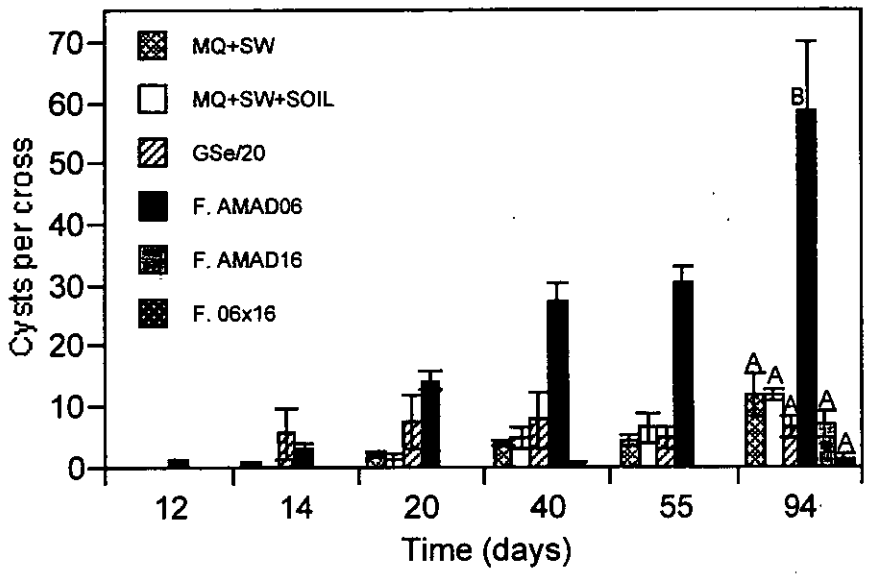

Figure 5.11: The effect of different culture derived filtrates on encystment of *Alexandrium minutum* (mean ± standard error). FSW+MQ = a 28 psu seawater control, FSW+MQ+soil = filtered seawater with added soil extract, F. AMAD06 and F. AMAD16 are filtrates from cultures of these two strains and F. 06x16 is a filtrate from a cross of the two strains. Treatments with the same letter were found to be not significantly different from one another ($p>0.05$, Tukey Test).

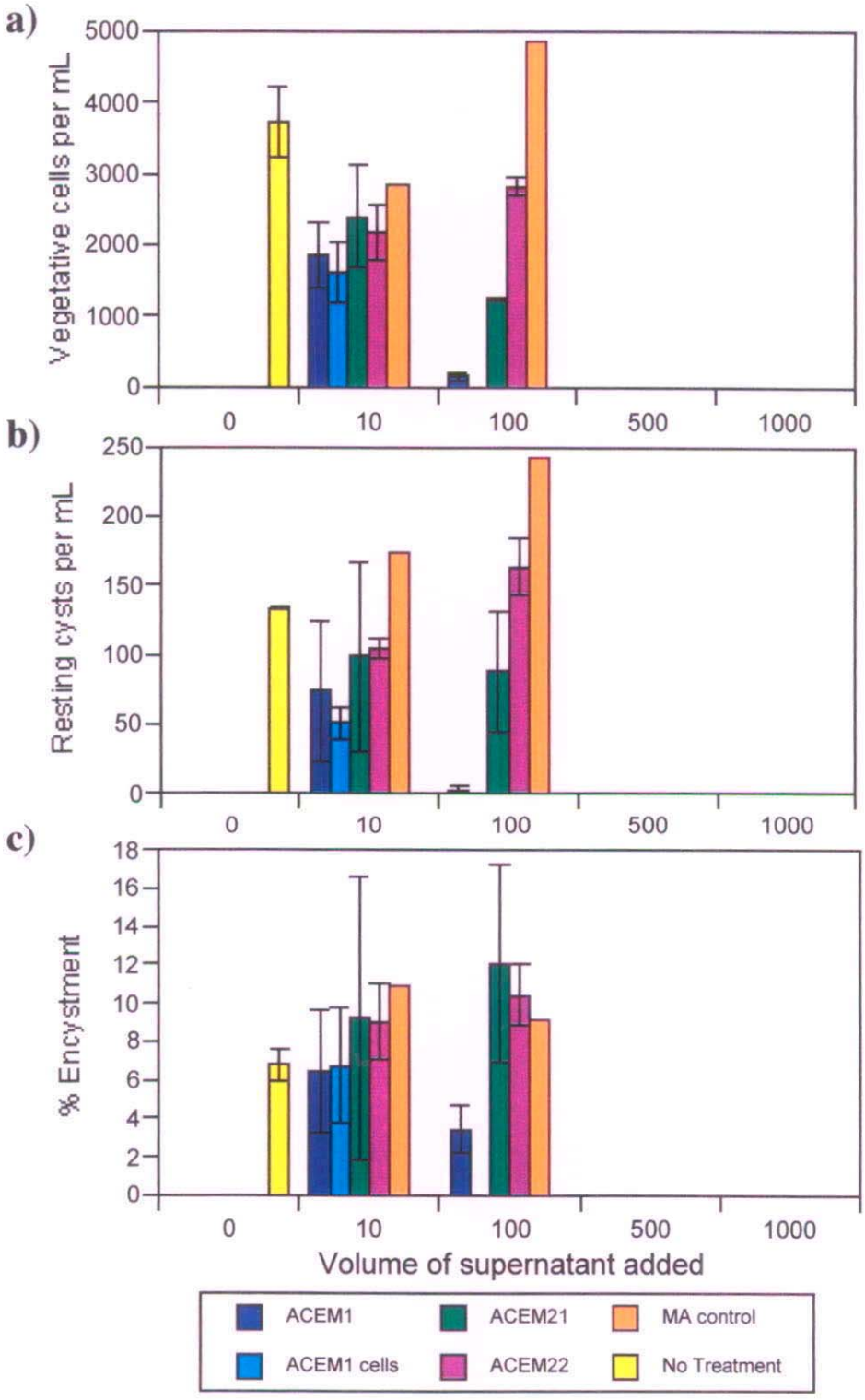

Figure 5.12: The effect of algicidal bacterial exudates of different species and supernatant volumes (in µL) on a) vegetative cells mL^{-1} b) resting cysts mL^{-1} and c) % encystment of *Gymnodinium catenatum* (mean ± standard error) after 25 days incubation. MA =1/10 marine agar. Treatments without error bars were unreplicated. Cell lysis occurred after the addition of 500 or 1000 µL of all bacterial supernatants and the marine agar controls.

5.7 EXCYSTMENT EXPERIMENTS

5.7.1 *GYMNODINIUM CATENATUM* - EFFECT OF ALGICIDAL BACTERIAL EXUDATES ON EXCYSTMENT

There was no detectable effect on either viability or excystment of *G. catenatum* resting cysts by any of the algicidal bacterial exudates or cells added (Fig. 5.13). There were no significant differences between treatments and controls on either day 8 or day 25 ($F_{6,14}$= 1.0, p=0.444; $F_{6,14}$= 0.4, p=0.892 respectively). In all treatments and controls, some resting cysts successfully germinated and some viable resting cysts remained after 25 days.

5.7.2 *ALEXANDRIUM MINUTUM* - EFFECT OF ALGICIDAL BACTERIA ON EXCYSTMENT

Qualitative observations suggest that cysts of *A. minutum* were not affected by addition of any of the bacterial strains. In all cases the resting cysts appeared viable with many successfully excysting after 27 days. In the ACEM1 treatment, once resting cysts had excysted, all observed germlings (presumed planomeiocytes) lysed. In the ACEM21 and ACEM22 treatments, and in the control, vegetative growth after excystment was successful. Germlings exposed to ACEM1 presumably lysed due to the continuing presence of the algicidal exudates. On day 29, two days after re-washing of one replicate from each treatment there was no evidence of excystment observed in any treatment. However, five days after re-washing ACEM21, ACEM22 and the control showed successful excystment and vegetative growth. The washed ACEM1 treatment replicate also showed evidence of successful excystment and vegetative growth although there appeared to be less vegetative cells than in the other treatments.

5.7.3 *GYMNODINIUM CATENATUM* - EFFECT OF ANAEROBIC CONDITIONS ON EXCYSTMENT

Exposure to anaerobic conditions inhibited excystment of *G. catenatum* resting cysts (Fig. 5.14a) with no detectable difference between nutrient deplete and nutrient replete conditions (+GSe and -GSe results were therefore pooled for further analysis). Anaerobic treatments had a significant effect on both the rate of excystment after removal from anaerobic conditions ($F_{3,4}$=91.857, p<0.001; Fig. 5.14b) and the total number of germinated cysts on day 51 at the termination of the experiment ($F_{3,4}$=18.123, p=0.009; Fig. 5.14a). Resting cysts that had not been placed in anaerobic conditions began to germinate immediately at a rate of 0.14 d^{-1} (Fig. 5.14b). Excystment of resting cysts placed in anaerobic conditions for 1 and 2 weeks was very low, but increased once returned to aerobic conditions (Fig. 5.14a). There was also very low excystment of resting cysts during the 4-week anaerobic treatment. However, once returned to oxygenated conditions, the rate of excystment in the 4 week treatment (0.28 d^{-1}) was significantly greater than all other treatments (p<0.05). Due to the higher excystment rate, after 3 weeks in oxygenated conditions the number of resting cysts

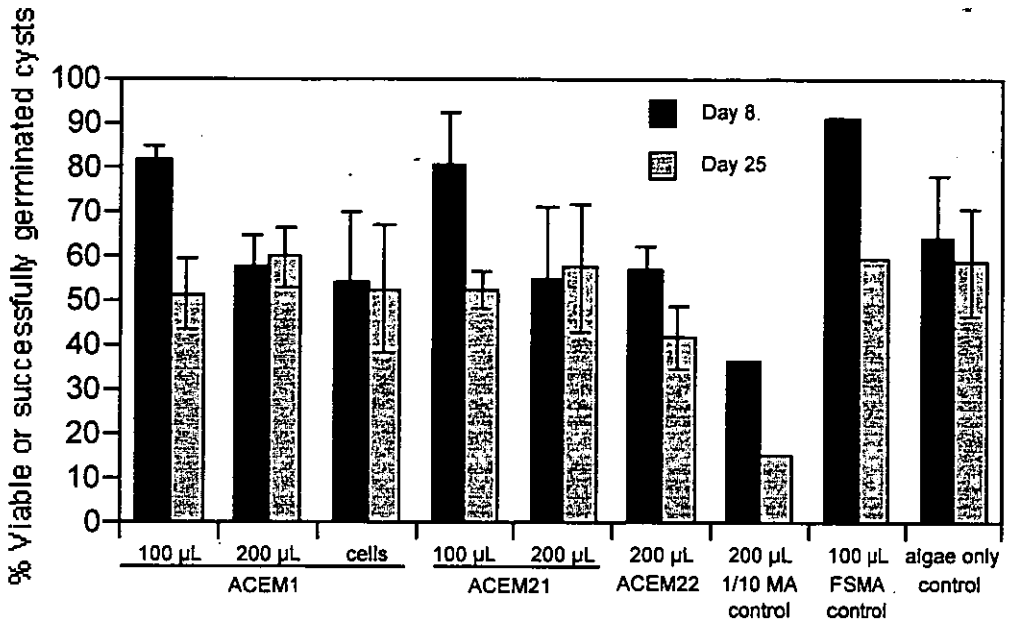

Figure 5.13: *Gymnodinium catenatum* - The effect of algicidal bacterial exudates of different bacterial species at different concentrations on the viability and germination of *Gymnodinium catenatum* resting cysts (mean ± standard error). ACEM1= *Pseudoalteromonas* sp., ACEM21= *Cytophaga lytica*, ACEM22= *Planococcus* sp. FSMA= full strength marine agar, MA 1/10 =1/10 marine agar. Differences between treatments were not significantly different ($p>0.05$ Tukey Test). Treatments without standard error bars were unreplicated.

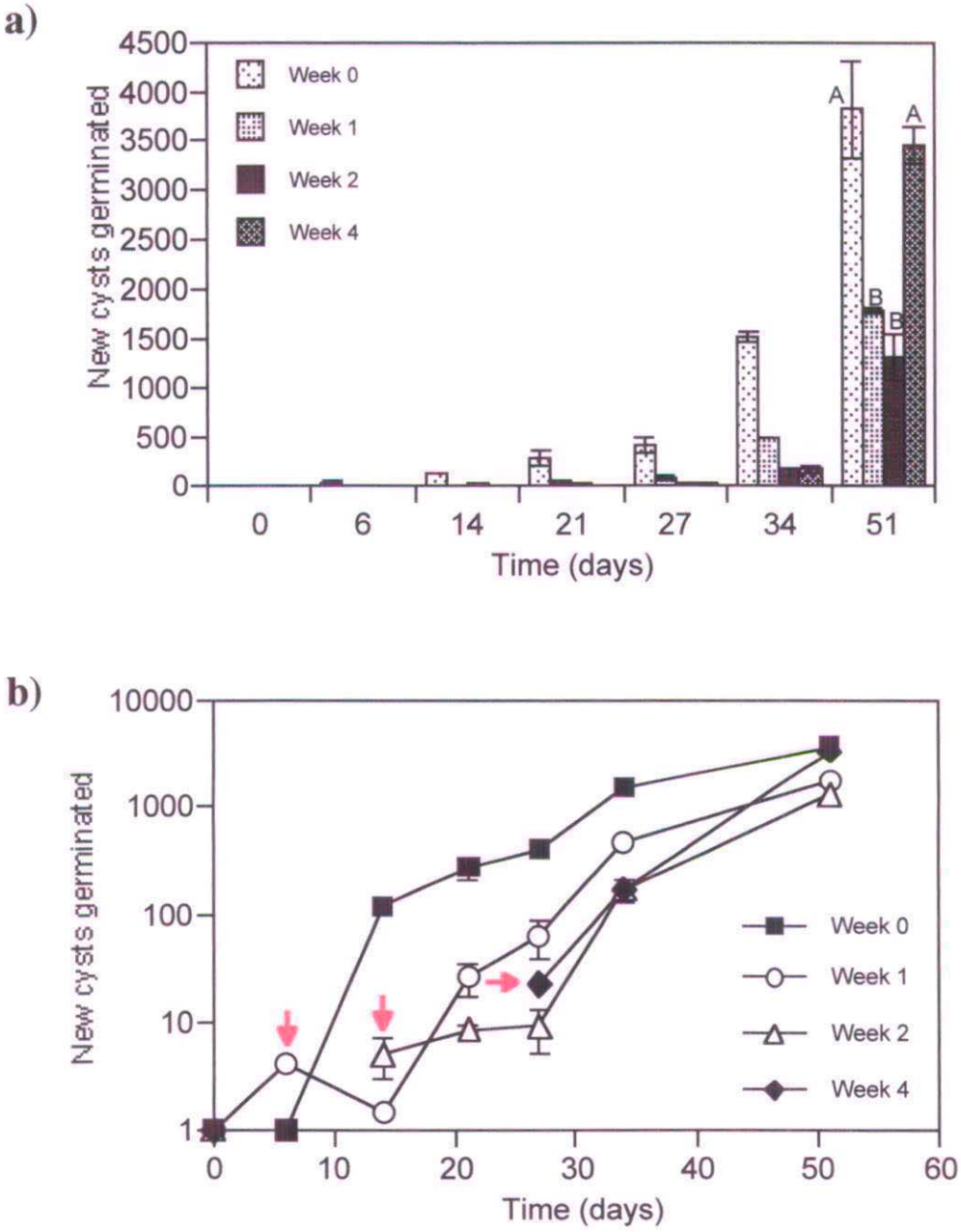

Figure 5.14: The effect of different periods of time (0, 1, 2, and 4 weeks) under anaerobic conditions on excystment of *Gymnodinium catenatum* a) (mean ± standard error, n=2). Treatments with the same letter were not significantly different from one another on day 51 ($p>0.05$, Tukey Test). b) red arrow indicates point at which treatment was removed from anaerobic conditions.

germinated in the 4 week anaerobic treatment was not significantly different to the 0 week treatment (p=0.812). Although the 1 and 2 week treatments had significantly lower excystment rates (0.20 and 0.22 d^{-1} respectively) than the 4 week treatment their excystment rates were significantly higher than the 0 week treatment. These results suggest that time spent in anaerobic conditions increased the rate of excystment once returned to aerobic conditions, and also that the greater the period of time (at least up to 4 weeks) the greater the effect.

5.7.4 *ALEXANDRIUM MINUTUM* - EFFECT OF LIGHT AND TEMPERATURE ON EXCYSTMENT

Light and temperature conditions were found to influence resting cyst excystment in *A. minutum* (Table 5.5). After three months under different light and temperature conditions, the three plates containing resting cysts that were placed under normal growth conditions (18 °C, 80 µmol photons PAR m^{-2} s^{-1}) all showed evidence of excystment, with empty resting cysts, vegetative growth and observed encystment in all 36 wells. Resting cysts in all other plates (4 °C in the dark, 4°C in dim light (2 µmol photons PAR m^{-2} s^{-1}), 18°C in the dark, and 25°C in blue light (20 µmol photons PAR m^{-2} s^{-1})) showed no evidence of excystment with intact resting cysts still present after the three month period in all cases (Table 5.5). One week after return to normal growth conditions, plates that had been at 4 °C in the dark and dim light contained motile vegetative cells resulting from resting cyst excystment in some wells (Table 5.5). The plate that had been in the dark at 18 °C for three months also contained motile vegetative cells after return to normal growth conditions for 1 week. There was no evidence of excystment in the plate that had been at 25 °C in blue light during the first week after return to normal growth conditions. After two weeks in normal growth conditions all plates showed evidence of excystment, including a high proportion of resting cyst excystment in all twelve wells of the 25 °C/blue light plate. Further excystment also occurred in the 4 °C dim light plate (Table 5.5).

Table 5.5: Results of temperature/light experiment on *A. minutum* excystment.

Treatment	Wells containing germinated cysts after 3 months at treatment conditions	Wells containing germinated cysts after return to normal growth conditions		
		1 week	2 weeks	4 weeks
4 °C dark (1 plate)	0/12	11/12	12/12	12/12
4 °C dim light (2) (1 plate)	0/12	2/12	7/12	10/12
18 °C dark (1 plate)	0/12	4/12	12/12	12/12
18°C light (80) (3 plates)	36/36	36/36	36/36	36/36
25 °C blue light (20) (1 plate)	0/12	0/12	12/12	12/12

In summary, darkness, low temperatures (4 °C) and high temperatures (25 °C) inhibited the excystment of *G. catenatum* resting cysts, but once transferred to the normal growth conditions it could be seen that these resting cysts were still viable. Whether the apparent high proportion of excystment of resting cysts which had been held at 25 °C in blue light was due to light quality or temperature was not determined. Excystment of resting cysts during the first two weeks in normal growth conditions was significantly greater ($p<0.05$, Fisher Exact Probability Test) in the plate that had been kept at 4 °C in complete darkness than in the one that had been in dim light at 4 °C (Table 5.5). These results suggest that storage in complete darkness may initially result in greater excystment success than storage in dim light.

Qualitative results from the temperature gradient experiment indicate that *A. minutum* can excyst at a wide variety of temperatures (Table 5.6). The first evidence of excystment occurred after 3 days at 20, 23 and 28 °C, after 4 days at 26 °C, after 6 days at 31°C, and after 7 days at 15 °C (very low excystment at 15 and 31 °C). After 10 days, many germinated resting cysts were observed at temperatures between 18 and 31 °C. One germling was seen in one of the 8°C treatment replicates after 18 days and after 2 months a large number of resting cysts had germinated at this temperature. No excystment was ever observed at 5 °C or 36 °C. When the 5 and 36 °C jars were placed under normal growth conditions, excystment was observed in the 5 °C jars but not in the 36 °C jar, suggesting that *A. minutum* resting cysts die at 36 °C.

Table 5.6: Results of temperature effect on Excystment of *A. minutum*

Temperature (°C)	Time until first observed excystment (days)	Comments
5	-	Excystment never observed but cysts viable
8	18	Only one germling observed after 18 days but many germinated cysts after 2 months
15	7	Low excystment initially but many germinated cysts after 18 days
18	8	Extensive excystment
20	3	Extensive excystment
23	3	Extensive excystment
26	4	Extensive excystment
28	3	Extensive excystment
31	6	Low excystment initially but many germinated cysts after 10 days
36	-	Excystment never observed and cysts non viable

DISCUSSION

5.8 SUMMARY OF ENCYSTMENT/EXCYSTMENT CUES INVESTIGATED

In this chapter, various environmental and endogenous factors were investigated for their influence on the encystment and excystment of *A. minutum* and *G. catenatum*. The results of this study along with previous studies of encystment and excystment in these two species are summarised in Table 5.7. Previous studies are briefly outlined below before discussing the findings of this study in detail.

Both *A. minutum* and *G. catenatum* have primarily heterothallic mating systems (Chapter 3 and Blackburn et al., 1989, 2001), i.e. sexual reproduction generally does not occur within clones, with compatible mating types being required for resting cyst formation. There is only one published study of encystment in *A. minutum* (Probert et al., 1998) and one of excystment (Cannon, 1993). The previous encystment study investigated the potential role of internal nutrient pools in encystment, and suggested that internal nitrogen depletion may be an encystment trigger, however, although planozygote formation was observed in this study resting cyst formation was not and so the results were inconclusive (Probert et al., 1998). Cannon (1993) studied the effects of different salinity, temperature, nutrient and light regimes on the excystment of *A. minutum* resting cysts collected from sediments in the Port River Estuary in South Australia. Cannon (1993) concluded that nutrients did not affect excystment and that the most favourable conditions for excystment were a temperature of 16 °C, salinity between 14 and 21 psu, and a light intensity between 14 and 25 µmol photons PAR m^{-2} s^{-1}. Field observations of *A. minutum* bloom dynamics in Kastela Bay, Croatia also suggested that an optimal temperature window, below 20 °C, is required for excystment of *A. minutum* (Marasovic et al., 1995). The minimum requisite dormancy period for *A. minutum* has been identified as approximately 4 weeks (Chapter 2).

A few direct studies on the encystment and excystment of *G. catenatum* have been carried out although excystment cues have received more attention than encystment cues (Table 5.7). Blackburn et al. (1989) found that *G. catenatum* encysted under nutrient deplete conditions, but that this was not essential for encystment. They also suggested that excystment of resting cysts could occur under a broad range of environmental conditions, in particular at low temperatures and light intensities although these were found to decrease the excystment rate. In a more detailed study of excystment in *G. catenatum* from Spain, Bravo and Anderson (1994) also found that darkness and temperatures below 11°C retarded excystment whereas growth medium did not effect excystment success. The requisite

dormancy period for *G. catenatum* was identified by Blackburn et al. (1989) to be as short as two weeks.

Table 5.7: Studies of encystment and excystment cues for *A. minutum* and *G. catenatum*; previous studies as well as results from this study. Underlined factors have been found to influence encystment or excystment.

Species	Encystment cues investigated	Excystment cues Investigated
Alexandrium minutum	internal nutrient pools (Probert et al., 1998)	salinity, temperature, light, nutrients and location of sediments (Cannon, 1993)
	temperature, nutrients, light, cell:cell interactions (this study)	algicidal bacteria, light, temperature, dormancy period, (this study)
Gymnodinium catenatum	nutrients (Blackburn et al., 1989)	temperature, light, dormancy period (Blackburn et al., 1989)
	temperature, nutrients, cell:cell interactions, algicidal bacteria, (this study)	high temperature (Hallegraeff et al., 1997)
		temperature, growth medium, darkness, (Bravo and Anderson, 1994)
		algicidal bacteria, anaerobic conditions, nutrients (this study)

5.9 TEMPERATURE

In both *A. minutum* and *G. catenatum*, temperature appears to play a key role in encystment, although it is uncertain which stage in the sexual reproduction process is affected (i.e. gametogenesis, gamete fusion or resting cyst formation). A defined 'temperature window' in which encystment can occur was found for both species in this study, 14 to 28 °C for *A. minutum* and 14 to 27°C for *G. catenatum* with the greatest percentage encystment at 20 and 27 °C respectively. For *G. catenatum* increased temperatures resulted in increased percentage encystment up to the temperature at which vegetative growth was no longer possible. This finding is supported by field data on *G. catenatum* in the Huon Estuary, Tasmania, where a significant positive correlation was found between the number of sexual stages present (resting cysts and planozygotes) and surface temperature ($r=0.440$ $n=121$, $p<0.01$ - see Chapter 6). An encystment temperature window that differs from the vegetative cell response to temperature has also been observed in other dinoflagellates. For example, encystment in *Gymnodinium nolleri* from Danish waters was found to have a temperature window of 13 to 33 °C with maximal encystment at 22 – 28 °C, (similar to the 20 – 27 °C range for maximal encystment of *G. catenatum* found in this study) although vegetative growth was possible from 6 – 33 °C (Ellegaard et al., 1998). The temperature window for

encystment identified for *A. tamarense* was 12 – 25 °C with a sharp peak of encystment at 21 °C while vegetative growth was possible between 7 and 25 °C and vegetative cells survived at 2.5°C (Anderson et al., 1984). Anderson et al. (1984) suggest that some metabolic process or processes unique to gamete formation, fusion, or encystment require a greater minimum temperature than that which supports cell division.

Temperature is also an important factor in excystment. *Gymnodinium catenatum* resting cyst excystment was retarded below 11 °C (Bravo and Anderson, 1994). Excystment of *A. minutum* was not detected at 5 °C and was low at 8 °C in this study. High temperatures also affected excystment of *A. minutum* with resting cysts becoming non-viable at 36°C. A previous study found that *G. catenatum* resting cysts died after one hour at 37.5 °C (Hallegraeff et al., 1997).

5.10 NUTRIENTS

Nutrient depletion (particularly nitrogen) has been suggested to be the cue for encystment in many dinoflagellate species (Chapter 1, Table 1.1) with the inference that encystment occurs once conditions become sub-optimal for growth. However, the results presented here on the encystment response of *G. catenatum* under different nutrient conditions showed that there was no significant relationship between initial nutrient levels and encystment. With initial nutrient levels ranging from full GSe medium (1978 µM KNO_3; 200 µM K_2HPO_4) to $GSe/_{1000}$ (1.978 µM KNO_3 nitrate; 0.2 µM K_2HPO_4 phosphate) the numbers of resting cysts produced did not differ significantly although there was a slight trend of greater encystment at lower nutrient concentrations. All treatments produced large numbers of resting cysts. The greatest resting cyst numbers were observed in $GSe/_{10}$ and $GSe/_{100}$ but these were not significantly different to other treatments. The results of this experiment were supported by observations of *G. catenatum* blooms in the Huon Estuary, where resting cysts were seen to be forming in the water column throughout two summer bloom events under highly variable nutrient conditions (1997/98 and 1998/99, Chapter 6).

The two experiments on the effect of nutrients on resting cyst formation in *Alexandrium minutum* yielded some interesting results which differed from those for *G. catenatum*. Both experiments investigated a similar range of initial concentrations of GSe medium but with slightly different experimental designs. The primary difference (other than the kind of culture vessel used) was that the first experiment had three times the irradiance of the second experiment (75 compared with 25 µmol photons PAR m^{-2} s^{-1}). In the lower irradiance experiment there was no evidence that nutrient depletion enhanced resting cyst formation, with the greatest initial nutrient concentration treatment ($GSe/_{20}$) producing a significantly greater number of resting cysts (Figs. 3a and 3b) than the other treatments. The number of

vegetative cells (based on fluorescence) was approximately twice that of the other two treatments ($GSe/_{100}$ and $GSe/_{1000}$) suggesting that the larger number of resting cysts was due to a larger pool of vegetative cells and ultimately compatible gametes. The petri dish experiment (greater irradiance) yielded very different results. Only the low nutrient ($GSe/_{50}$ or less) treatments produced significant numbers of resting cysts with $GSe/_{50}$, $GSe/_{100}$ and GSe without nitrate and phosphate producing the greatest numbers of resting cysts. This response is more compatible with the concept of resting cysts being produced during bloom decline in response to nutrient stress. It seems clear from these results that nutrient depletion under certain conditions (greater irradiance) can enhance encystment in *A. minutum*.

Nutrient concentrations were found to have no detectable influence on excystment of *G. catenatum* in the anaerobic experiment in this study. Other studies have also shown no nutrient effect on excystment (Bravo and Anderson, 1994 for *G. catenatum*; Cannon, 1993 for *A. minutum* using cysts collected from the field).

One possible limitation of the experimental results on nutrient effects (and in other encystment experiments) is that all strains were pre-conditioned in $GSe/_{20}$ for approximately 1 week before being exposed to the various treatments. If nutrient depletion triggers gametogenesis, this may have already occurred under these conditions, obscuring the effect of the treatments in the various experiments. However, this method was standardised across experiments and in many cases a significant effects were observed, suggesting that if this is a confounding factor it is a minor one.

5.11 CELL:CELL INTERACTIONS/PHEROMONE-LIKE SUBSTANCES

The cell:cell contact required for sexual reproduction in dinoflagellates could arise from random encounters between compatible mating types or alternatively some kind of cell:cell communication may occur by means of pheromone-like substances (Wyatt and Jenkinson, 1997). In both cases the physical environment (e.g. water column stability and currents) and dinoflagellate swimming speed would influence the probability of cell:cell contact. If cell:cell contact is random, then the other primary factor controlling encystment would be cell density, with increased cell density increasing the chance of contact between compatible gametes. If cell:cell communication occurs, then although cell density is still important, the efficiency with which compatible gametes make contact would be significantly enhanced. The idea that sexual reproduction in dinoflagellates may be facilitated through cell:cell communication is not a new one. Since von Stosch (1973) first described the 'dancing gametes' of *Gymnodinium pseudopalustre* and *Woloszynskia apiculata*, there has been speculation that cell:cell communication in dinoflagellates may occur through pheromone-like substances (Pfiester, 1989; Wyatt and Jenkinson, 1997).

While the study of cell:cell communication and pheromones is currently restricted to speculation based on observations in the dinoflagellates, it is relatively well studied in other microalgae, macroalgae and in other protozoa. In particular, the dinoflagellates have many characteristics in common with the ciliates (Phylum Protozoa) (Pfiester and Anderson, 1987) and it has been suggested that they may be more closely related than previously supposed (Taylor, 1980). In the ciliates, pheromones have been found to have several different modes of action. In *Euplotes raikovi*, pheromones released by complementary mating types (non-self pheromones) induce sexual behaviour while self pheromones either have no effect (Stock et al., 1999) or have even been found to promote vegetative growth (Vallesi et al., 1995).

Pheromone-like substances in dinoflagellates could act at various different stages in the life cycle, and their efficacy and function is likely to be linked to cell density given the limitations in distance over which a chemical compound can act due to the process of diffusion (Wyatt and Jenkinson, 1997). Indeed it is possible that cell density may act as a trigger for the release of pheromone-like substances, which then either function by triggering gametogenesis or attracting compatible gametes to one another. Cell:cell communication could also influence the development of a planozygote into a resting cyst and the location at which that resting cyst is ultimately deposited (Holt et al., 1994; Wyatt and Jenkinson 1997). The apparently non-random deposition of *A. minutum* resting cysts in culture (see Chapter 2) suggests that this may indeed occur in some species.

The experiments carried out on the effect of various filtrates on encystment in this study yielded some compelling results in support of cell:cell communication in dinoflagellates. In the *G. catenatum* bloom filtrate experiment, encystment was significantly enhanced by the addition of filtrate from an actively encysting bloom population of *G. catenatum*. However, in the second experiment using water taken from the same location in non-bloom conditions there was no effect and in fact this treatment yielded the fewest number of resting cysts. It is possible that other factors were present in the water, which were not produced by *G. catenatum* that may enhance encystment and more detailed characterisation of the field samples, in particular the concentrations of the major nutrients, would therefore be instructive. However, I believe, this study shows we should be looking for a pheromone-like substance which plays a role in encystment. Results of the culture filtrate experiments for *G. catenatum* were somewhat equivocal with a significant effect in only one of the experiments. These results are complicated by the low level of selfing observed in strain GCDE08 (see Chapter 3), which may confuse an encystment signal. The uncertainty of the effect that a selfing strain might have on cell:cell communication was the rationale for repeating the experiment with different non-selfing strains. The results of the second

experiment in which significant differences were detected between filtrates should therefore be given more weight than the first. In the second *G. catenatum* culture filtrate experiment, the filtrate of one strain had a greater effect on encystment than the other strain filtrate, or the cross filtrate (although not significantly different to the control). The results suggest that both strains of *G. catenatum* may be producing a chemical signal, as the encystment response was not significantly different for the two strain filtrates, but interestingly the cross filtrate produced significantly fewer resting cysts. It is possible that an interaction occurs between strains or between pheromones in the cross, which results in inhibition of encystment. Such a response could take the form of a change in the chemical signal or, as in the ciliate *Euplotes raikovi*, (Vallesi et al., 1995) the presence of self-pheromones for both strains may induce vegetative growth, counteracting the encystment response.

The effect of culture filtrates was particularly dramatic in *A. minutum*. In this experiment the AMAD06 culture filtrate treatment resulted in the production of more than five times the resting cysts of any other treatment. These results suggest that in this species one strain may produce an encystment signal while the other strain acts as an acceptor – passive and active strains – ones which produce pheromone-like substances and ones which respond to these compounds in some way either by gametogenesis or by gamete attraction. Passive and active strains are known from other algal groups such as the desmids (Brandham, 1967; Coesel and de Jong, 1986) and *Chlamydomonas* (Tsubo, 1961; Goodenough, 1991). Such a passive/active system is one explanation for the observed highly significant effect of AMAD06 filtrate on the encystment of *A. minutum*. The fact that the cross filtrate, which must also contain some of the same compounds, did not elicit an encystment response may indicate uptake of the pheromone by passive cells in the cross prior to filtration. If the pheromone were not produced in high concentrations, this would result in a much reduced concentration of the compound in the cross filtrate.

If, as the results suggest, cell:cell communication is occurring in these species and potentially in other dinoflagellates, this has major implications for dinoflagellate ecology. Aside from confirming the presence and nature of these compounds, two other key questions need to be answered:

- What is the response? – At which stage of sexual reproduction does cell:cell communication occur? Do these factors facilitate gametogenesis or is their primary function one of attraction between compatible gametes or triggering the development of planozygotes into resting cysts?

- Does cell:cell communication influence bloom dynamics through different signals, either inhibiting or triggering vegetative growth or encystment?

To answer these key ecological questions more research is required.

5.12 ALGICIDAL BACTERIA

Algicidal bacteria have the potential to play an important role in structuring phytoplankton communities and many species have been found which are able to lyse harmful algal species (Doucette et al., 1998, 1999; Fukami et al., 1996; Imai et al., 1993; Lovejoy et al., 1998). In recent studies in the Huon Estuary, six algicidal species from various taxonomic groups were identified (Skerratt, 2001). Vegetative cells of both *A. minutum* and *G. catenatum* were found to be vulnerable to these bacterial species (with the exception of ACEM22, which did not lyse *A. minutum* vegetative cells). Total cell lysis was observed within 2 hours of exposure to exudates of the bacterial strains (bacterial numbers required for algal lysis were generally $10^6 - 10^8$ cells L^{-1}; Skerratt, 2001). The effect of algicidal bacteria on resting cysts has not previously been studied. In this study, the three bacterial species tested did not affect excystment or viability of resting cysts of *G. catenatum* and *A. minutum*, although the algicidal compounds did lyse germlings after excystment. The resistance of resting cysts to bacteria that lyse vegetative cells may therefore contribute to long-term survival of resting cysts in the sediments. The abundance of algicidal bacteria in sediments is unknown, although one of the algicidal species identified from the Huon Estuary (*Pseudoalteromonas* sp. – ACEM4) formed biofilms and was more common in the sediments than in the water column (Skerratt, 2001).

As resting cysts are resistant to algicidal bacteria, encystment would be a potentially powerful ecological strategy for avoiding algicidal bacteria. Adachi et al., (1999) showed that natural planktonic bacterial assemblages (which were not identified) enhanced encystment of *A. catenella* in culture. However, the natural bacterial assemblage used was associated with an *A. tamarense* bloom in which encystment was not monitored and their conclusion that these bacteria effect bloom dynamics in the natural environment was confounded by this and other uncontrolled factors. In the present study the presence of algicidal bacterial exudates showed no significant effect on encystment of *G. catenatum* (*A. minutum* was not tested). That is, although the process of encystment has the potential to effectively protect *G. catenatum* cells from algicidal bacteria the presence of algicidal bacteria does not induce encystment.

5.13 ANAEROBIC CONDITIONS

All dinoflagellate species studied to date have shown inhibition of resting cyst excystment under anaerobic conditions (e.g. *Alexandrium tamarense*, *Scrippsiella* sp., *Gonyaulax polyedra*, *G. rugosum*, and *G. verior* - Anderson et al., 1987; *Gymnodinium* spp. *Protoperidinium* spp., *Scrippsiella* spp. - Montani et al., 1995; *Peridinium* sp. – Endo and

Nagata, 1984). *Gymnodinium catenatum* is no exception as excystment under anaerobic conditions was also low in this study. The inhibition of excystment under anaerobic conditions, and enhanced germination once returned to aerobic conditions, which was observed in this study, suggests that anaerobic sediments may provide ideal regions for accumulation of resting cysts in 'resting cyst beds'. Regions with anaerobic sediments should therefore be investigated for resting cyst density as potential bloom sources.

5.14 SUMMARY

From this study and those carried out by other researchers (e.g. Blanco, 1995; Bravo and Anderson, 1994; Ellegaard et al., 1998; Kremp and Anderson, 2000; Sgrosso et al., 2001) it is clear that the processes of encystment and excystment involve a complex interplay of biological and environmental factors, which vary in importance from species to species and act at different stages in the life cycle. The experiments presented here support the concept that encystment is not just a response to nutrient depletion during bloom decline. Nutrient depletion can be important at times, for some species, under some conditions, but is certainly not the only factor influencing encystment. Nutrients, temperature, irradiance, and cell:cell communication may all act to induce encystment, possibly in combination with themselves and with other as yet unobserved factors. Excystment is also caused by the interaction of various factors. It is highly dependent on temperature and on oxygen concentrations in sediments as well as being influenced by light and subject to the endogenous dormancy period. Further research is required in order to fully understand the induction of both encystment and excystment, incorporating both field and laboratory studies and developing methods to explore the specific stages in the life cycle affected by different cues. In particular, the confirmation of the presence of pheromone-like substances and their characterisation would be extremely beneficial to this field of research, as would a more detailed understanding of the role of cell density in initiating sexual reproduction. The environmental implications of the results of these experimental studies are discussed in Chapter 7.

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REFERENCES

Adachi M, Kanno T, Matsubara T, Nishijima T, Itaura S, Yamaguchi M (1999) Promotion of cyst formation in the toxic dinoflagellate *Alexandrium* (Dinophyceae) by natural bacterial assemblages from Hiroshima Bay, Japan. Marine Ecology Progress Series 191: 175–185.

Anderson DM, Wall D (1978) Potential importance of benthic cysts of *Gonyaulax tamarensis* and *G. excavata* in initiating toxic dinoflagellate blooms. Journal of Phycology 14: 224–234.

Anderson DM, Chisholm SW, Watras CJ (1983) Importance of life cycle events in the population dynamics of *Gonyaulax tamarensis*. Marine Biology 76: 179–189.

Anderson DM, Kulis DM, Binder BJ (1984) Sexuality and cyst formation in the dinoflagellate *Gonyaulax tamarensis* : Cyst yield in batch cultures. Journal of Phycology 20: 418–425.

Anderson DM, Taylor CD, Armbrust EV (1987) The effects of darkness and anaerobiosis on dinoflagellate cyst excystment. Limnology and Oceanography 32: 340–351.

Blackburn SI, Hallegraeff GM, Bolch CJ (1989) Vegetative reproduction and sexual life cycle of the toxic dinoflagellate *Gymnodinium catenatum* from Tasmania, Australia. Journal of Phycology 25: 577–590.

Blackburn SI, Bolch CJ, Haskard KA, Hallegraeff GM (2001) Reproductive compatibility among four global populations of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). Phycologia 40: 78–87.

Blanco J (1995) Cyst production in four species of neritic dinoflagellates. Journal of Plankton Research 17: 165–182.

Braarud T (1945) Morphological observations on marine dinoflagellate cultures. Avhandling av det Norske Vitenskapelige Akademi, Oslo 1943-3: 1–18.

Brandham PE (1967) Time-lapse studies of conjugation in *Cosmarium botrytis* II. Pseudoconjugation and an anisogamous mating behaviour involving chemotaxis. Canadian Journal of Botany 45: 483–493.

Bravo I, Anderson DM (1994) The effects of temperature, growth medium and darkness on excystment and growth of the toxic dinoflagellate *Gymnodinium catenatum* from northwest Spain. Journal of Plankton Research 16: 513–525.

Cannon JA (1990) Development and dispersal of red tides in the Port River, South Australia. In Graneli E, Sundstrom B, Edler L, Anderson DM (eds), Toxic Marine Phytoplankton. Elsevier Science Publishing Co. Inc., New York: 110–115.

Cannon JA (1993) Excystment of the toxic dinoflagellate, *Alexandrium minutum*, from sediments in the Port River, South Australia. In Smayda TJ, Shimizu Y (eds) Toxic Phytoplankton Blooms in the Sea. Elsevier Science Publishers B.V. Amsterdam: 109–114.

Coesel PFM, de Jong W (1986) Vigorous chemotactic attraction as a sexual response in *Closterium ehrenbergii* Meneghini (Desmidiaceae, Chlorophyta). Phycologia 25: 405–408.

Costas E, Gonzalez Gil S, Aguilera A, Lopez Rodas V (1993) An apparent growth factor modulation of marine dinoflagellate excystment. Journal of Experimental Marine Biology and Ecology 166: 241–249.

Destombe C, Cembella A (1990) Mating-type determination, gametic recognition and reproductive success in *Alexandrium excavatum* (Gonyaulacales, Dinophyta), a toxic red-tide dinoflagellate. Phycologia 29: 316–325.

Doblin MA (1998) Importance of selenium and humic substances from land runoff in the development of *Gymnodinium catenatum* blooms. PhD thesis, 1998, University of Tasmania, 133 pp.

Doucette GJ, McGovern ER, Babinchak JA (1999) Algicidal bacteria active against *Gymnodinium breve* (Dinophyceae). I. Bacterial isolation and characterisation of killing activity. Journal of Phycology 35: 1447–1454.

Doucette GJ, Kodama M, Franca S, Gallacher S (1998) Bacterial interactions with Harmful Algal Bloom Species: Bloom ecology, toxigenesis and cytology. In Anderson DM, Cembella AD, Hallegraeff GM (eds), Physiological Ecology of Harmful Algal Blooms. NATO ASI series, Springer-Verlag, Berlin, Heidelberg, Vol. G41: 619–647.

Ellegaard M, Kulis DM, Anderson DM (1998) Cysts of Danish *Gymnodinium nolleri* Ellegaard et Moestrup Sp. Ined. (Dinophyceae) - studies on encystment, excystment and toxicity. Journal of Plankton Research 20: 1743–1755.

Endo T, Nagata H (1984) Resting and excystment of cysts of *Peridinium* sp. (Dinophyceae). Bulletin of Plankton Society of Japan 31: 23–33.

Fukami K, Sakaguchi K, Kanou M, Nishijima T (1996) Effect of bacterial assemblages on the succession of blooming phytoplankton from *Skeletonema costatum* to *Heterosigma akashiwo*. In Yasumoto T, Oshima Y, Fukuyo Y (eds), Harmful and toxic algal blooms. Intergovernmental Oceanographic Commission, UNESCO: 335–342.

Goodenough UW (1991) *Chlamydomonas* mating interactions. In Dworkin M (ed), Microbial Cell-Cell Interactions. American Society for Microbiology, Washington DC: 71–112.

Hallegraeff GM, Bolch CJ (1991) Transport of toxic dinoflagellate cysts via ships' ballast water. Marine Pollution Bulletin 22: 27–30.

Hallegraeff GM, Steffensen DA, Wetherbee R (1988) Three estuarine Australian dinoflagellates that can produce paralytic shellfish toxins. Journal of Plankton Research 10: 533–541.

Hallegraeff GM, Marshall JA, Valentine J, Hardiman S (1998) Short cyst-dormancy period of an Australian isolate of the toxic dinoflagellate *Alexandrium catenella*. Marine and Freshwater Research 49: 415–420.

Hallegraeff FM, Valentine JP, Marshall JA, Bolch CJ (1997) Temperature tolerances of toxic dinoflagellate cysts: application to the treatment of ships' ballast water. Aquatic Ecology 31: 47–52.

Holt JR, Merrell JR, Seaborn DW, Hartranft JL (1994) Population dynamics and substrate selection by three Peridinium species. Journal of Freshwater Ecology 9: 17–128.

Huber G, Nipkow F (1922) Experimentelle utersuchungen uber die entwicklung von *Ceratium hirundinella* O. F. M. Zeitschrift fur Botanik 14: 337–371.

Huber G, Nipkow F (1923) Experimentelle untersuchungen uber die entwicklung und formbildung von *Ceratium hirundinella* O. Fr. Mull. Flora New Series 16: 114–215.

Imai I, Ishida Y, Hata Y (1993) Killing of marine phytoplankton by a gliding bacterium Cytophaga sp. isolated from the coastal sea of Japan. Marine Biology 116: 527–532.

Ishikawa A, Taniguchi A (1996) Contribution of benthic cysts to the population dynamics of *Scrippsiella* spp. (Dinophyceae) in Onagawa Bay, Northeast Japan. Marine Ecology Progress Series 140: 169–178.

Jameson, I.D. Blackburn, S.I., Thompson, P.A. (in prep.) The effect of temperature on akinete differentiation, heterocyst formation and vegetative growth in 6 strains of the toxic cyanobacteria *Anabaena circinalis* from Australian Waters.

Kremp A (2000a) Morphology and excystment pattern of the resting cyst of *Peridiniella catenata* (Dinophyceae) from the Baltic Sea. Phycologia 39: 183–186.

Kremp A (2000b) Distribution, dynamics and in situ seeding potential of *Scrippsiella hangoei* (Dinophyceae) cyst populations from the Baltic Sea. Journal of Plankton Research 22: 2155–2169.

Kremp A, Anderson DM (2000) Factors regulating excystment of resting cysts of the spring bloom dinoflagellate *Scrippsiella hangoei* from the northern Baltic Sea. Journal of Plankton Research 22: 1311–1327.

Lovejoy C, Bowman JP, Hallegraeff GM (1998) Algicidal effects of a novel marine *Pseudoalteromonas* isolate (Class proteobacteria, gamma subdivision) on harmful algal bloom species of the genera *Chattonella, Gymnodinium* and *Heterosigma*. Applied and Environmental Microbiology 64: 2806–2813.

Marasovic I, Nincevic Z, Odzak N (1995) The effect of temperature on blooms of *Lingulodinium polyedra* and *Alexandrium minutum* in Kastela Bay. In Lassus P, Arzul G, Erard E, Gentien P, Marcaillou C (eds) Harmful Marine Algal Blooms. Technique et Documentation, Lavoisier, Intercept Ltd: 187–192.

Montani S, Ichimi K, Meksumpun S, Okaichi T (1995) The effects of dissolved oxygen and sulfide on excystment of the cysts of some different phytoflagellates. In Lassus P, Arzul G, Erard E, Gentien P, Marcaillou C (eds), Harmful Marine Algal Blooms. Technique et Documentation, Lavoisier, Intercept Ltd., Paris: 627–632.

Nordli E (1951) Resting spores in *Gonyaulax polyedra* Stein. Nytt Magasin for Naturvidenskapene 88: 207–212.

Nuzzo L, Montresor M (1999) Different excystment patterns in two calcareous cyst-producing species of the dinoflagellate genus Scrippsiella. Journal of Plankton Research 21: 2009–2018.

Oshima Y, Itakura H, Lee K-C, Yasumoto T, Blackburn SI, Hallegraeff G (1993) Toxin production by the dinoflagellate *Gymnodinium catenatum*. In Smayda TJ, Shimizu Y (eds), Toxic phytoplankton blooms in the sea. Elsevier Science Publishers BV, Amsterdam: 907–912.

Pfiester LA (1989) Dinoflagellate Sexuality. International Review of Cytology 114: 249–272.

Pfiester LA, Anderson DM (1987) Dinoflagellate reproduction. In Taylor FJR (ed), The Biology of Dinoflagellates. Blackwell Scientific Publications, Oxford: 611–648.

Pfiester LA, Timpano P, Skvarla JJ, Holt JR (1984) Sexual reproduction and meiosis in *Peridinium inconspicuum* Lemmerman (Dinophyceae). American Journal of Botany 71: 1121–1127.

Probert IP, Erard-le Denn E, Lewis J (1998) Intracellular nutrient status as a factor in the induction of sexual reproduction in a marine dinoflagellate. In Reguera B, Blanco J, Fernandez ML, Wyatt T, (eds), Harmful Algae. Xunta de Galicia and Intergovernmental Oceanographic Commission of UNESCO: 343–344.

Rengefors K, Anderson DM (1998) Environmental and endogenous regulation of cyst excystment in two freshwater dinoflagellates. Journal of Phycology 34: 568–577.

Sgrosso S, Esposito F, Montresor M (2001) Temperature and daylength regulate encystment in calcareous cyst-forming dinoflagellates. Marine Ecology Progress Series 211: 77–87.

Skerratt JH (2001) Marine microbial interactions in a Tasmanian estuary. PhD Thesis 2001, University of Tasmania, 269 pp.

Stock C, Kruppel T, Key G, Lueken W (1999) Sexual behaviour in *Euplotes raikovi* is accompanied by pheromone-induced modifications of ionic currents. Journal of Experimental Biology 202: 475–483.

Taylor FJR (1980) On dinoflagellate evolution. BioSystems 13: 65–108.

Tsubo Y (1961) Chemotaxis and sexual behaviour in *Chlamydomonas*. Journal of Protozoology 8: 114–121.

Tyler MA, Coats DW, Anderson DM (1982) Encystment in a dynamic environment: deposition of dinoflagellate cysts by a frontal convergence. Marine Ecology Progress Series 7: 163–178.

Vallesi A, Giuli G, Bradshaw RA, Luporini P (1995) Autocrine mitogenic activity of pheromones produced by the protozoan ciliate *Euplotes raikovi*. Nature 376: 522–524.

Von Stosch HA (1973) Observation on vegetative reproduction and sexual life cycles of two freshwater dinoflagellates, *Gymnodinium pseudopalustre* Schiller and *Woloszynskia apiculata* sp. nov. British Phycological Journal 8: 105–134.

Wyatt T, Jenkinson IR (1997) Notes on *Alexandrium* population dynamics. Journal of Plankton Research 19: 551–575.

CHAPTER 6

BLOOM DYNAMICS OF THE TOXIC DINOFLAGELLATE *GYMNODINIUM CATENATUM* (DINOPHYCEAE) IN THE HUON ESTUARY, SOUTH-EAST TASMANIA, AUSTRALIA

INTRODUCTION

There have been numerous studies of the spatial and temporal variability of phytoplankton in estuarine systems both in Australia and overseas (e.g. Thompson, 1998; Ault et al., 2000; Pinckney et al., 1998; Sin et al., 1999). Estuarine phytoplankton communities are complex and composed of dynamic, multispecies assemblages characterised by high diversity and rapid successional shifts in species composition in response to environmental changes (Gallegos, 1992; Pinckney et al., 1998). A variety of physical (temperature, water column stability, salinity), chemical (macronutrient and micronutrient concentrations), and biological (grazing, competition, bacterial degradation, parasites, viruses) factors are known to influence phytoplankton dynamics (Gallegos and Jordan, 1997; Lewitus et al., 1998; Sin et al., 1999). Mass proliferations of phytoplankton – 'phytoplankton blooms' or 'algal blooms' – are a prominent feature of estuarine systems. Bloom forming phytoplankton species play a key role in estuaries, including increased biomass production and enhanced energy transfer through the food web (Smayda, 1980). In temperate regions, blooms generally occur in spring and summer coinciding with the seasonal temperature and light increases (Cole and Cloern, 1987).

The dynamics of many individual phytoplankton species of significance in estuarine systems have been investigated, in particular those that form blooms with deleterious effects on other components of the food web or on humans (harmful algal blooms - HABs) (e.g. Anderson, 1997). These HAB species can be divided into three categories: 1) Species that are essentially harmless but form dense blooms causing fish and invertebrate kills due to oxygen depletion; 2) Species which produce potent toxins that can cause illness or death, either in grazers, or are bioaccumulated in grazers and consequently cause illness or death further up the food chain (e.g. in humans and other mammals); 3) Species that are not toxic but are physically harmful e.g. through damage to, or clogging of, fish gills (Hallegraeff, 1993).

In recent years, increased dependence on the marine environment for food, and the growth of the aquaculture industry worldwide has brought many toxic algal species to the attention of environmental mangers and the general public. There is evidence to suggest that there has been a significant increase in the frequency and distribution of HABs over the last 30 years (Smayda, 1990; Hallegraeff, 1993). Factors such as increased eutrophication and the transport of organisms around the world through ballast water have been implicated as two of the primary causes of this (Hallegraeff, 1993). There is now a general consensus that there are more toxic algal species, more algal toxins, more fisheries resources affected, more food-web disruption and more economic losses from HABs than ever before (Anderson and Garrison, 1997).

Knowledge of the factors that stimulate or inhibit growth and bloom development of harmful algal species is essential for effective HAB management. Because algal bloom dynamics are a complex interplay of physical, chemical and biological interactions, their study requires an integrated multidisciplinary approach. Up until the mid 1990s much HAB research focussed on the detection and monitoring of toxic or otherwise harmful species rather than on their ecology. In recent years there has been an increase in the number of autecological studies of HAB species, resulting in a shift away from studies which regard algal blooms simply as high chlorophyll to investigations of the unique physiology and ecology of individual species. The implementation of such programs as ECOHAB (the USA directive Ecology and Oceanography of HABs) and GEOHAB (the IOC/SCOR directive Global Ecology and Oceanography of HABs) has been important in fostering this shift.

As part of an autecological approach to HAB research and management there is a need for studies of the ecology of HABs to incorporate all of the life stages of a species, including understanding the causative factors of life cycle transitions (Wyatt and Jenkinson, 1997). Many HAB species reproduce both vegetatively and sexually. Over the last 30 years, sexual life cycles, incorporating a resting stage, have been identified in many dinoflagellates, including many of the toxic species (see Chapter 1, Table 1.1). The role of resting cysts in dinoflagellate bloom dynamics has been investigated in a relatively limited number of species, including *Alexandrium tamarense* (Anderson and Keafer, 1985, 1987), *Amphidinium carterae* (Sampayo, 1985), *Gymnodinium pseudopalustre* (Tyler and Heinbokel, 1985), *Gymnodinium sanguineum* (Voltolina, 1993), and *Scrippsiella trochoidea* (Ishikawa and Taniguchi, 1996). For some species resting cysts have been shown to play a crucial role in bloom initiation through synchronised excystment (e.g. *Alexandrium tamarense*, Anderson and Keafer, 1985) while in other species bloom decline is triggered by mass encystment (e.g. *Gymnodinium pseudopalustre*, Tyler and Heinbokel, 1985). Resting cysts have also been shown to maintain a perennial vegetative population until favourable conditions for bloom development are present (e.g. *Scrippsiella trochoidea*, Ishikawa and Taniguchi, 1996).

The toxic dinoflagellate *Gymnodinium catenatum* forms recurrent blooms in the Huon and Derwent Estuaries of south-east Tasmania, Australia (Hallegraeff et al., 1995). This chapter describes the bloom dynamics of *G. catenatum* in the Huon Estuary over a period of three years from 1996 to 1999. The first two years of the study were conducted in collaboration with the CSIRO Huon Estuary Study. The final report of this study 'Huon Estuary Study: Environmental research for integrated catchment management and aquaculture' was published in 2000 (CSIRO Huon Estuary Study Team, 2000; hereafter cited as HEST, 2000)

and includes preliminary results from this PhD project. The third year's data was collected in collaboration with J. Skerratt (PhD student, University of Tasmania).

6.1 *GYMNODINIUM CATENATUM*

The toxic dinoflagellate *Gymnodinium catenatum* was first recorded from south-east Tasmania in 1980 (Hallegraeff and Sumner, 1986) and is believed to have been introduced into the region in the 1970s (McMinn et al., 1997). Since then it has formed toxic blooms in many years, causing significant economic losses to the shellfish industry in the Huon Estuary and adjacent waters. *Gymnodinium catenatum* blooms in the region are highly variable with respect to biomass, toxicity and geographic extent (Figs. 6.1 and 6.2, Hallegraeff et al., 1995).

Gymnodinium catenatum produces paralytic shellfish toxins or PSTs. These PSTs bioaccumulate in shellfish such as mussels and oysters and can result in paralytic shellfish poisoning if consumed by humans and other mammals (Yentsch, 1984). Since 1986 the Tasmanian Government has operated a biotoxin-monitoring program according to USFDA (United States Food and Drug Authority) guidelines. These guidelines stipulate closure of shellfish farms to harvesting when levels of toxin in shellfish exceed 80 μg saxitoxin equivalents per 100 g shellfish tissue. Blooms of *G. catenatum* have resulted in closures of shellfish farms in the Huon Estuary for periods of up to nine months in some years. A bloom of *G. catenatum* is here defined as greater than 10,000 cells L^{-1} which is the level at which shellfish (e.g. mussels and oysters) consuming *G. catenatum* reach detectable toxin levels (Hallegraeff et al., 1995).

Gymnodinium catenatum has a very characteristic appearance (see Chapter 1, Fig. 1.2a), forming chains commonly up to 64 cells long and occasionally longer and swimming with a whirling snake-like motion. The primary means of reproduction of *G. catenatum* is vegetative cell division (binary fission), giving an exponential increase in cell numbers when environmental conditions are appropriate for growth. *Gymnodinium catenatum* also reproduces sexually (Blackburn et al. 1989). The process of sexual reproduction begins with gametogenesis. The gametes then fuse to form a planozygote, which is larger and more heavily pigmented than the vegetative cells. The planozygote loses its flagella and forms a rounded resting cyst or hypnozygote (see Chapter 1, Fig. 1.2b) which sinks to the sediments. The resting cyst of *G. catenatum* is also quite distinctive (see Chapter 1, Fig. 1.2b). It is spherical, pigmented brown, and is one of few species that produce a resting cyst with microreticulations on the surface (i.e. a surface composed of hundreds of 1-3μm polygons which reflect several features of the motile stage) and also contains distinct dark red accumulation bodies (usually 1 or 2) (Anderson et al., 1988). When conditions are

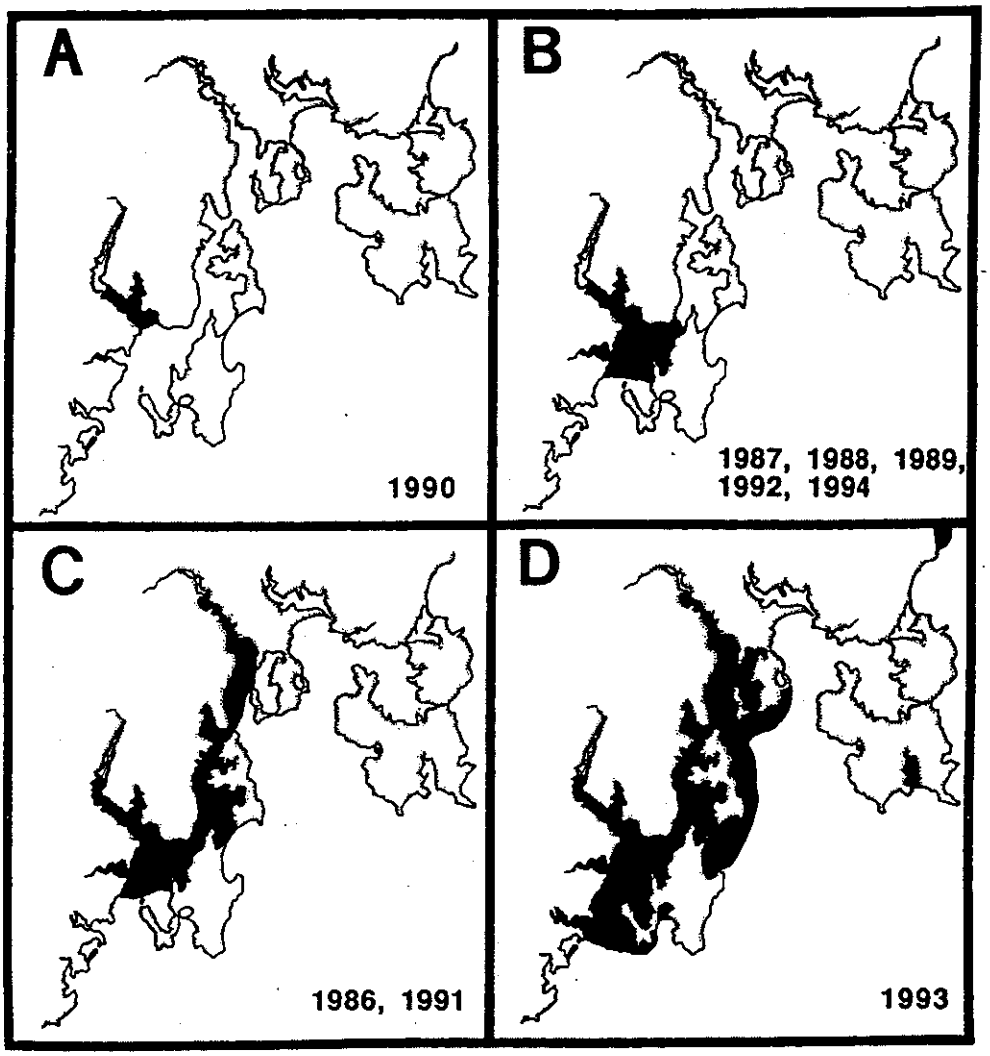


Figure 6.1: Spatial extent of *G. catenatum* blooms in south eastern Tasmania based on shellfish toxicity (reproduced from Hallegraeff et al., 1995).

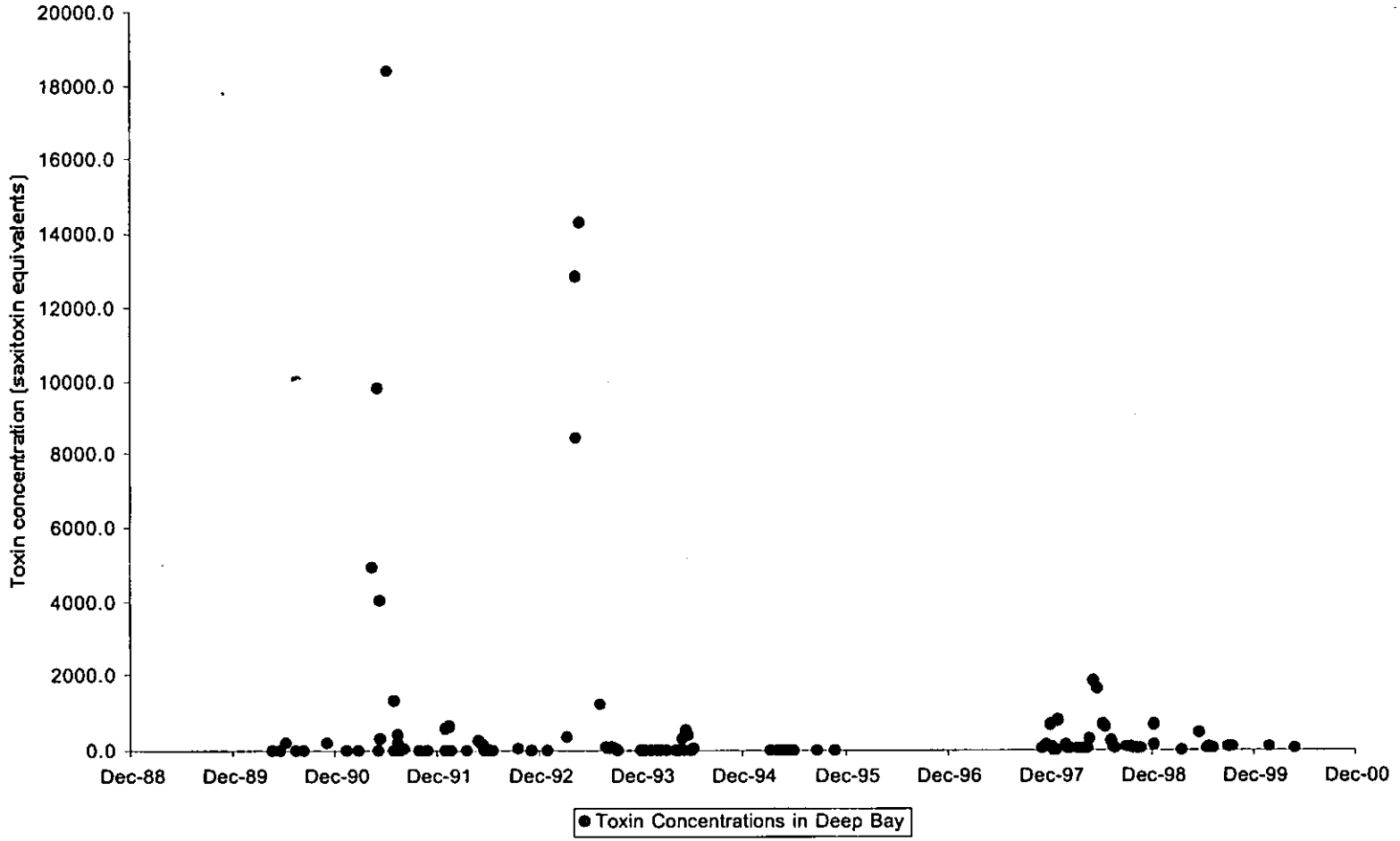


Figure 6.2: Shellfish toxin concentrations in the Huon Estuary from 1988 to 2000 as determined through both HPLC and mouse bioassay (the higher value is recorded where both tests were carried out on a single sample). Data provided by R. Brown (Manager Tasmanian Shellfish Quality Assurance Program, Tasmanian Department of Health and Human Services).

appropriate this resting cyst germinates to give rise to a germling or planomeiocyte, which divides, recommencing vegetative growth (Chapter 1, Fig. 1.1 and Blackburn et al., 1989)

Gymnodinium catenatum has been most extensively studied from Australian and Spanish populations (Hallegraeff and Fraga, 1998). The toxin profile of *G. catenatum* is well characterised. *Gymnodinium catenatum* produces primarily toxins of the N-sulfocarbomyl group (regardless of geographic origin) with more than 90% of the toxins composed of C1-C4 with trace amounts of dcSTX, GTX3 and dcGTX2 (Oshima et al., 1993). Tasmanian isolates differ from other regions by also containing 13-de-oxydecarbomyl toxins (Oshima et al., 1993). The pigment, fatty acid and sterol compositions of *G. catenatum* have also been described (Hallegraeff et al., 1991). *Gymnodinium catenatum* strains isolated from one of the blooms in the Huon Estuary during this study (strains GCW5-97 and GCHUN2-97, see Chapter 3, Table 3.1) were analysed for pigment composition and are described by Clementson et al. (1998). The primary pigments in this species are chlorophylls *a*, c_1+c_2, peridinin and diadinoxanthin, with smaller amounts of dinoxanthin, diatoxanthin and β,β carotene (Hallegraeff et al., 1991; Clementson et al., 1998).

There has been considerable research carried out on the physiology of *G. catenatum* (e.g. Blackburn et al., 1989; Bravo and Anderson, 1994; Flynn et al., 1996), and the micronutrient selenium and humic substances have been identified as important for growth of Tasmanian strains (Doblin et al., 1999a, 1999b, 2000). The genetic composition and sexual compatibility of different global populations of *G. catenatum* has also been investigated (Bolch et al., 1999; Blackburn et al., 2001).

Various ecological studies of *G. catenatum* have also been undertaken, including a single study of the ecology of *G. catenatum* for the Australian population by Hallegraeff et al. (1995). Their study investigated inter-annual bloom variation based on historical information of toxin data and physical parameters such as river flow, temperature and wind speeds. Bloom initiation and dynamics have been investigated for Spanish populations of *G. catenatum* by various authors (Bravo and Anderson, 1994; Fraga, 1996). There is an ongoing debate in the study of *G. catenatum* regarding the mechanism of bloom formation and the importance of resting cysts in this process. Blackburn et al. (1989), in a study of the life history of Tasmanian *G. catenatum*, found that resting cysts can germinate under a surprising variety of conditions and that planomeiocytes (the life stage immediately following excystment) are rarely observed in water column field samples. Because of these observations they suggested that resting cysts are unlikely to play a major role in the seasonal timing and abundance of *G. catenatum* in Tasmanian waters. This view has also been supported by other authors (Hallegraeff et al., 1995; Hallegraeff and Fraga, 1998).

In *G. catenatum* blooms of the Spanish Rias, there is some evidence that resting cysts make an important contribution to bloom formation. Temperature and light levels in the Rias during the period from winter through till summer are such that resting cysts are unable to germinate. This could potentially result in synchronised resting cyst germination in the early summer when conditions are also most suitable for vegetative growth (Bravo and Anderson, 1994). Contrary evidence from the Spanish Rias suggests that the resting cysts serve only to maintain a vegetative population at a low level until water column conditions are suitable for growth and also that offshore vegetative populations may provide the inoculum for blooms (Fraga, 1996; Gomez et al., 1996). The Spanish ecosystem differs from the Tasmanian ecosystem in that there are much stronger oceanic influences including periods of strong upwelling and the riverine inputs during bloom periods are insignificant (Hallegraeff and Fraga, 1998). The extent of sexual reproduction in blooms, and whether encystment of blooms has the potential to contribute to bloom decline is unknown for either population.

Prior to the period of time covered in this study, the last major *G. catenatum* bloom in the Huon Estuary occurred in 1993, followed by a small bloom in 1994 (Hallegraeff et al., 1995). There were no blooms or records of toxicity after this for three years (including the first year of monitoring as part of the CSIRO Huon Estuary study) until late November 1997.

6.2 THE HUON ESTUARY

The Huon Estuary is located in south-east Tasmania, an island to the south of mainland Australia (Fig. 6.3) between latitude 42° 45' S and 43° 45' S, in a maritime climate that is dominated by zonal westerlies that produce changeable, cool temperate conditions (HEST, 2000). The general physical characteristics of the estuary are as follows; approximately 39 km in length, 4.5 km wide at the mouth, a surface area of 44.4 km^2, a volume of 1.38 km^3, mean tidal range of 0.9 m and a typical tidal excursion of ± 150 m. Maximum tidal velocities are on the order of 0.02 ms^{-1} and winds are predominantly from the WSW (HEST, 2000).

The Huon Estuary supports an expanding aquaculture industry, including farming of Atlantic Salmon (*Salmo salar*), Blue Mussels (*Mytilus edulis*) and Pacific Oysters (*Crassostrea gigas*). The Huon Estuary is also an important region for agriculture (grazing, dairies, and orchards), and tourism. The catchment of the estuary incorporates wilderness regions of buttongrass plains and eucalypt forests as well as forestry operations, and extensive agriculture in the lower catchment (HEST, 2000).

The main tributary of the estuary, the Huon River, has high levels of humic substances and a mean annual flow of 87 $m^3 s^{-1}$ measured at Frying Pan Creek, upstream from Judbury (Fig.

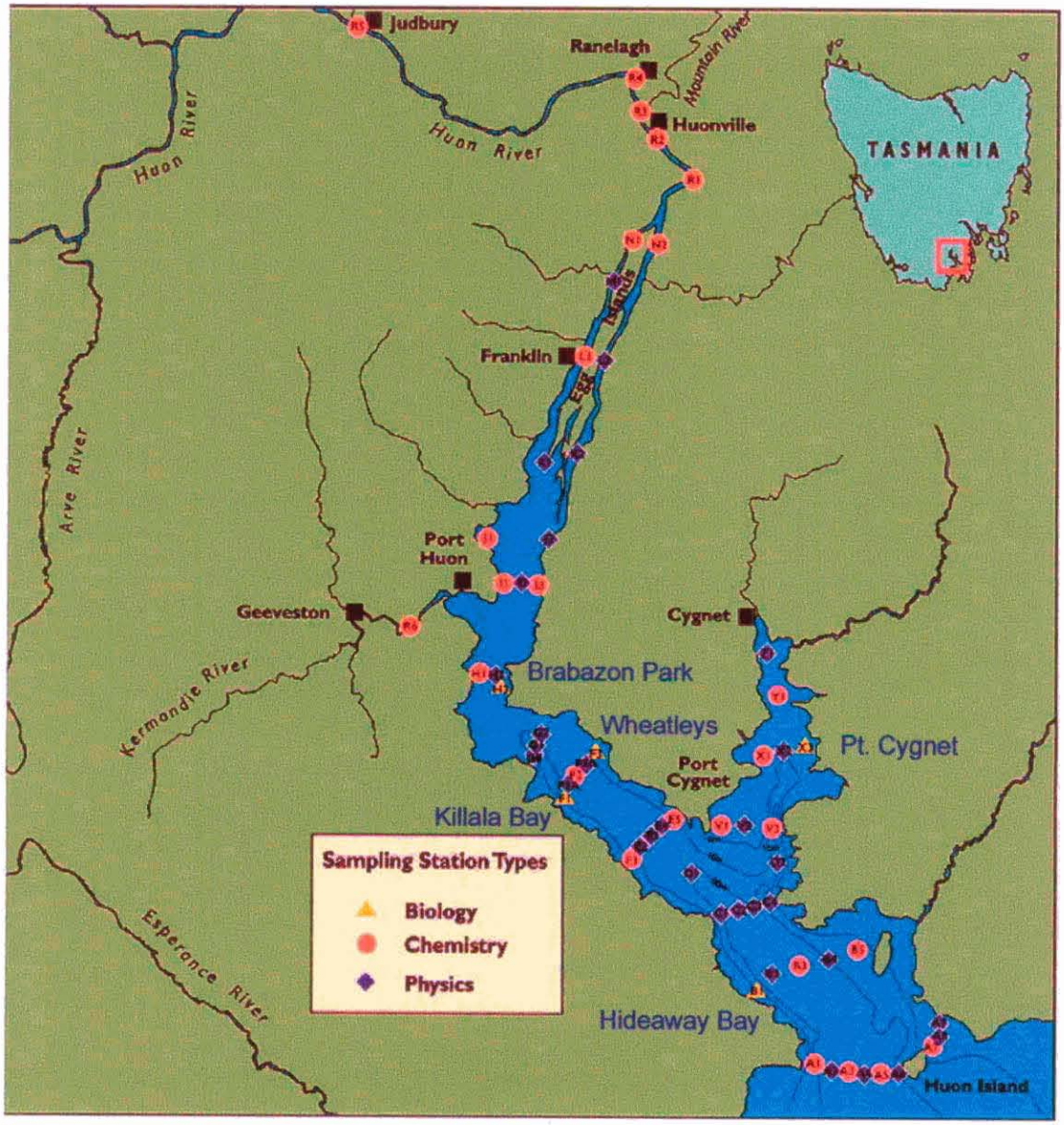


Figure 6.3: Map of the Huon Estuary indicating sampling sites for the Huon Estuary Study (site names for biological sites in blue) and further monitoring, along with other important features of the region (reproduced from HEST, 2000).

6.3). Smaller tributaries also flow into the estuary, the Kermandie River and Mountain River are the most significant of these (Fig. 6.3). The flow of the Huon River and marine input, form a classic salt wedge estuary with the top layer being strongly affected by the humic laden freshwater flow and the deeper waters being dominated by marine characteristics (HEST, 2000). The physics of the estuary result in high flushing rates with residence times of a few days (Table 6.1).

Table 6.1: Flushing times for the Huon Estuary and its different zones for low and high river flow (Reproduced from HEST, 2000).

	Upper Estuary		Upper + Middle Estuary		Upper + Middle + Lower Estuary	
River Flow ($m^3 s^{-1}$)	25	250	25	250	25	250
Surface Layer	1.1 d	0.2 d	0.8 d	0.2 d	1.3 d	0.6 d
Surface + Bottom Layers	2.4 d	0.4 d	5.8 d	1.1 d	5.6 d	2.5 d

Surface salinity data for the Huon Estuary indicate that there is a tendency for the fresh waters from the Huon River and other tributaries to flow down the eastern coast of the upper estuary and the north-eastern coast of the lower estuary (Woodward et al., 1992; HEST, 2000). This effect is most likely a combination of the predominance of the winds from the WSW (HEST, 2000) and the effect of the rotation of the earth (Dyer 1997).

6.2.1 NUTRIENTS IN THE HUON ESTUARY

Most nutrients in the Huon Estuary undergo strong seasonal variations in concentration (Table 6.2). Nitrate is influenced by biological activity, is strongly depleted in surface waters (often below detection) in spring, summer and early autumn, and has elevated levels later in autumn and in winter. Nitrate is present throughout the year in bottom (a few metres above the sediment) marine waters (HEST, 2000). Nitrite in the estuary has broadly similar seasonal and mixing trends to nitrate although at an order of magnitude lower concentration. Phosphate is also markedly affected by biological activity following a similar seasonal cycle to nitrate but phosphate is depleted more gradually than nitrate and not too as great a degree. This is not unexpected: if microalgae assimilate N and P at Redfield ratios (16:1) then P is in excess relative to N in the Huon Estuary (HEST, 2000).

Particulate nitrogen and phosphorus have uniformly low concentrations throughout the estuary independent of season (<1.5–5 μM and typically <0.1–0.3μM respectively). Total N is influenced by DON and has an inverse relation with salinity due to the high DON input from the Huon River (HEST, 2000), while total P increases with salinity down the estuary. Both total P and total N are also strongly influenced by particulate N during periods of high biomass (i.e. during microalgal blooms). Low nitrate:phosphate ratios in the Huon Estuary

indicate nitrogen limitation of microalgal production (below 3 at most estuarine sites in 1997/98) (HEST, 2000).

Ammonia concentrations in the Huon Estuary are generally low (<1μM). Bottom waters have frequent increased concentrations of ammonia, particularly in summer. This bottom ammonia is most likely a result of remineralisation of organic nitrogen in the remains of dead microalgae deposited on the estuary floor after the crash of dense blooms (HEST, 2000).

Silicate also has an inverse relation with salinity in the Huon Estuary, reflecting the primarily terrestrial source of this nutrient (HEST, 2000). The Huon River and the Kermandie River, a smaller tributary of the estuary, are both important sources of silicate in the estuary.

Table 6.2: Concentration ranges of nutrients and other water-quality parameters during the Huon Estuary Study (1996-1998) from estuarine surveys and weekly/fortnightly monitoring (reproduced from HEST, 2000).

Water-Quality measurements	Estuarine ranges (all sites) (Concentrations, μM)[a]
Nitrate + Nitrite	<0.05 – 7.2
Nitrite	<0.03-2.25
Total ammonia	<0.02-2.6
Total dissolved N	<0.5-26
Total N	<0.5-45
Dissolved reactive P	<0.03-0.71
Total dissolved P	<0.08-1.86
Total P	<0.08-3.16
Dissolved reactive Si	<0.5-106
Dissolved oxygen	9.1-370.0
DO (% saturation)	3.6-163
SPM (mg kg^{-1})[b]	0.1-12.6

[a] Concentrations in μM, except where indicated otherwise
[b] Suspended particulate matter (= nonfilterable residue) retained on 0.45μm filter

6.2.2 PHYTOPLANKTON PIGMENTS IN THE HUON ESTUARY

Phytoplankton pigment composition was measured by HPLC for a period of two years (1996 to 1998) as part of the Huon Estuary Study (HEST, 2000). Pigment composition can be used to identify the phytoplankton taxa present in a sample. Some pigments relate specifically to a particular algal Class and are termed 'marker pigments' (Jeffrey and Vesk, 1997). Some marker pigments are found exclusively in one Class (e.g. alloxanthin is only found in the Cryptophyta). Other pigments are primary pigments of one algal Class but may also be found in low concentrations in algae from other Classes (e.g. fucoxanthin is considered a

marker pigment for diatoms but is also found in some dinoflagellates, chrysophytes and raphidophytes) (Jeffrey et al., 1997) (Table 6.3).

Results of pigment analyses from the Huon Estuary Study found that from October 1996 to June 1997 the main marker pigments found in the estuary were fucoxanthin and alloxanthin, indicating dominance by diatoms and cryptophytes. Elevated levels of 19' hexanoyloxyfucoxanthin (haptophytes) were also occasionally observed (including a 3 week period in December 1996 / January 1997). A significant contribution of zeaxanthin (cyanobacteria) was observed at the beginning of the study, from October 1996 to March 1997, with occasional high contributions. However, zeaxanthin rarely contributed to the phytoplankton biomass after March 1997. Peridinin (a dinoflagellate marker) was a minor component of the pigment composition during the first year of the study but made a large contribution in almost all samples after September 1997, and was the dominant or even sole marker pigment in dinoflagellate bloom periods from 9 December 1997 to 13 January 1998 and from 28 April 1998 to 3 June 1998 (Fig. 6.4) (HEST, 2000).

Table 6.3: Algal pigments found in samples from this study and the algal taxa in which the pigment has been detected (adapted from Jeffrey et al., 1997).

Pigment	Algal Division/ Class
Chlorophyll *a*	All photosynthetic algae (except prochlorophytes)
Chlorophyll *b*	Chlorophytes, Prasinophytes, Euglenophytes, symbiotic prochlorophytes
Chlorophyll c_1	Diatoms, some Prymnesiophytes, some freshwater Chrysophytes, Raphidophytes
Chlorophyll c_2	Most diatoms, Cryptophytes, Dinoflagellates, Prymnesiophytes, Chrysophytes Raphidophytes
β,β carotene	All algae except cryptophytes and rhodophytes
β,ε carotene	Cryptophytes, prochlorophytes, rhodophytes, green algae
Alloxanthin	Cryptophytes
19'butanoyloxyfucoxanthin	Some Prymnesiophytes, one Chrysophyte, several dinoflagellates
Diadinoxanthin	Diatoms, Dinoflagellates, Euglenophyta, Prymnesiophytes Chrysophytes Raphidophytes
Diatoxanthin	Euglenophyta, Bacillariophyta, Dinophyta, Prymnesiophyceae Chrysophyceae Raphidophyceae
Dinoxanthin	Dinoflagellates
Fucoxanthin	Diatoms, Prymnesiophytes Chrysophytes Raphidophytes, several dinoflagellates
19'hexanoyloxyfucoxanthin	Prymnesiophytes, several dinoflagellates
Lutein	Chlorophytes, Prasinophytes
Neoxanthin	Chlorophytes, Prasinophytes, Euglenophytes
Peridinin	Dinoflagellates
Pheophytin *a*	Zooplankton fecal pellets, sediments
Prasinoxanthin	Some Prasinophytes
Violaxanthin	Chlorophyceae, Prasinophyceae, Eustigmatophytes
Zeaxanthin	Cyanophytes, Prochlorophytes, Rhodophytes, Chlorophytes, Eustigmatophytes

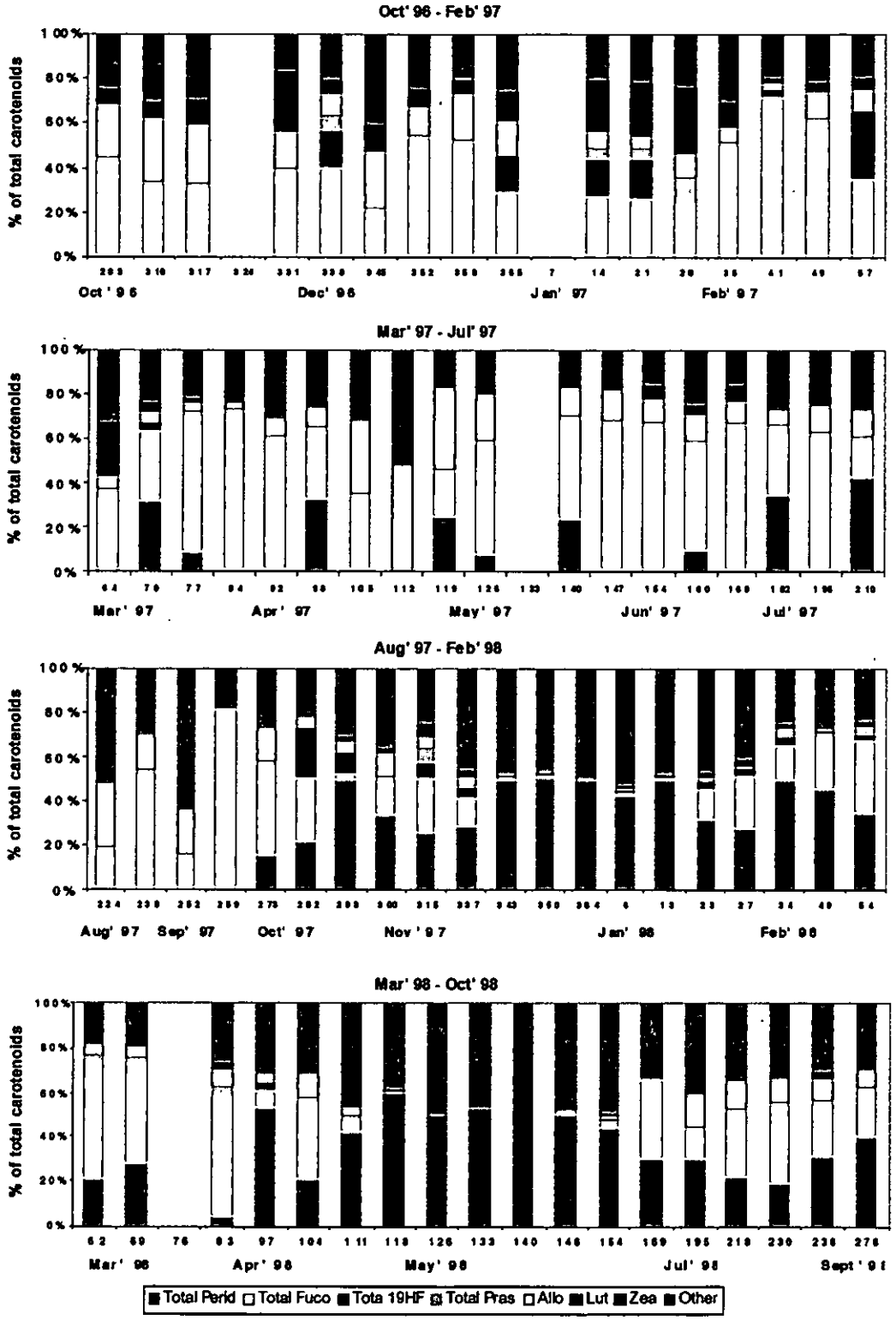


Figure 6.4: Marker pigment composition for depth-integrated samples from Killala Bay for the first two years of the study (reproduced from HEST, 2000). Perid = peridinin, Fuco = fucoxanthin, 19HF = 19'hexanoyloxyfucoxanthin, pras = prasinoxanthin, allo = alloxanthin, lut = lutein, zea = zeaxanthin and other = other carotenoids.

6.3 CHAPTER OUTLINE

In this chapter the bloom dynamics of phytoplankton in the Huon Estuary, south-east Tasmania are described, with particular attention to the toxic dinoflagellate *Gymnodinium catenatum*. The chapter is divided into three sections whose results are presented separately.

Section 1: Phytoplankton dynamics of the Huon Estuary. This section describes phytoplankton bloom dynamics in the Huon Estuary from 1996 to 1999 and includes analysis of pigment and nutrient composition further to the Huon Estuary Study.

Section 2: Dynamics of the toxic dinoflagellate *G. catenatum* (Dinophyceae) in the Huon Estuary. This section focuses on the dynamics of *G. catenatum* in the Huon Estuary from 1996 to 1999.

Section 3: Life cycle transitions in populations of the toxic dinoflagellate *G. catenatum* (Dinophyceae) in the Huon Estuary. This section focuses on the dynamics of life cycle transitions observed during blooms of *G. catenatum*.

MATERIALS AND METHODS

Unless otherwise indicated analyses were conducted by N. Parker. Field sampling in the first two years was conducted in collaboration with the Huon Estuary Study team and in the third year in collaboration with J. Skerratt.

6.4 FIELD SAMPLING METHODS

As part of the CSIRO Huon Estuary Study (HES) (July 1996 – August 1998), phytoplankton dynamics in the Huon Estuary were investigated through three sampling regimes that covered different spatial and temporal scales; quarterly spatial surveys, weekly monitoring, and automated continuous profiling (HEST, 2000). Although the data presented here were acquired primarily from the weekly monitoring and the automated continuous profiler, the other sampling regimes are described briefly to give context for references to the Huon Estuary Study report (HEST, 2000).

Temporal coverage of the estuary included ten 'spatial surveys,' which were conducted at approximately 3-month intervals. During these spatial surveys 63 geographically fixed sites throughout the estuary (Figure 6.3) were sampled according to their status as physical, chemical or biological sites: at all 63 sites, physical parameters (conductivity/salinity, temperature and depth) and fluorescence were measured through CTD casts. Thirty of these 'physical' sites were further identified as 'chemical' sites with water collection for chemical analysis of nutrients and other water quality parameters and phytoplankton net tows. Of the

30 chemical sites, 5 sites were further identified as 'biological' sites at which detailed analysis of phytoplankton was carried out.

In order to resolve shorter-term phytoplankton dynamics, weekly to fortnightly sampling was carried out at the five biological sites. The biological sites were Hideaway Bay (B1), Port Cygnet (X3b), Wheatleys (F3), Killala Bay (F1), and Brabazon Park (H3) (Fig. 6.3). The study of *G. catenatum* dynamics was focussed at these five sites.

At Killala Bay (F1) and Hideaway Bay (B1), automated *in situ* instrumentation made once per hour vertical profiles from the surface to approximately 3 m off the bottom (20 m and 35 m at Killala Bay and Hideaway Bay respectively), of salinity, temperature and fluorescence. The profiler took scans every 2 seconds with 0.25 m depth resolution, (binned into 0.5 metre depth intervals). Each cast at Killala Bay took approximately 4.5 minutes and approximately 6 minutes at Hideaway Bay. The rate of descent and ascent was 0.125 m s^{-1}. These instruments provided information on rapid changes in water column structure and phytoplankton biomass.

6.4.1 PHYSICAL PARAMETERS

Temperature measurements during the HES were obtained from the CTD trace (Seacat® SBE 19). During further monitoring (August 1998 to August 1999) temperature measurements were made using a conductivity meter (WTW® LF196).

Salinity measurements from bottle samples during the HES were made using a salinometer (Guildline® 8400B) or obtained from CTD traces (Seacat® SBE 19). During further monitoring (August 1998 to August 1999) salinity measurements were made using a conductivity meter (WTW® LF196).

Secchi depth was measured at all biological sites during spatial surveys and weekly monitoring. An estimate of the light extinction coefficient (k_d) was calculated from secchi depth using Equation 1 (Holmes, 1970).

$$K_d = \left(\frac{1.13}{SD}\right) + 0.1 \qquad \text{Equation 1}$$

Where SD = secchi depth.

6.4.2 WATER AND PHYTOPLANKTON COLLECTION

Net samples were taken at all chemical sites during spatial surveys and all biological sites during weekly monitoring for qualitative analysis of phytoplankton. A vertical net tow through the upper $^2/_3$ of the water column – equivalent to 12 m at Hideaway Bay, Killala Bay

and Port Cygnet, 9 m at Wheatleys and 8 m at Brabazon Park – was taken using a 20 µm mesh net (Australian Filter Specialists) with a mouth diameter of 230 mm. The net was deployed and retrieved at approximately 1 ms^{-1}. The concentrated sample in the cod-end was transferred to a 70 mL polystyrene jar and stored in cool dark conditions for return to the laboratory. The volume filtered by the net was approximately 45 L m^{-1}.

Integrated water column samples of the upper $^2/_3$ of the water column were collected using a weighted 12 m long clear plastic hose (Tygon® tubing), with an internal diameter of 20 mm. Samples were transferred to a 5 L pre-rinsed container, mixed, and subsamples taken for chlorophyll analysis and phytoplankton counts. Samples for chlorophyll analysis were stored in cool, dark conditions for return to the laboratory. Samples for phytoplankton counts were transferred to 1 L Karsten® bottles and fixed by the addition of 2 mL of Lugol's iodine solution (Throndsen, 1978). Lugol's fixed samples were stored in cool, dark conditions for return to the laboratory.

Discrete depth water samples were collected from three depths at all chemical (spatial surveys) and biological (weekly monitoring) sites (surface = 0 m, mid-depth = 2 m and bottom = 2 m above the sediment) using a Niskin bottle. A subsample of 1 L was transferred from the Niskin bottle to a polypropylene bottle (Kartell®) and stored in the dark on ice, until return to the laboratory for nutrient analysis. Another subsample of 1 L was also taken from each Niskin bottle sample, transferred to a plastic bottle and stored in cool, dark conditions until return to the laboratory for analysis of pigment composition.

6.4.3 FURTHER PHYTOPLANKTON MONITORING AUGUST 1998 – AUGUST 1999

Regular monitoring of phytoplankton dynamics in the Huon Estuary continued from the end of the Huon Estuary Study in August 1998 until August 1999, conducted by N. Parker and J. Skerratt. Sites regularly occupied were reduced to three – Port Cygnet (X3B), Wheatleys (F3) and Killala Bay (F1) – these sites were sampled at approximately 3-weekly intervals. At the three sites, surface water temperature and salinity, pycnocline depth, temperature and salinity below the pycnocline, and secchi depth were measured. Niskin Bottle samples at surface (0 m) mid-depth (2 m) and bottom (2 m above sediment) depths were taken for nutrient analyses and chlorophyll concentrations. A vertical net tow was taken for phytoplankton observations and a water column integrated sample was also taken for phytoplankton observations and enumeration of *G. catenatum* cells – *G. catenatum* sexual stages were enumerated in all integrated samples. Methods for the processing of samples for nutrient analysis, chlorophyll determinations and phytoplankton enumeration were the same as those used in the Huon Estuary Study as outlined below.

6.5 LABORATORY METHODS

6.5.1 PHYTOPLANKTON OBSERVATIONS, IDENTIFICATION AND ENUMERATION

Vertical net tow samples were examined for qualitative observations of living phytoplankton. Phytoplankton from all net samples were observed within 24 hours of collection using a Leitz Labovert inverted microscope. The sample was thoroughly mixed and a subsample poured into a 55 mm petri dish (approximately half of the net sample). Phytoplankton were identified to species or genus and observations recorded of relative abundance (present, common, dominant or bloom). General 'cell health' observations were also recorded, including observations of motility, cell integrity, the presence of bacteria, evidence of sexual reproduction, and grazing.

Lugol's fixed integrated water column samples (approximately 1L) were concentrated down to a volume of approximately 10 mL by serial settling in measuring cylinders. 1 mL of the concentrated sample was then transferred to a Sedgewick-Rafter Cell (Guillard, 1978) and phytoplankton identified to genus or species level and enumerated using a Lietz® Labovert inverted microscope. Flagellates in the nanoplankton group (2-20 μm) were grouped, as positive identifications could not be made using the inverted light microscope. Species counts during the first two years of the study were conducted by P. Bonham (CSIRO Marine Research) and by N. Parker in the third year. Sexual stages of *G. catenatum* were enumerated by N. Parker for all three years.

6.5.2 SPECTROPHOTOMETRIC CHLOROPHYLL DETERMINATION

Chlorophyll concentrations were measured by spectrophotometry (by the Huon Estuary Study Team in the first two years and by N. Parker and J. Skerratt in the third year). Samples of approximately 1 L were filtered onto 47 mm diameter Whatman GF/F filters, rinsed with filtered seawater (FSW), placed in cryovials (Nalgene®) and then frozen in liquid nitrogen until analysis. To extract the pigments, the filters were covered with 100% acetone, vortexed and sonicated in an ice water bath for 15 minutes. Water was then added to the acetone such that the extract mixture was 90:10 acetone:water (vol:vol) and sonicated once more. The extracts were then centrifuged through a Biorad® column to remove the filter paper. Samples were analysed on a GBC® UV/Vis 916 scanning spectrophotometer for the determination of chlorophylls *a*, *b* and *c*, measuring the absorption at 630, 647, 664 and 750 nm and calculated using the equations of Jeffrey and Humphrey (1975).

The determination of other pigment concentrations by HPLC was carried out by the Huon Estuary Study Team. This method followed the chlorophyll extraction procedure above, extracts were then filtered through a 0.2 μm membrane filter (Whatman®, anatope) prior to

analysis by HPLC using Waters® components, comprising a 600 controller, 717 plus refrigerated autosampler and a 996 photo-diode array detector. Pigments were separated using a stainless steel 25 cm x 4.6 mm I.D. column packed with ODS2 of 5 µm particle size (SGE) with gradient elution as described in Wright et al. (1991). The separated pigments were detected at 436 nm and identified against standard spectra using Waters Millennium software. Concentrations of chlorophyll *a*, chlorophyll *b*, β,ε carotene and β,β carotene in sample chromatograms were determined from standards (Sigma) and all other pigment concentrations were determined from standards of purified pigments obtained from algal cultures.

6.5.3 NUTRIENT ANALYSIS

50 mL of the water sample for nutrient analysis was transferred to a 70 mL bottle and frozen at -20 °C without filtration. An additional 50 mL of sample was filtered through acid washed (10% Hydrochloric acid) and milliQ® water rinsed Poretics® 0.2µm membrane filters in acid washed Millipore® 45 mm filtration rigs and then also transferred to a 70 mL bottle and frozen at -20 °C prior to analysis.

Two 10 mL aliquots of unfiltered sample were transferred to 10 mL polypropylene tubes and stored at 4°C for silicate analysis as silicate polymerises when frozen (Macdonald et al., 1986).

Two 10 mL aliquots of filtered sample were transferred to 10 mL polypropylene tubes and stored at -20°C for ammonia analysis. Ammonia concentrations were analysed by R. Watson by a newly developed flow injection method modified from Jones (1991) (Watson et al., unpublished data).

Nutrient concentrations were measured on a Technicon® AAII autoanalyser by K. Berry. The autoanalyser has four channels, one for each of the nutrients – silicate, nitrate/nitrite, nitrite and phosphate (Grasshof, 1976; Cowley et al., 1999). Standard flow-analysis techniques (Plaschke, 1999) were modified slightly for the determination of nutrients. Methods used for nutrient determinations are described in detail by Cowley et al. (1999) and Latham (2001).

6.6 STATISTICAL METHODS

Various factors have been identified as influencing phytoplankton dynamics including biological, chemical and physical factors. A number of statistical techniques were employed to investigate these relationships, starting with pair-wise correlation coefficients calculated between the entire suite of biological, chemical and physical parameters measured during the study (Table 6.4) using the statistical software SPSS (version 10.0, SPSS Inc.).

A mixture of other univariate and multivariate statistics were applied to the dataset. One-way ANOVAs of seasonally averaged data were calculated using SPSS (Version 10.0). Correspondence Analysis (CA) Principal Component Analysis (PCA) and Canonical Correspondence Analysis (CCA) were performed using Canoco for Windows (version 4.0). Short-term variability was smoothed by calculating seasonal averages of each parameter for each biological site ((B1, X3B, F1, F3, H3). Seasons were defined as bracketed by the dates of the equinoxes and solstices (e.g. spring lasts from September 22 to December 21). Transformations (either log or square root) were performed where necessary to remove skewness in the data

Table 6.4: Biological, chemical and physical factors measured during the study for which correlation coefficients were calculated.

Biological parameters	Chemical Parameters	Physical Parameters
G. catenatum vegetative and sexual cell concentrations	Nitrate/nitrate (filtered and unfiltered) Surface, mid-depth and bottom	Temperature, surface and mid-depth
Potential grazer concentrations	Phosphate (filtered and unfiltered) surface, mid-depth and bottom	Salinity, surface and mid-depth
Pigment concentrations, chlorophyll, peridinin and fucoxanthin	Silicate (filtered and unfiltered) surface, mid-depth and bottom	Secchi depth and light extinction coefficient
Bloom species cell concentrations	Ammonia (surface, mid-depth and bottom)	River flow (Frying Pan Creek)
	Dissolved oxygen	Rainfall – Geeveston and Franklin

6.6.1 CORRESPONDENCE ANALYSIS (CA)

Seasonal variation in bloom species abundance, pigments and nutrients were each analysed using Correspondence Analysis, an ordination technique that is often used when variable response curves can be assumed to be unimodal (Swadling et al., 2000). The biological sites within the estuary were treated as replicate seasonal samples (5 sites for the first two years and 3 for the third year). Some variables were included as passive due to missing data points, which skew the CA results. These variables did not influence the distribution of points but their relationship to other variables could still be determined. For the bloom species CA, the abundance of six algal groups (*G. catenatum* vegetative cells, *Ceratium* spp., *Chaetoceros* spp., *Pseudonitzschia* spp., *Polykrikos schwartzii* and small flagellates) were analysed. Cell counts of bloom species were log transformed and the abundance of *G. catenatum* sexual stages was included as a passive variable. The pigment CA, included 23 marker pigments (rare pigments downweighted). In the nutrient CA, 8 nutrients from 3 depths were analysed, and ammonia and dissolved oxygen (also from 3 depths) were included as passive variables due to gaps in the dataset.

6.6.2 PRINCIPAL COMPONENTS ANALYSIS (PCA)

Seasonal variation in physical parameters was examined by Principal Components Analysis. PCA is an ordination technique used to reduce the dimensionality of multivariate data sets and enable graphical presentation of the relationships between factors (Swadling et al., 2000). Four physical variables were included in the analysis; surface salinity, mid-depth salinity, surface temperature and light extinction coefficient (estimated from secchi depth using Equation 1). A further three physical variables were analysed as passive variables; average flow (measured at frying pan creek), total rain (seasonal average of rainfall from Geeveston and Franklin) and bottom salinity. No transformations were required for physical variables.

6.6.3 CANONICAL CORRESPONDENCE ANALYSIS (CCA)

Variation in biological parameters (bloom species and pigments) was analysed in relation to environmental parameters (chemical and physical variables) by Canonical Correspondence Analysis (CCA). CCA is a multivariate direct gradient analysis technique in which the ordination axes are constrained to be linear combinations of environmental variables (Ter Braak, 1986). CCA was used to identify which environmental variables could directly account for variations observed in the biological data. The minimal number of explanatory variables to be included in the ordinations explaining statistically significant ($p \leq 0.05$) proportions of variation in the biological data were identified by the forward selection option of CCA in Canoco (Version 4.0), which is analogous to the forward selection process used in step-wise regression (Ter Braak, 1990). Canonical coefficients (significance judged by approximate t-tests) were examined to estimate the relative contributions of the individual environmental variables to the ordination axes (Pienitz et al., 1995).

Seasonal averages of pigment data (all 23 marker pigments with rare species down-weighted, first two years only) and bloom species data (all three years) were analysed by CCA. Including nutrient concentrations at all three depths in the CCA resulted in high variance inflation factors. Three separate CCAs were therefore calculated for all physical variables plus nutrients at each depth. Six algal groups were included in the bloom species CCA - *G. catenatum* vegetative cells, *Ceratium* spp., *Polykrikos schwartzii*, *Chaetoceros* spp., *Pseudonitzschia* spp., and mixed small flagellates. *Gymnodinium catenatum* sexual cells was included as a passive variable due to gaps in the dataset and the influence of environmental variables on this parameter addressed separately (see below). Four physical variables were included in the bloom species and pigment CCAs: surface salinity, mid-depth salinity, surface temperature, and light extinction coefficient. The difference in salinity between surface and mid-depth was initially included but eventually excluded due to colinearity with other variables.

Six (bloom species and pigment CCAs) or eight (sexual stage CCAs) chemical variables from three depths were included in the CCA analyses. These variables were; nitrate+nitrite, nitrite, total dissolved nitrogen, phosphate, total dissolved phosphorus, silicate, total nitrogen and total phosphorus. Total nitrogen and total phosphorus were only included in the sexual stage CCAs.

The subset of the dataset for which sexual stages were enumerated was also analysed by CCA. Data from F1 (Killala Bay) and F3 (Wheatleys) were averaged and each sampling date for the three years (for which nutrient data were also collected) was included in the CCA. Six other algal groups were included with *G. catenatum* sexual cells in the analysis – *G. catenatum* vegetative cells, *Ceratium* spp., *Polykrikos schwartzii*, *Chaetoceros* spp., *Pseudonitzschia* spp., and mixed small flagellates. Six physical variables were included in the analysis – average rainfall from Geeveston and Franklin, light extinction coefficient calculated from secchi depth, average flow from frying pan creek, cumulative flow for 3 weeks prior to sampling, surface salinity and surface temperature.

6.7 SUPPORTING DATASETS

Mean daily Huon River flow measured at Frying Pan Creek, upstream of Judbury (Figure 6.3) was provided by the Tasmanian Department of Primary Industry, Wildlife and the Environment (DPIWE). The Tasmanian Bureau of Meteorology provided daily rainfall data from Geeveston and Franklin and wind speeds from Cape Bruny, and the Tasmanian Shellfish Quality Assurance Program, Tasmanian Department of Health and Human Services provided shellfish toxicity data determined by both HPLC and mouse bioassay.

RESULTS SECTION 1: PHYTOPLANKTON DYNAMICS

6.8 CHLOROPHYLL CONCENTRATIONS

Chlorophyll *a* concentrations indicated low over-wintering biomass in the estuary, with less than 1.0 mg m^{-3} mean chlorophyll *a*. Biomass increased in spring and through summer and decreased again in autumn (Fig. 6.5). Patterns of chlorophyll *a* concentrations were generally similar between the five biological sites although the magnitude of the concentrations differed (Fig. 6.5). A one-way ANOVA of seasonal log chlorophyll *a* indicated that chlorophyll *a* levels were significantly different between seasons ($F_{11,41}$=11.50, $p<0.01$). Post hoc Tukey Tests showed that summer and autumn mean chlorophyll *a* concentrations were significantly lower in 1996/97 than in either 1997/98 or 1998/99 and were not significantly different to winter chlorophyll levels in that year (Fig. 6.6). The presence of dinoflagellates (in particular *G. catenatum*) substantially increased mean seasonal chlorophyll *a* concentrations (Fig. 6.6). The maximum integrated chlorophyll *a*

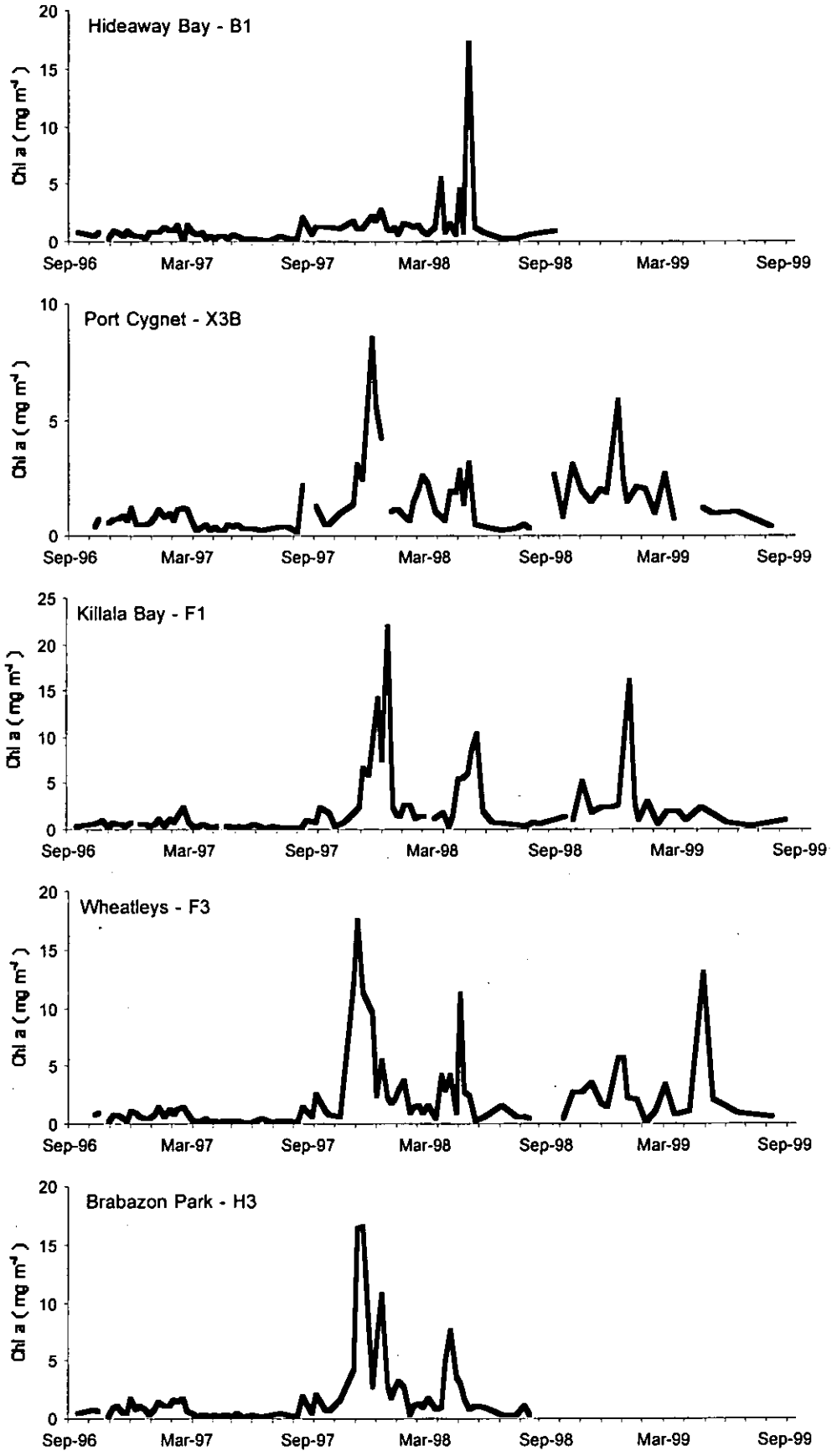


Figure 6.5: Chlorophyll *a* from integrated samples at all five biological stations versus time for the three years of the study (Hideaway Bay and Brabazon Park were only sampled during the first two years).

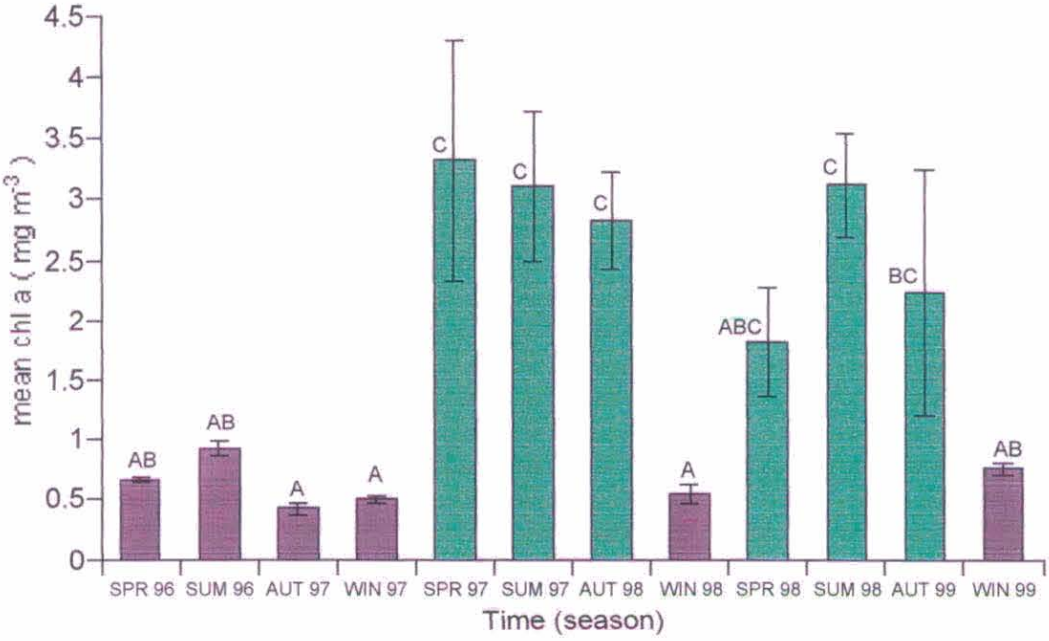


Figure 6.6: Seasonal mean chlorophyll *a* (± se). Green bars indicate seasons in which *G. catenatum* blooms were recorded.

concentration recorded was 21.9 mg m^{-3} at Killala Bay on 13 January 1998 during a bloom of *G. catenatum*.

6.9 DYNAMICS OF BLOOM SPECIES

Five microalgal groups were found to be dominant either numerically or in terms of biomass during the three year study (Fig. 6.7). These were the small flagellates (2-20 µm, incorporating various algal classes including prasinophytes, cryptophytes and prymnesiophytes – based on pigment composition and personal observations), *Pseudonitzschia* spp., *Chaetoceros* spp., *Ceratium* spp. and *Gymnodinium catenatum*. The small flagellates were present in high numbers throughout the year although their contribution to biomass was small. The other taxa showed clear 'bloom' events of elevated cell numbers compared to their background levels (Fig. 6.7). The dynamics of these 'bloom forming species' were generally consistent between the five biological sites although short time lags and biomass differences between sites were observed (Fig. 6.7).

Low biomass over winter was observed in all three years of the study. In 1996/97 the low biomass continued through the spring and summer. The large spring blooms of diatoms observed in the two latter years were not detected in 1996/97, although a gap in sampling may have missed such a bloom. Dinoflagellates were almost absent from the water column from the beginning of the study in July 1996 until February 1997. In the second and third years of the study large spring diatom blooms were followed by dinoflagellate blooms dominated by the toxic species *G. catenatum* (Fig. 6.7). The summer *G. catenatum* dominated blooms were followed by mixed diatom/dinoflagellate blooms (*Chaetoceros* spp., *Pseudonitzschia* spp. and *Ceratium* spp.) in the late summer, and a second *G. catenatum* bloom in the autumn. Occasional blooms of *Polykrikos schwartzii* were also observed; *P. schwartzii* is a heterotrophic dinoflagellate that was observed feeding on *Gymnodinium catenatum*.

Figure 6.8 shows the distribution of seasonally averaged samples from the five biological sites with respect to the dominant algal groups (including *P. schwartzii* with the other 5 groups) as determined by correspondence analysis (CA). Sites within seasons within years generally clustered together, reflecting the estuary wide nature of the observed phytoplankton dynamics. Clustering of samples from a particular site over the period of the study was not observed, indicating a strong seasonal dynamic. Samples from 1996 clustered separately from the other two years. The 1996 cluster of samples occurred to the right of the diagram, reflecting a stronger contribution by flagellates and diatoms in these samples. Considerable overlap of samples was observed between seasons in years 1997 and 1998. Autumn and winter of 1997 were clustered closer to the right of the diagram (dominated by

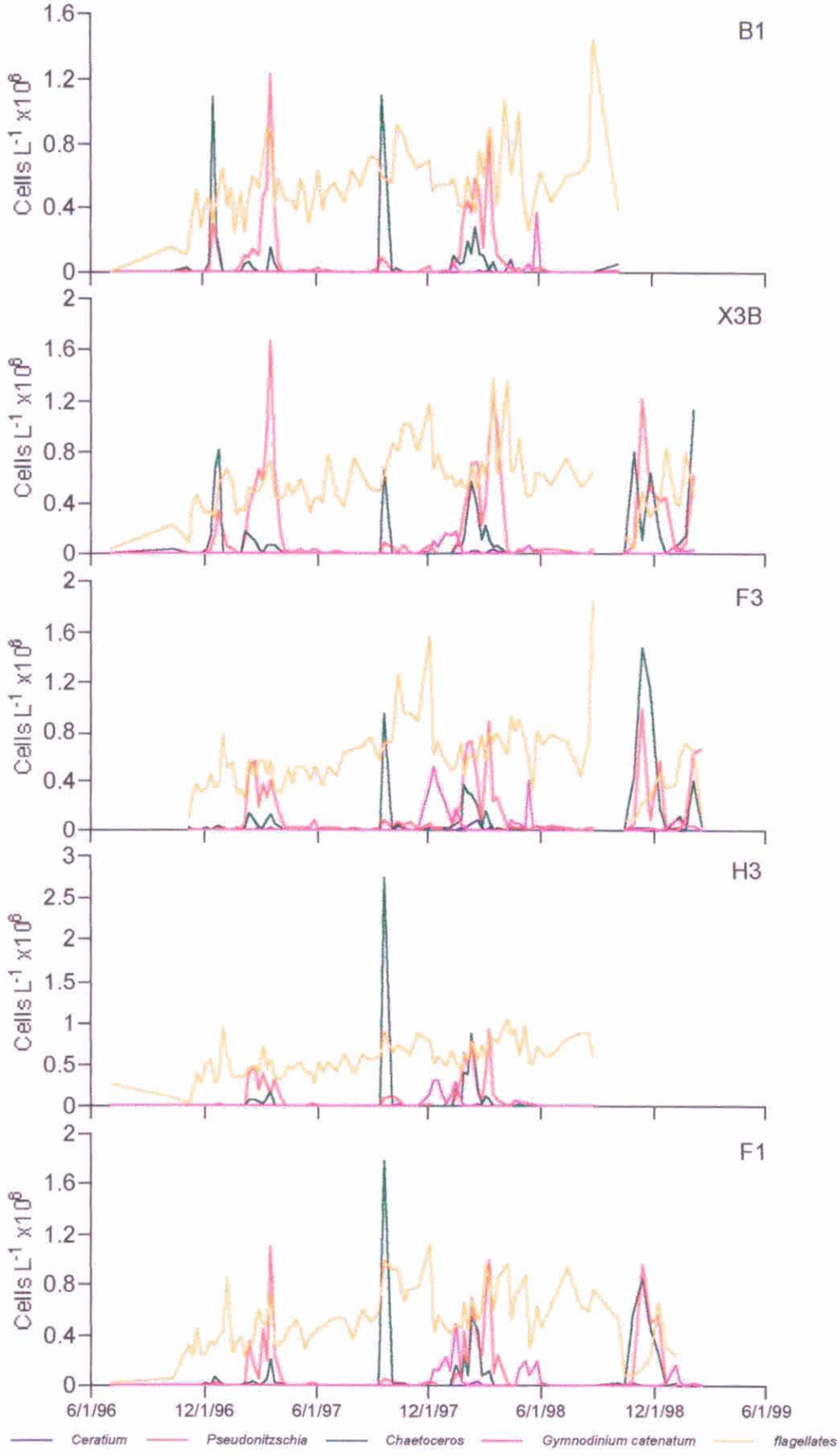


Figure 6.7: Cell densities of the main bloom forming taxa observed in the Huon Estuary during the three year study at the five biological sites Hideaway Bay (B1), Port Cygnet (X3B), Wheatleys (F3), Brabazon Park (H3) and Killala Bay (F1). Hideaway Bay and Brabazon Park were only sampled in the first two years of the study.

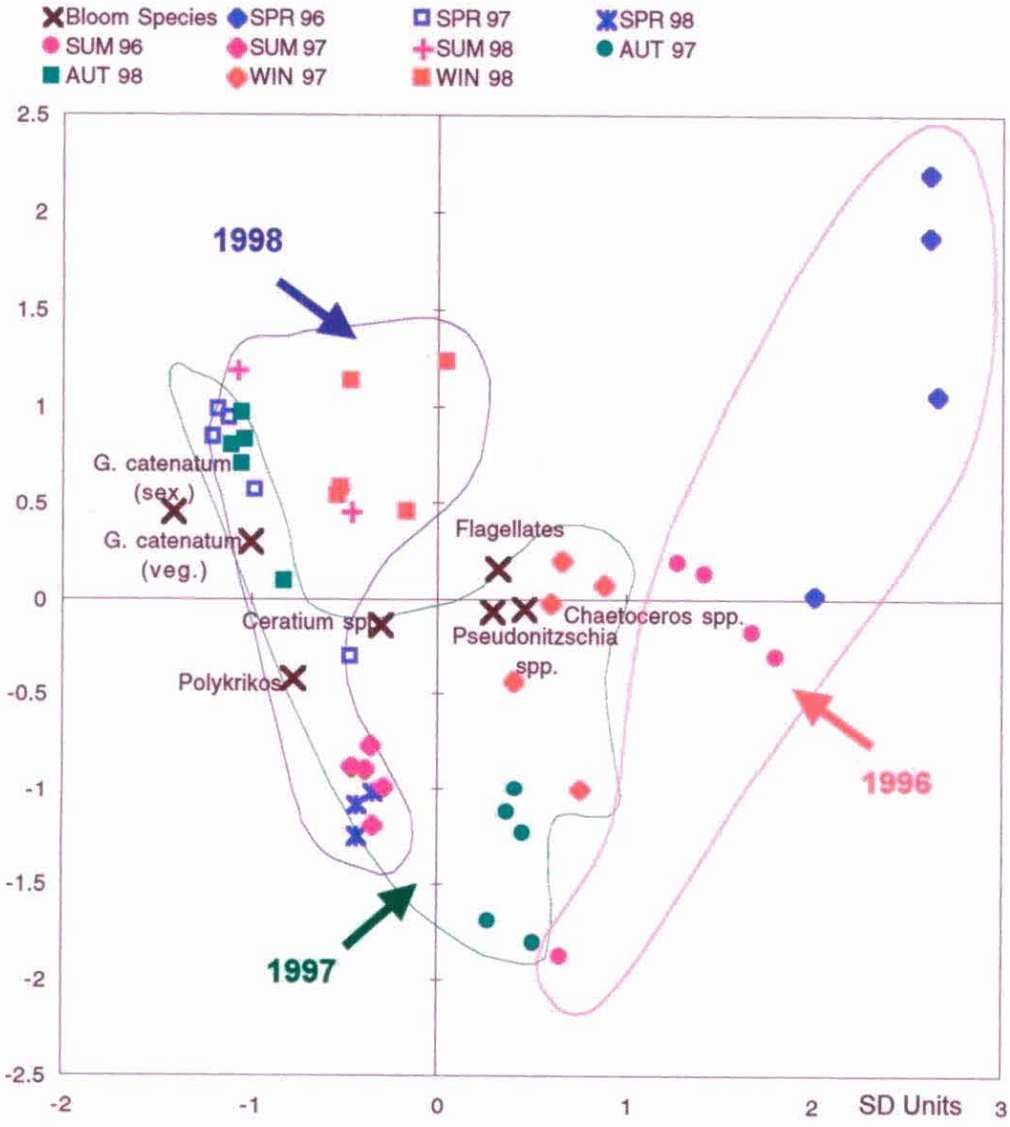


Figure 6.8: Correspondence analysis (CA) of phytoplankton taxa over 10 seasons. Samples represent seasonal averages for an individual site. *Gymnodinium catenatum* (sexual stages) was included as a passive variable and therefore does not effect the distribution of samples. Lines encircle all data from a particular year (colour coded). All cell counts were log transformed.

diatoms and flagellates) with all subsequent seasons in 1997 and 1998 clustered to the left of the diagram and dominated by dinoflagellates.

6.10 DYNAMICS OF MARKER PIGMENTS

General dynamics of marker pigments are further described in Section 6.2.2 and Figure 6.4. These data were also analysed by CA for comparison with results of CA analysis of the major bloom species (Section 6.9). Pigment concentrations reflect the entire phytoplankton biomass rather than just the dominant species, and also give an indication of the relative contribution of different taxa to overall biomass. In particular pigment composition is useful for determining the contribution to the phytoplankton community of species that are too small for identification by light microscopy. Figure 6.9 shows the distribution of seasonally averaged sites with respect to pigment composition as determined by CA. Concentrations of marker pigments were only analysed in the first two years (nine seasons) as part of the Huon Estuary Study (HEST, 2000). Observed patterns were similar to those seen in the dominant bloom species CA (Fig. 6.8). Clustering of samples from a particular site over the period of the study was not observed. The separation of the 1996 seasons from the 1997 and 1998 seasons was more pronounced than in the bloom species CA and may reflect the high biomass contribution of dinoflagellates relative to cell numbers in the latter two years. All winter samples and autumn 1997 samples clustered together in the upper right sector of the diagram and were influenced by marker pigments for cryptophytes, green algae, chrysophytes and diatoms. The 1996 sites clustered towards the lower right sector of the diagram and were influenced by marker pigments for a variety of flagellates (prymnesiophytes, chlorophytes, prasinophytes, green algae, and cyanobacteria). The remainder of samples clustered to the left of the diagram and were influenced by dinoflagellate pigments, in particular those present in *G. catenatum* (chlorophylls c_1+c_2, peridinin, diadinoxanthin, dinoxanthin and diatoxanthin). Chlorophyll *a* and its allomer occur in all algae and β,β carotene in most, this was reflected by their position close to the origin of the diagram. Interestingly, the chlorophyll *a* epimer pigment, which is also found in most algae was more closely associated with the dinoflagellate pigments. Chlorophyll *a* epimer generally occurs in low concentrations and may therefore be more readily detected in the high biomass *G. catenatum* blooms. Phaeophytin *a*, which is a chlorophyll degradation product associated with grazing was also clustered with the dinoflagellate pigments.

6.11 NUTRIENT DYNAMICS

Nutrient concentrations in the Huon Estuary were analysed using CA (for a more general description of nutrient dynamics in the estuary see Section 6.2.1). Figure 6.10 shows the distribution of nutrients relative to the seasonally averaged sites as determined by CA. The

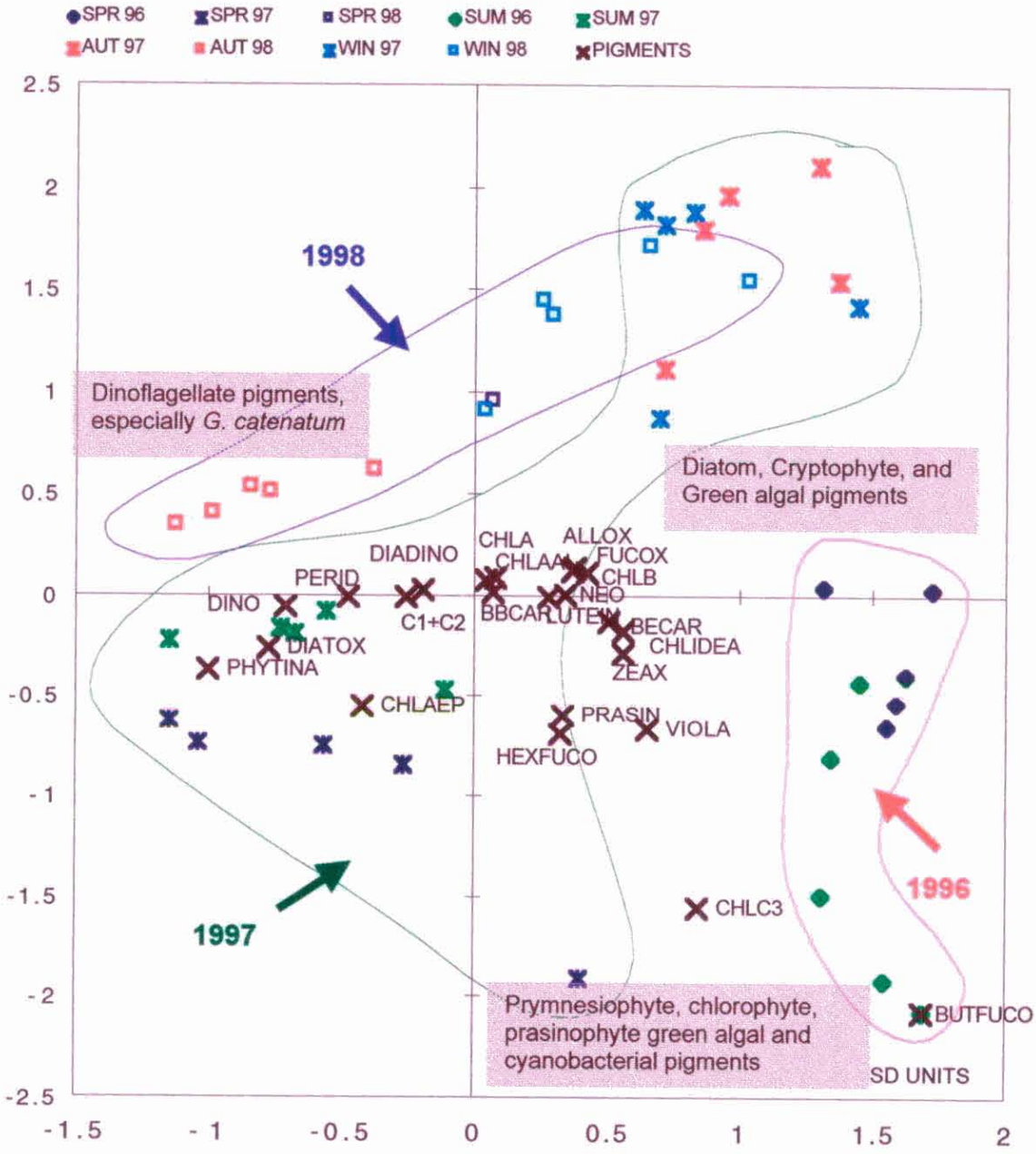


Figure 6.9: Correspondence analysis (CA) of 23 marker pigments over 9 seasons. Samples represent seasonal averages for an individual site. Pigments to the left of the diagram are associated with dinoflagellates, and to the right with diatoms and flagellates (grey text boxes indicate algal groups associated with pigments in that section of the diagram). Lines encircle all data from a particular year (colour coded). Pigment abbreviations are: ALLOX = alloxanthin, BECAR = β,ε-carotene, BBCAR = β,β carotene BUTFUCO = 19'butanoyloxyfucoxanthin, CHLA = chlorophyll *a*, CHLB = chlorophyll *b*, C1+C2 – chlorophyll c_1+c_2, CHLC3 = chlorophyll c_3, CHLAEP = chlorophyll *a* epimer, CHLAAL = chlorophyll *a* allomer, CHLIDEA = chlorophyllide *a*, DIADINO = diadinoxanthin, DIATOX = diatoxanthin, DINO = dinoxanthin, FUCOX = fucoxanthin, HEXFUCO = 19'hexanoyloxyfucoxanthin, LUTEIN = lutein, NEO = neoxanthin, PERID = peridinin, PHYTINA = phaeophytin a, PRASIN = prasinoxanthin, VIOLA = violaxanthin, ZEAX = zeaxanthin,

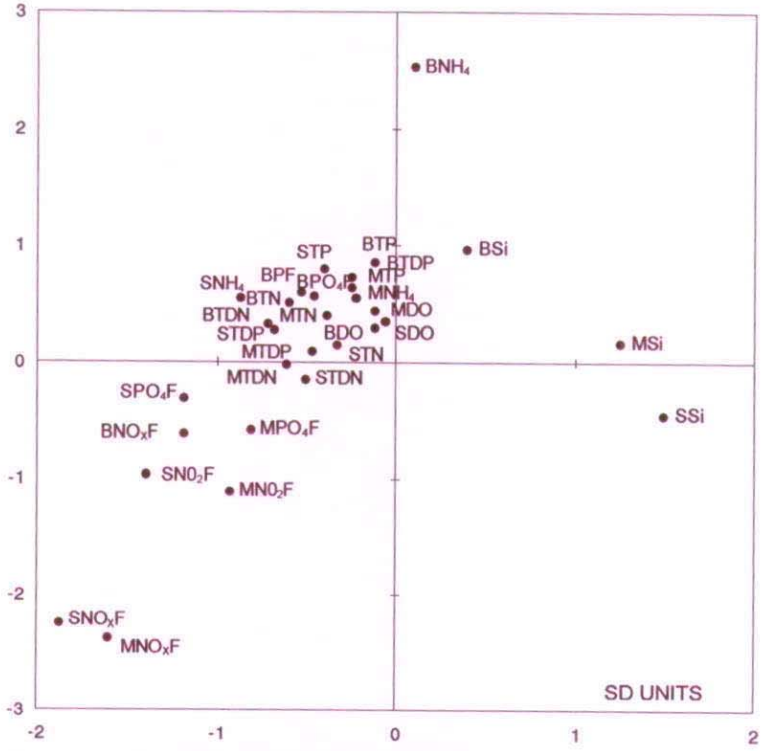


Figure 6.10: Correspondence analysis (CA) of 8 nutrients from three depths over 11 seasons (see Fig. 6.11 for position of samples with respect to nutrients). Nutrient variables have been multiplied by a factor of 4 to prevent crowding. Dissolved oxygen (DO) and ammonia (NH_4) were included as passive variables due to gaps in the datasets for these variables, therefore they do not influence the distribution of samples. Nutrient prefixes S, M and B indicate surface, mid-depth and bottom samples respectively. Nutrient abbreviations are; Si = silicate, NO_xF = filtered nitrite+nitrate, NO_2F = filtered nitrite, PO_4F = filtered phosphate, TDN = total dissolved nitrogen, TDP = total dissolved phosphorus, TN = total nitrogen and TP = total phosphorus.

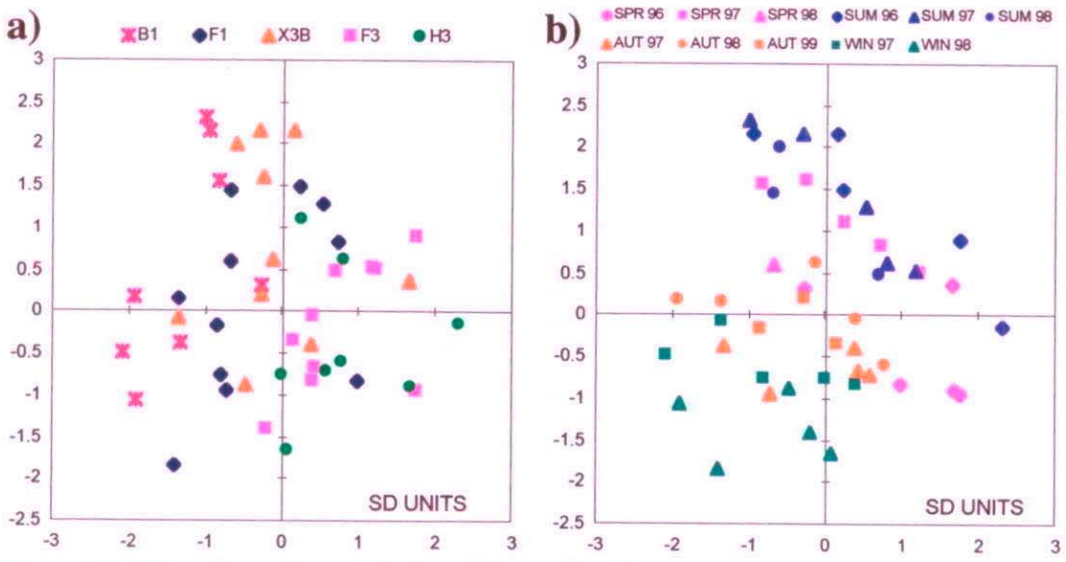


Figure 6.11: Correspondence analysis (CA) of 8 nutrients from three depths over 11 seasons (see Fig. 6.10 for nutrient variables). Samples represent seasonal averages for an individual site. a) separation of samples by site and b) separation of samples by season. Site codes are B1 = Hideaway Bay, F1 = Killala Bay, X3B = Port Cygnet, F3 = Wheatleys, H3 = Brabazon Park. Season abbreviations are SPR = spring, AUT = autumn, SUM = summer, and WIN = winter.

distribution of seasonally averaged sites in relation to nutrients within the estuary showed clustering of sites from different years (Fig. 6.11a), in contrast to the bloom species and pigment CA analysis, which did not (Figs 6.8 and 6.9). Site clusters progressed from the most oceanic site (B1 – Hideaway bay) at the left of the diagram to the site with the strongest riverine influence (H3 – Brabazon Park) at the right (Figure 6.11a). This horizontal axis was primarily influenced by silicate concentrations in surface and mid depth waters. The ocean to river mouth trend is consistent with observations from the Huon Estuary Study of silicate being inversely proportional to salinity, and suggests a primarily terrestrial origin for silicate in the system (HEST, 2000). Within individual seasons, samples were less closely clustered than for bloom species and pigment CAs, however samples from the same seasons in different years were more closely clustered than in the other two CAs (Fig. 6.11b). The distribution of winter and autumn samples was largely driven by high nitrite, nitrate+nitrite, phosphate, and total dissolved nitrogen, while spring and summer samples were more closely associated with total nitrogen, total phosphorus, and total dissolved phosphorus. Bottom ammonia did not cluster with ammonia at other depths or with any other nutrients; this was a passive variable and therefore did not influence the distribution of samples but indicates that this nutrient has different dynamics to the others.

6.12 PHYSICAL CHARACTERISTICS OF THE HUON ESTUARY

6.12.1 SECCHI DEPTH

Secchi depth measurements were taken at all sites during weekly/fortnightly sampling for the three year study and ranged between 0.6 and 10.5 m with an overall mean secchi depth for the biological sites of 4.2 (Table 6.5). The light extinction coefficient (k_d), estimated from secchi depth using equation 1 (Holmes, 1970), varied between 0.21 and 1.98 with an overall mean for the biological sites of 0.5 (Table 6.5).

Table 6.5: Mean and range for all secchi depth (SD) and light extinction coefficient (k_d) measurements from the five biological sites. Sites with the same letter were not significantly different from one another ($p>0.05$, Tukey Test)

	Brabazon Park (H3)	Wheatleys (F3)	Killala Bay (F1)	Port Cygnet (X3B)	Hideaway Bay (B1)	Overall Mean
SD (m) mean	2.6^{a}	3.0^{a}	5.0^{c}	4.2^{b}	5.9^{d}	4.2
SD (m) range	0.7 – 6.5	0.60 – 8.50	1.2 – 10.0	0.9 – 9.0	1.3 – 10.5	0.6 – 10.5
k_d (m^{-1}) mean	0.67^{a}	0.64^{a}	0.38^{bc}	0.46^{b}	0.34^{c}	0.5
k_d (m^{-1}) range	0.27 – 1.71	0.23 – 1.98	0.21 – 1.04	0.23 – 1.36	0.21 – 1.00	0.21 – 1.98

Secchi depths were significantly different between sites ($F_{4,424}$=40.46, p<0.001) (Table 6.5). Hideaway Bay (B1) secchi depths were significantly deeper than any of the other biological sites, reflecting the greater oceanic influence at the mouth of the estuary (p<0.05, Tukey Test), Killala Bay had the next deepest secchi depths followed by Port Cygnet (both significantly different to Hideaway Bay). Brabazon Park (H3) and Wheatleys (F1) have the strongest influence from the Huon River and had significantly shallower secchi depths than all other biological sites (not significantly different to one another). The significant difference between Killala Bay and Wheatleys, which are directly across the estuary from one another, highlights the stronger influence of the Huon River on the north/northeast side of the Huon Estuary (Table 6.5). Secchi depth did not vary significantly between seasons ($F_{11,40}$=0.69 p=0.733) and was highly positively correlated with surface salinity (r=0.588, p<0.01), indicating that freshwater input, primarily from the Huon River, is a determining factor in light attenuation.

6.12.2 PCA ANALYSIS OF PHYSICAL VARIABLES

The physical characteristics of the Huon Estuary are described in general terms in the introduction (Section 6.2). Physical variables were analysed by Principal Components Analysis (PCA). Figure 6.12 shows the distribution of seasonally averaged physical variables by site as determined by PCA. PCA axis 1 explained 81 % of the variation in the physical data, with PCA axis 2 accounting for a further 13 %. The variables that were associated with axis 1 included surface salinity and the light extinction coefficient (estimated from secchi depth) while surface temperature was more closely associated with axis 2. The distribution of seasonally averaged site data showed a separation of seasons along axis 2, driven by surface temperature (Fig. 6.12a). Sites were separated along axis 1 in association with surface salinity and light extinction coefficient with samples from the marine end site (B1 - Hideaway Bay) situated to the left of the diagram and samples from the site furthest up the river (H3 - Brabazon Park) to the right of the diagram (Fig. 6.12b). There was no clear pattern of samples associated with either years or seasons within years.

RESULTS SECTION 2: *GYMNODINIUM CATENATUM* DYNAMICS

6.13 *GYMNODINIUM CATENATUM* BLOOM DYNAMICS

Gymnodinium catenatum cells were absent or below detectable levels (<5 x 10^{-3} cells L^{-1} in net samples) in the water column between July 1996 and March 1997 at all five biological sites (see Fig. 6.4 for site locations). In April 1997 *G. catenatum* cells were first recorded in the water column in low numbers (Fig. 6.13a). Cell numbers increased during October and November 1997, with the earliest bloom concentrations (>10,000 cells mL^{-1}) being detected

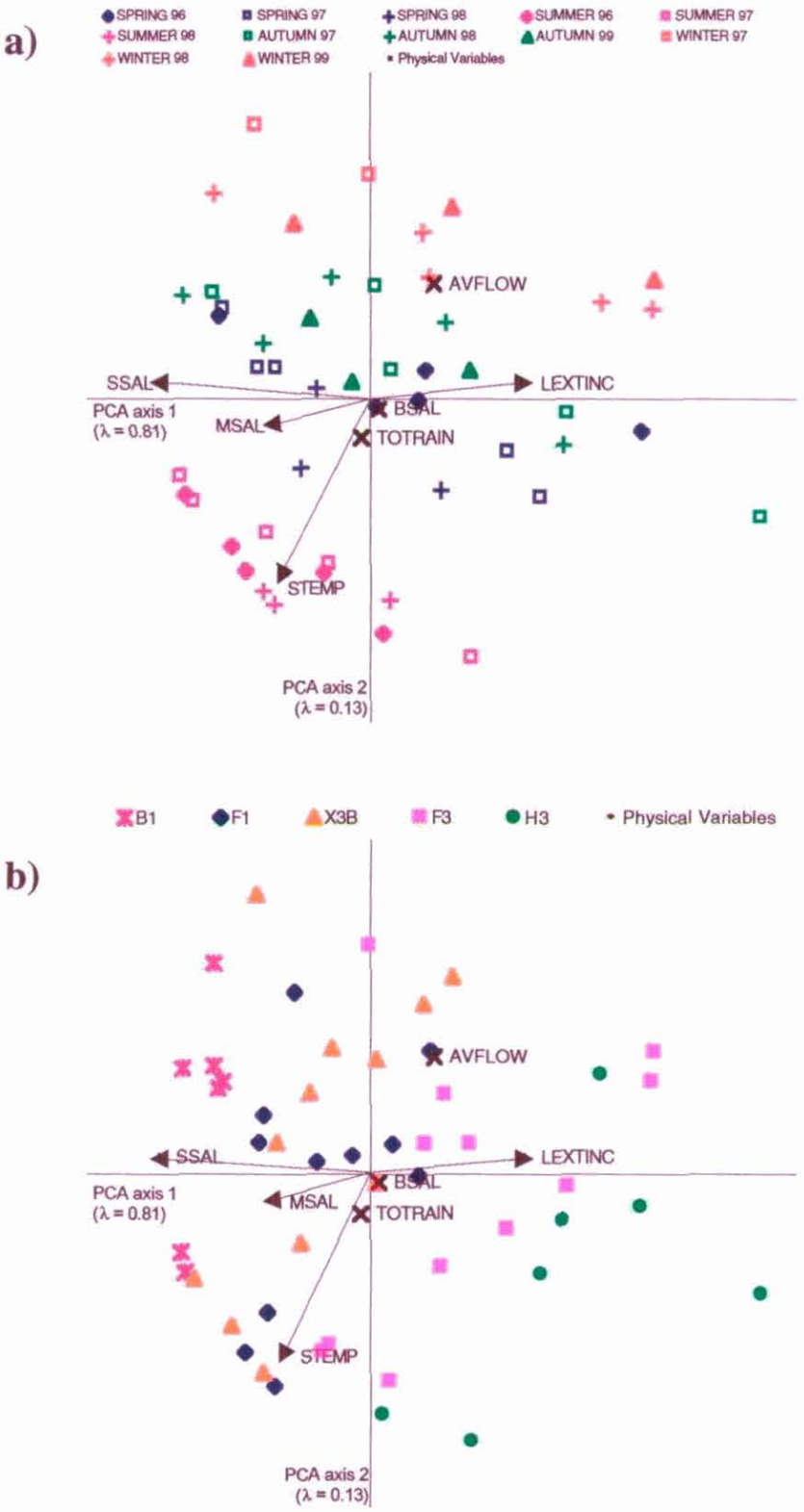


Figure 6.12: Principal Components Analysis (PCA) of the five physical variables measured for five sites over three years. The three passive variables are represented by an X without an arrow. a) separation of seasons and b) separation of sites within the estuary. Physical variable abbreviations are; SAL = salinity, TOTRAIN = rainfall measured from Geeveston and Franklin, AVFLOW = average daily flow measured at Frying Pan Creek, STEMP = surface water temperature, LEXTINC = light extinction coefficient estimated from secchi depth.

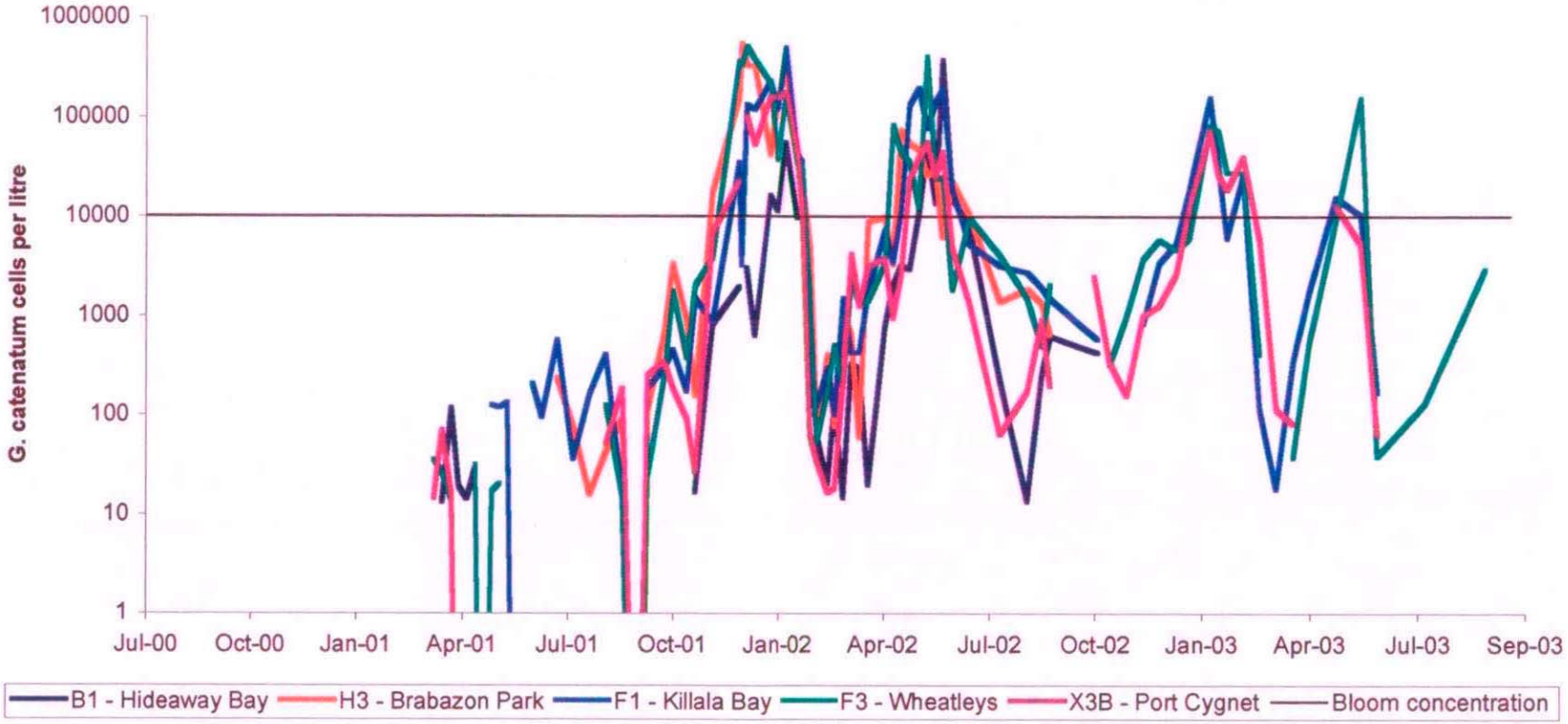


Figure 6.13a: *Gymnodinium catenatum* cell densities throughout the study (log scale) at the five biological sites for the first 2 years and at X3B, F1 and F3 for the third year.

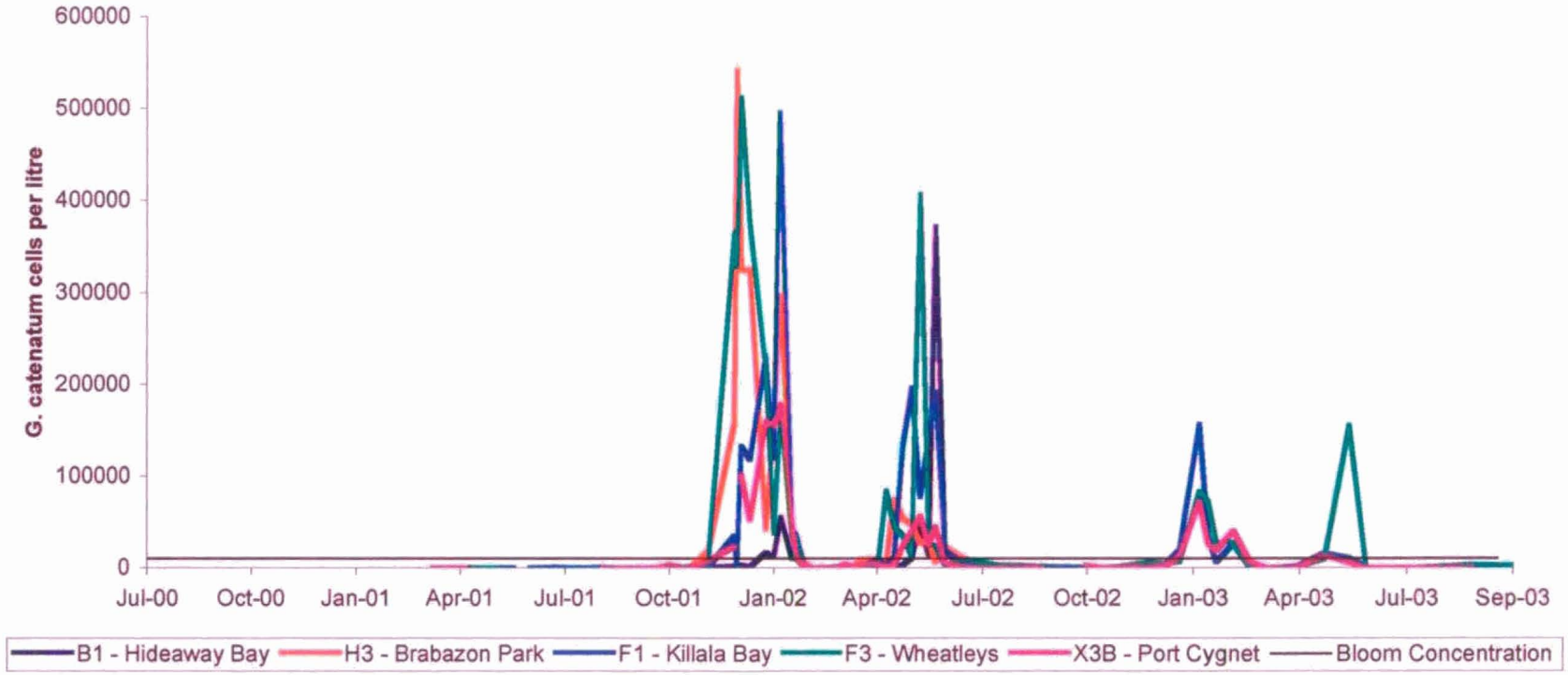


Figure 6.13b: *Gymnodinium catenatum* cell densities throughout the study (linear scale) at the five biological sites for the first 2 years and at X3B, F1 and F3 for the third year.

in the mid estuary. By early December 1997 cell densities were up to 5.5 x 10^5 cells L^{-1} (Fig. 6.13b) and the bloom was distributed throughout the lower part of the estuary (detected in net tows from a spatial survey).

Plankton net tows taken during the high biomass summer blooms were covered with a colourless mucilage (personal observation), made up of polysaccharides and proteins (P. Mansour, unpublished data). In periods of strong river flow during the summer bloom the lower salinity surface layer was up to 3 m deep and *G. catenatum* cells showed evidence of osmotic stress with cells becoming rounded and lysing. The summer bloom declined in mid January 1998, but was followed by a second bloom in the autumn that began in mid-April and continued through until late June 1998 (Figs. 6.13a,b). Cell densities were lower than during the summer bloom (Figs. 6.13a,b) although the maximum cell density (observed at Wheatleys – F3) was similar (4.0 x 10^5 cells L^{-1}) to the peak cell concentration of the summer bloom (observed at Brabazon Park – H3).

Low cell densities of *G. catenatum* were observed throughout the following winter (Fig. 6.13a). Another bloom of *G. catenatum* developed in the summer of 1998/99, this bloom began in mid December 1998 and continued through till early February 1999 with lower cell densities than the previous summer bloom (Fig. 6.13a and 6.13b) with a maximum observed cell concentration of 1.6 x 10^5 cells L^{-1}at Killala Bay (F1). A fourth bloom was recorded in the autumn of 1999 beginning in mid April and continuing through till mid May. The autumn 1999 bloom had lower cell densities compared with the previous autumn bloom (Fig. 6.13a and 6.13b) with a maximum of 1.6 x 10^5 cells L^{-1} at Wheatleys (F3) and with much lower cell densities observed at the other two sites, Killala Bay (F1) and Port Cygnet (X3B) (Fig. 6.13a and 6.13b). The amount of mucilage produced during autumn blooms appeared to be less than in the summer blooms at similar *G. catenatum* cell densities.

The net growth rate of *G. catenatum* during bloom development, calculated from the mean of all biological sites, was similar for the four blooms observed, ranging between 0.08 and 0.10 d^{-1}. Much higher growth rates were calculated over shorter time periods, as high as 0.9, which were most likely due to advection of cells into the region rather than in situ growth, since the maximum growth rate recorded in laboratory studies is 0.33 d^{-1} (Blackburn et al., 1989).

Gymnodinium catenatum cells at Hideaway Bay, the site with the strongest marine influence, regularly appeared less 'healthy' than cells in the mid estuary and also tended to have shorter chain lengths (personal observations). Hideaway Bay is more exposed to oceanic influences, is the deepest site (35 m) and tended to have greater turbulence than the other biological sites (HEST, 2000).

6.14 SHELLFISH TOXICITY AND *GYMNODINIUM CATENATUM*

Toxin concentrations of mussels growing either in the inter-tidal zone or on longlines, as determined either through a mouse bioassay or by HPLC, were obtained from R. Brown (Manager Tasmanian shellfish quality assurance program, Department of Health and Human Services) for the period of the study (1996-2000) (Fig. 6.14). One of the sites regularly monitored for shellfish toxicity in the Huon Estuary is Deep Bay, where a Mussel longline lease is situated. The sampling site X3B was also located in Deep Bay, adjacent to this mussel farm, and toxin levels from this site were compared with vegetative cell concentrations. Prior to bloom formation in November of 1997 there had been no positive toxicity results from the Huon Estuary since June 1994 (Fig. 6.2). Toxicity levels at site X3B were elevated as soon as cell numbers of *G. catenatum* increased in the water column in November 1997. Toxin concentrations in the summer bloom reached 821 saxitoxin equivalents per 100 g shellfish tissue during the peak of the bloom period. A drop in *G. catenatum* cell numbers in mid December coincided with a drop in toxin concentration, suggesting a high depuration (clearance) rate of toxins from mussels during this period. As the bloom declined, toxin concentrations also fell and were below farm closure levels by February (80 saxitoxin equivalents). With the start of the autumn 1998 bloom, toxin concentrations increased again to a peak of 1845 saxitoxin equivalents per 100g shellfish tissue. This was more than double that of the summer bloom although the maximum observed *G. catenatum* cell concentrations were three times less in the autumn bloom than in the summer bloom and relative abundance of species were similar. Shellfish also remained toxic for longer after the *G. catenatum* bloom declined than in the summer bloom. The continued high shellfish toxicity through July and even into September, with low *G. catenatum* cell concentrations, suggests that shellfish depuration rates were lower at this time.

6.15 VERTICAL MIGRATION OF *G. CATENATUM*

Vertical migration of *G. catenatum* was observed at Killala Bay using the automated profiling system described in Section 6.4. *Gymnodinium catenatum* dominated the biomass during both summer and autumn blooms and the fluorescence maxima recorded by the automated profiling system was therefore known to correspond to *G. catenatum* cells during these periods. Fluorescence traces showed a strong diurnal vertical migration of up to 20 m d^{-1} in both summer and autumn blooms (Figs. 6.15, 6.16), corresponding to a swimming speed of between 1.5 and 6.0 m h^{-1} with downward swimming velocity exceeding upward swimming velocity (Fig. 6.15).

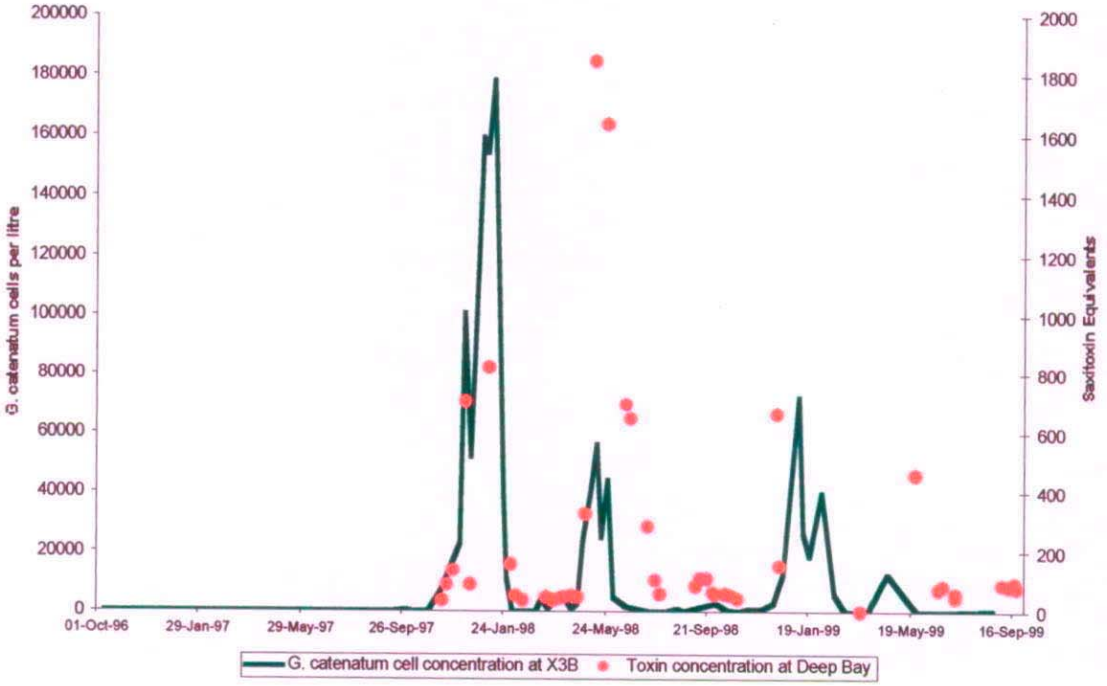


Figure 6.14: *Gymnodinium catenatum* cell densities at site X3B and shellfish toxin concentrations of mussels on longlines in Deep Bay. Toxin data were provided by R. Brown (Manager Tasmanian Shellfish Quality Assurance Program, Tasmanian Department of Health and Human Services).

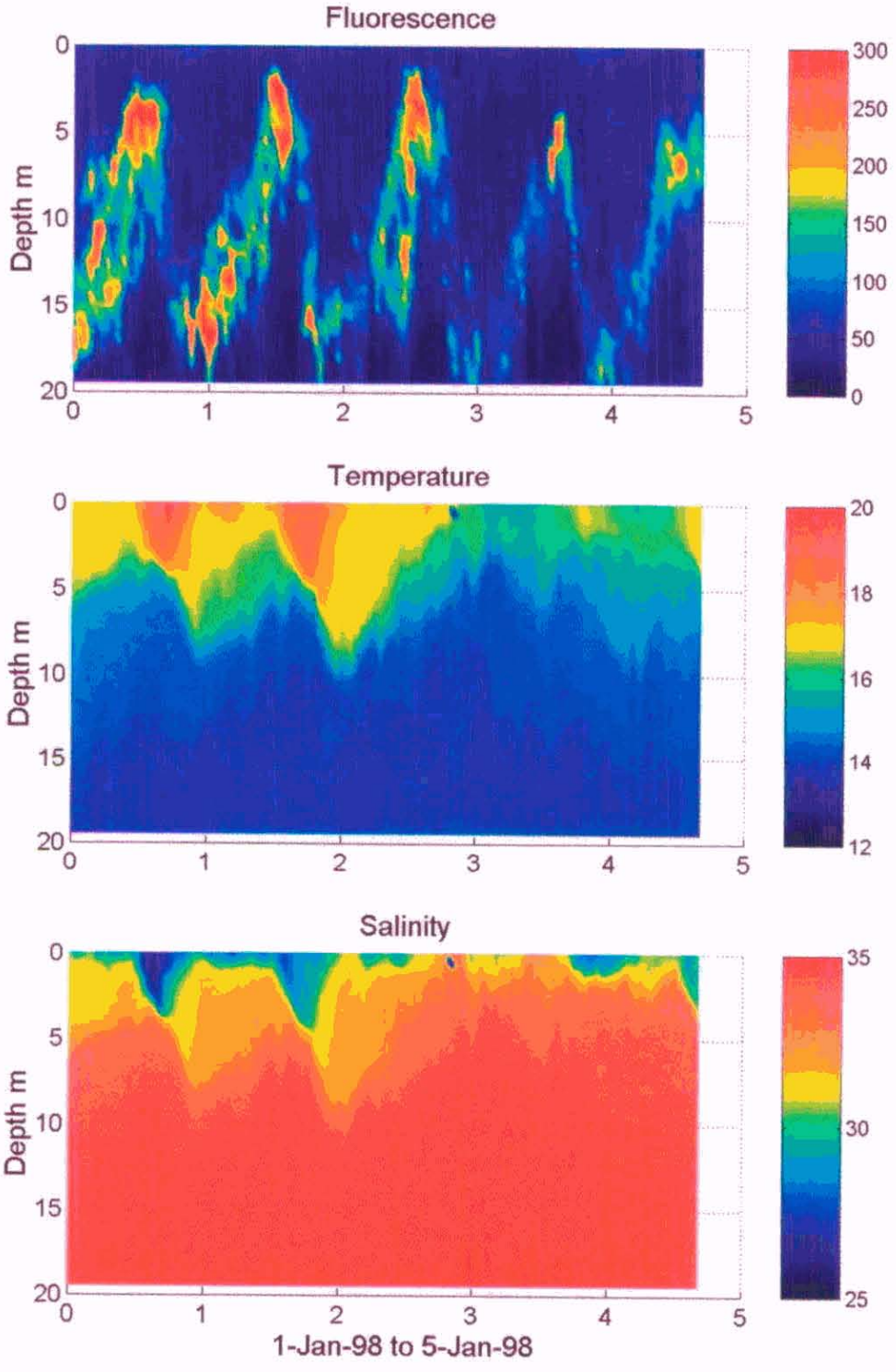


Figure 6.15: Time-depth sections of fluorescence, temperature and salinity at Killala Bay, 01 to 05 January, 1998 (reproduced from HEST, 2000).

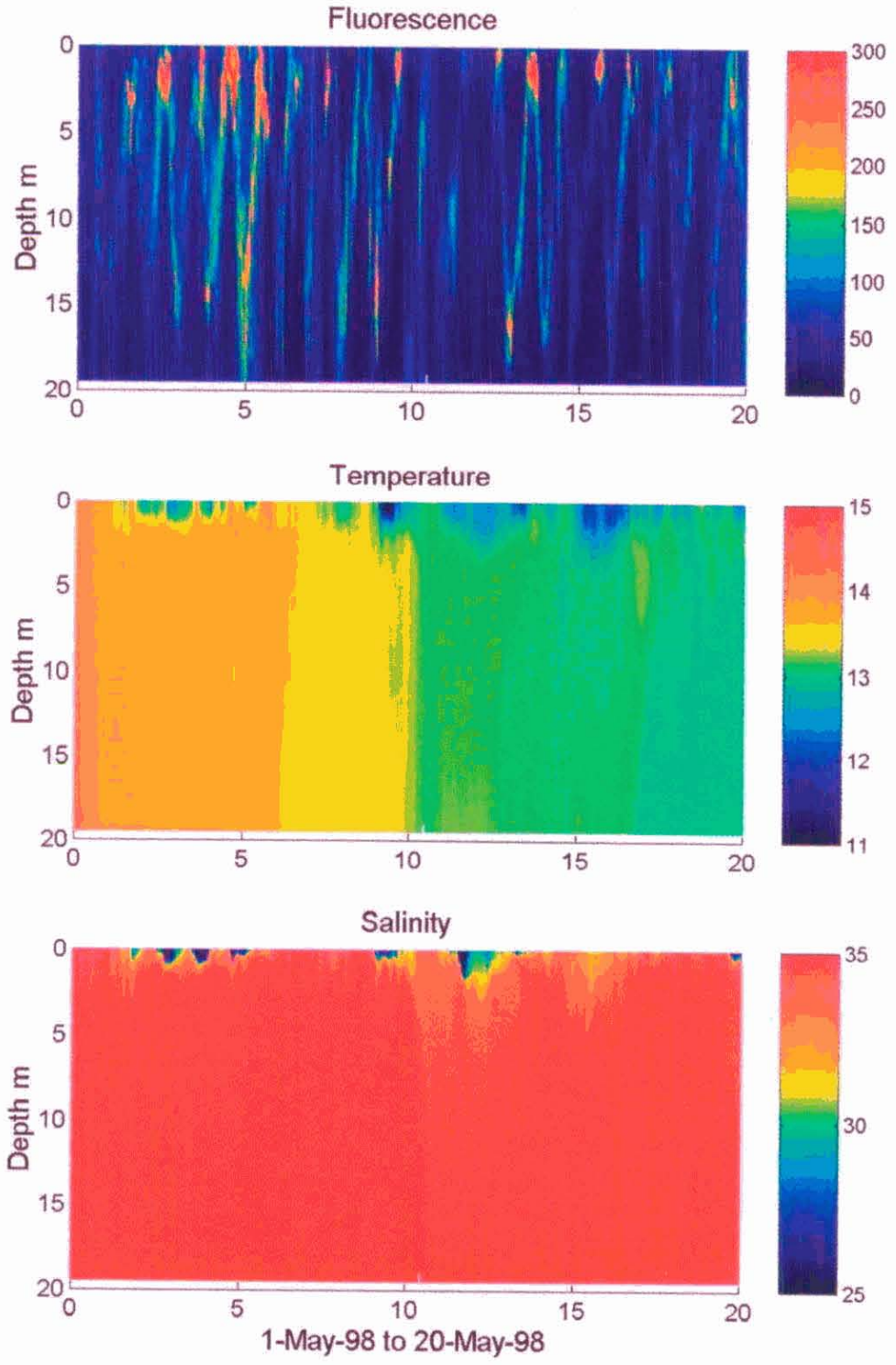


Figure 6.16: Time-depth sections of fluorescence, temperature and salinity at Killala Bay, 01 to 20 May, 1998 (Reproduced from HEST, 2000).

6.16 GRAZER DYNAMICS

Various grazers of microalgae were identified by microscopy in both net and integrated samples throughout the study. These included ciliates, tintinnids, rotifers, cladocerans, appendicularians, copepods and the heterotrophic dinoflagellate *Polykrikos schwartzii* (Figs. 6.17 and 6.18). Several of these organisms were observed actively grazing on *G. catenatum*. *Polykrikos schwartzii* was the most commonly observed *G. catenatum* grazer and a peak in the cell concentration of this species coincided with the summer bloom of 1997/98 at Wheatleys, Killala Bay and Port Cygnet (Fig. 6.19). However, blooms of *P. schwartzii* also occurred during times of low *G. catenatum* cell density and cells were observed engulfing other dinoflagellate and diatom species, suggesting that this species is largely an opportunistic feeder (personal observations). During sampling in 1998/99 other identified grazers were enumerated (Fig. 6.20). Densities of 'other grazers' showed greater variability between sites than did *P. schwartzii* (Fig. 6.20). There was no concurrent increase in these 'other grazer' concentrations with increased *G. catenatum* cell concentrations suggesting that these grazers were less likely to influence bloom dynamics than *P. schwartzii*.

6.17 NUTRIENT CONCENTRATIONS IN *G. CATENATUM* BLOOMS

Nutrients in the Huon Estuary during the three years of the study underwent strong seasonal cycles (see Section 6.2.1 and Fig. 6.11). Nutrient dynamics were similar, although differing in magnitude at the three biological sites sampled during the three year study (Wheatleys, Killala Bay and Port Cygnet; Figs 6.21 – 6.24). *Gymnodinium catenatum* cell densities were negatively correlated with mid-depth nitrate+nitrite and phosphate (Figs 6.21 and 6.22) and positively correlated with total nitrogen and total phosphorus at mid-depth ($p<0.01$), the latter reflecting the significant contribution of high biomass blooms to particulate N and P (Figs 6.23 and 6.24).

Nutrient conditions in the estuary during the four *G. catenatum* blooms differed (Figs 6.21 and 6.22). Surface macronutrients during summer blooms (1997/98, 1998/99) were relatively low at the time of bloom initiation with surface nitrate+nitrite of less than 1 μM and phosphate concentrations of less than 0.5 μM. Deep nitrate+nitrite was also low in the summer 1997/98 bloom (1-2 μM) although bottom ammonia values were relatively high (about 1μM). Bottom nutrients were depleted during the summer 97/98 bloom (not measured in 98/99). The autumn blooms of both years were also initiated at relatively low surface nutrient concentrations although slightly greater than the summer blooms. Elevated concentrations of phosphate, nitrate and nitrate/nitrite were present at depth relative to surface waters throughout the autumn blooms, particularly in 1999.

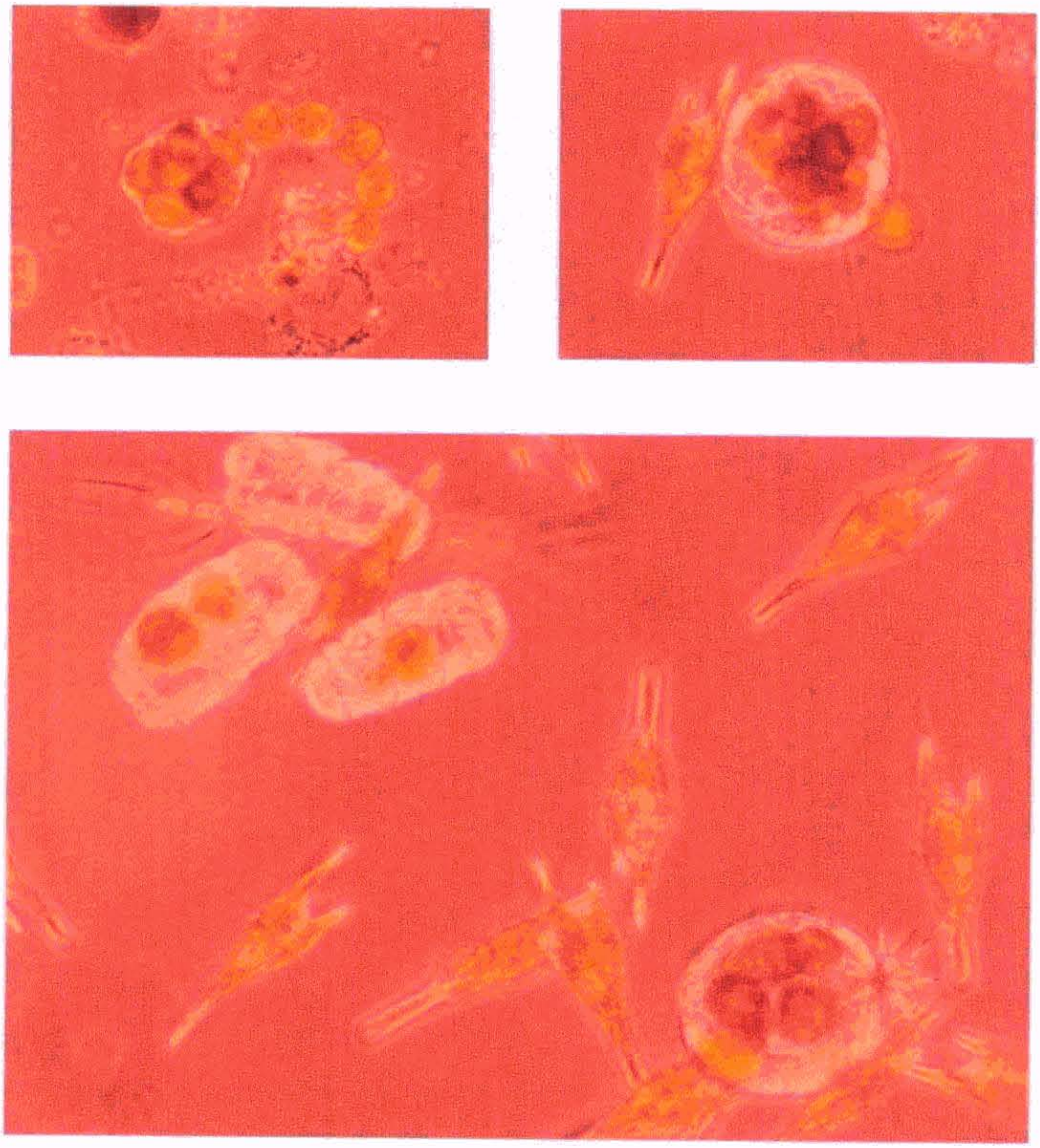


Figure 6.17: *Polykrikos schwartzii* consuming *Gymnodinium catenatum* during the summer bloom of 1997/98 in the Huon Estuary.

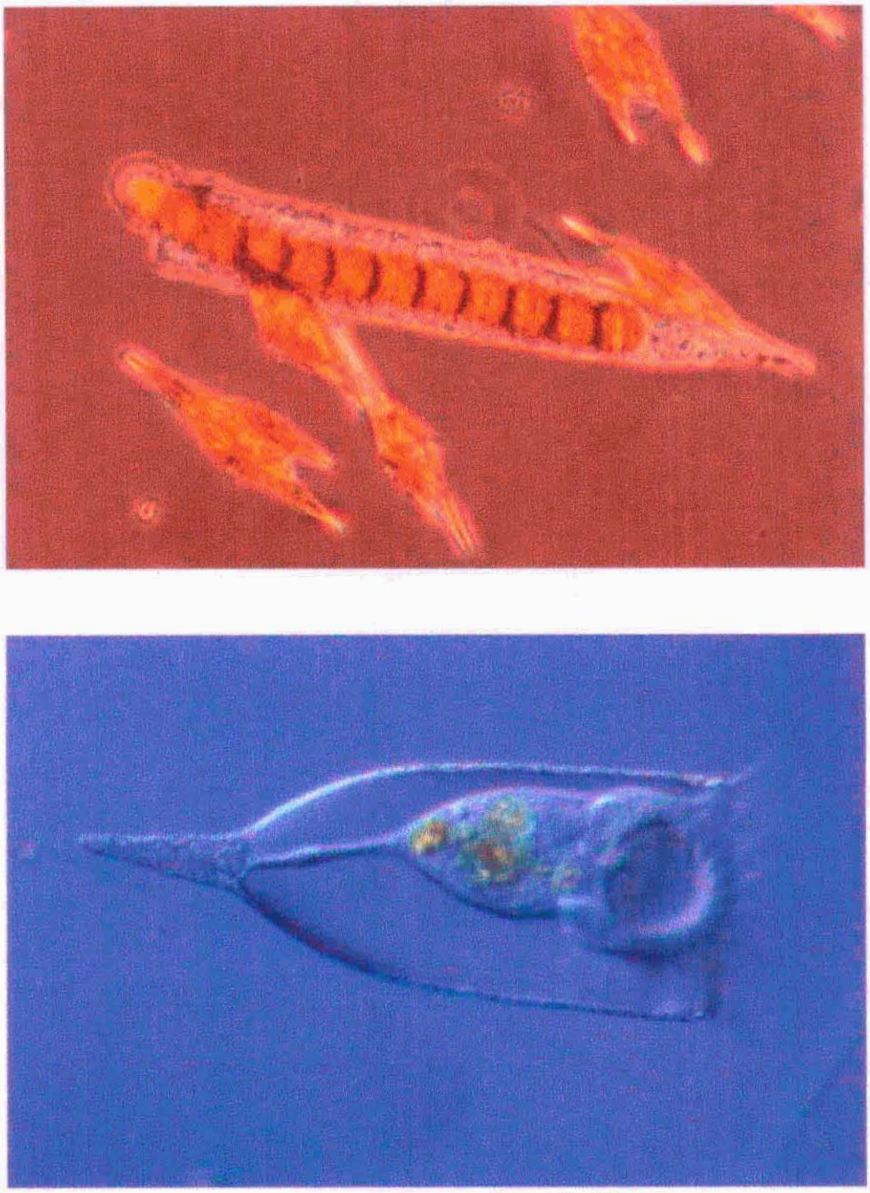


Figure 6.18: Two species of tintinnid observed consuming *Gymnodinium catenatum* during the summer bloom of 1997/98 in the Huon Estuary.

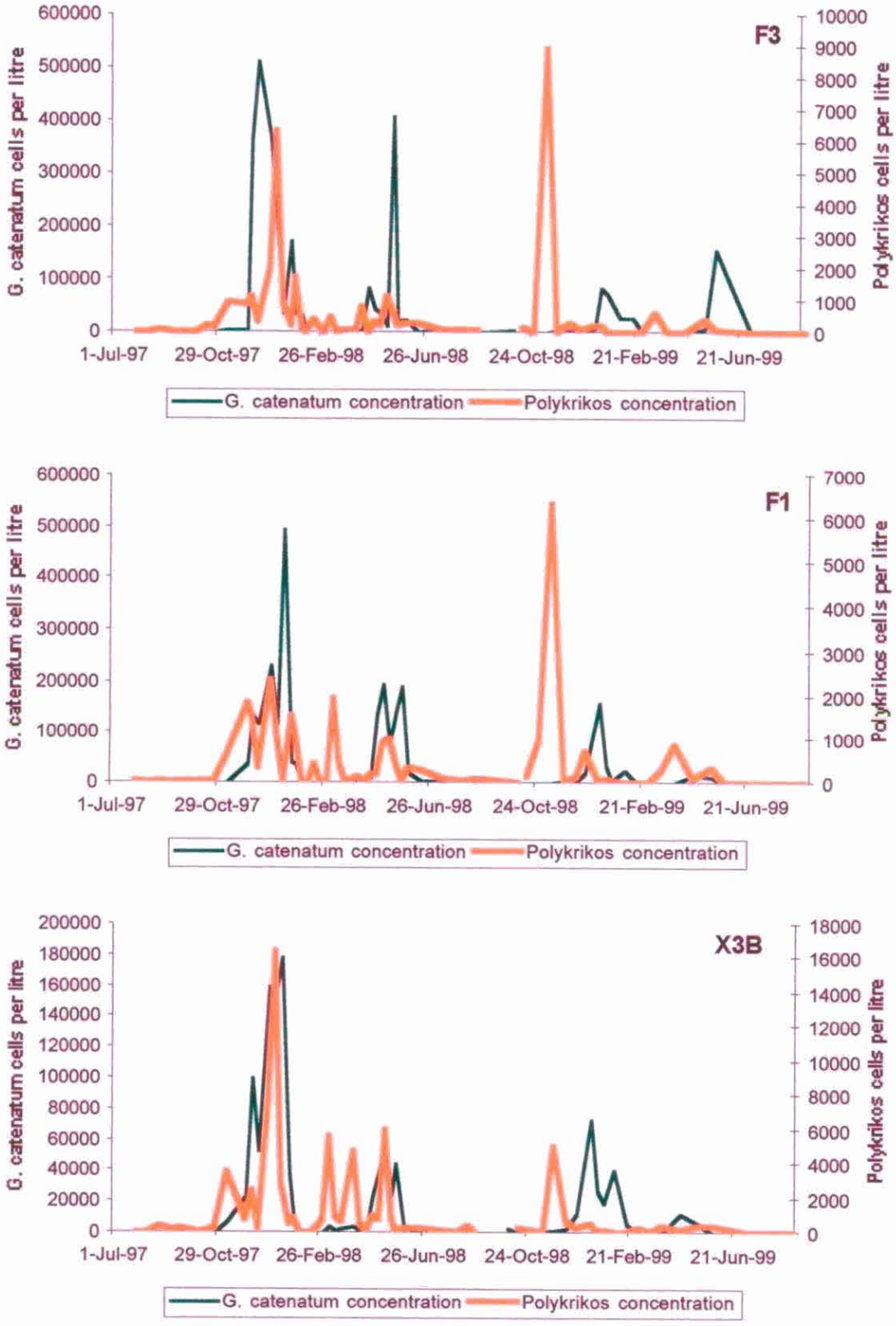


Figure 6.19: Cell densities of the heterotrophic dinoflagellate *Polykrikos schwartzii* and *Gymnodinium catenatum* over time at F3 - Wheatleys, F1 - Killala Bay, and X3B - Pt. Cygnet.

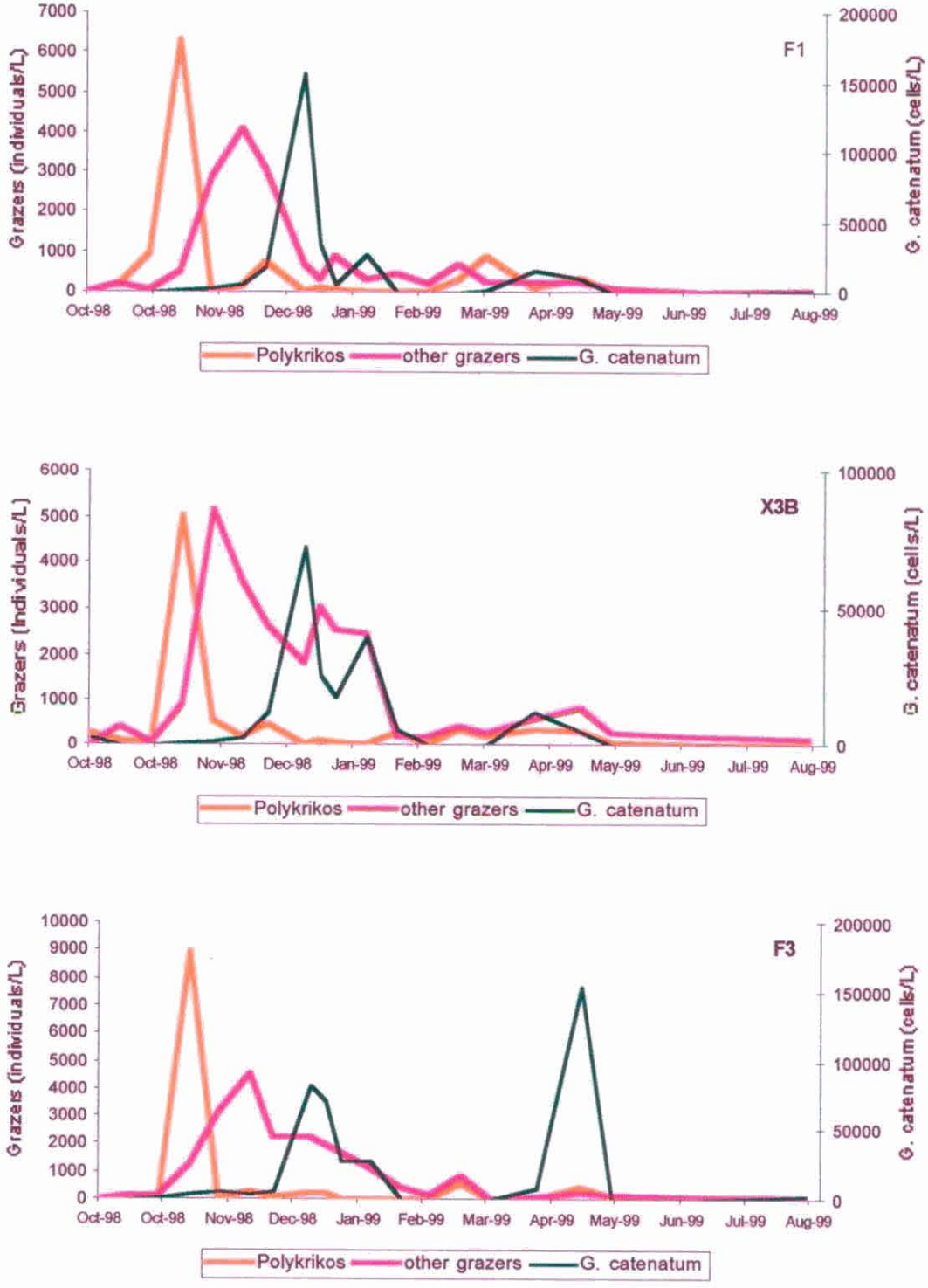


Figure 6.20: Cell densities of the heterotrophic dinoflagellate *Polykrikos schwartzii*, 'other grazers' (tintinnids, ciliates) and *Gymnodinium catenatum* from October 1998 to August 1999 at Wheatleys (F3), Killala Bay (F1) and Pt. Cygnet (X3B).

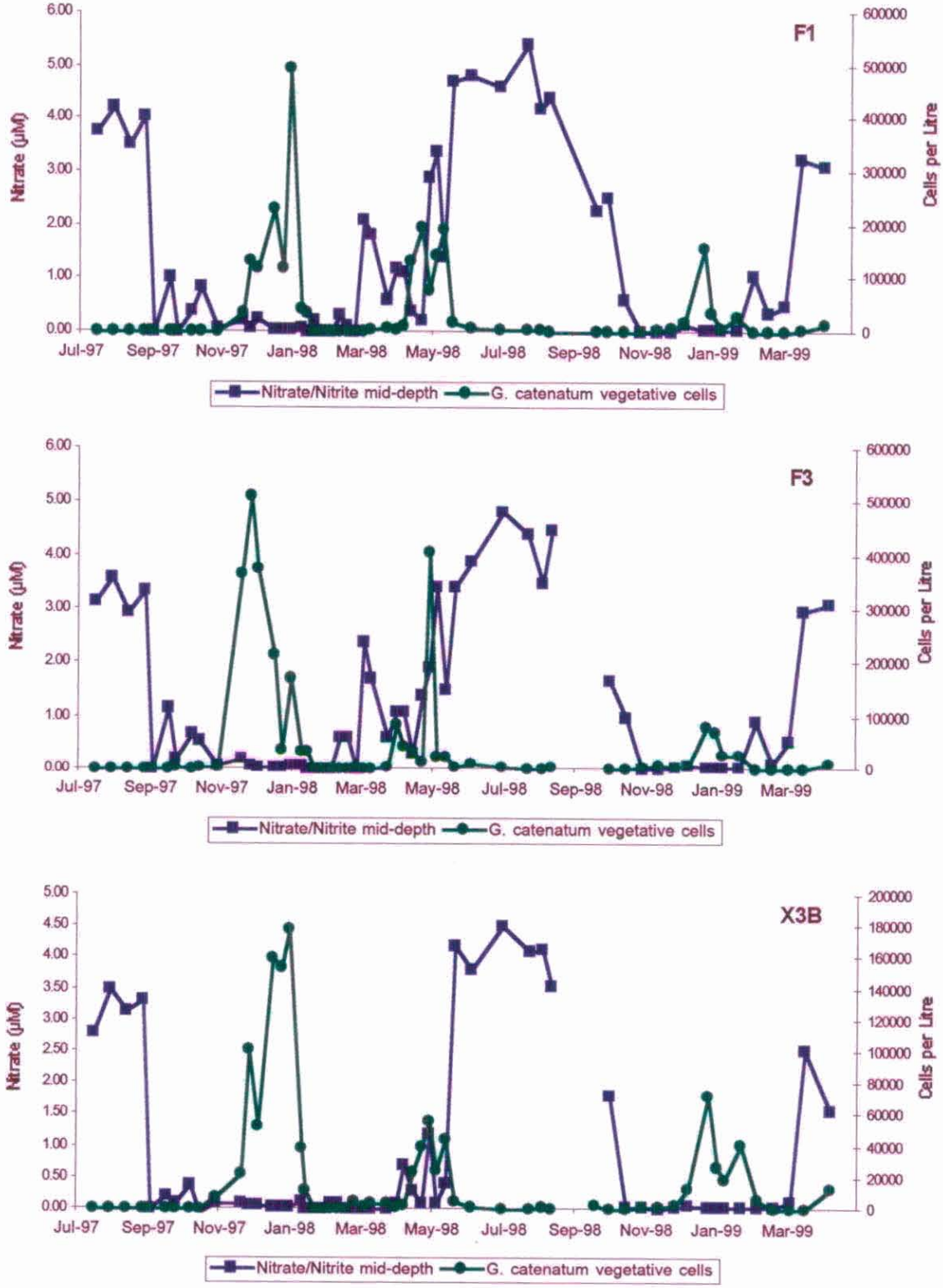


Figure 6.21: Vegetative cell densities of *Gymnodinium catenatum* compared with nitrate/nitrite concentrations measured at mid-depth from F1 (Killala Bay), F3 (Wheatleys) and X3B (Pt. Cygnet) during the second two years of the study.

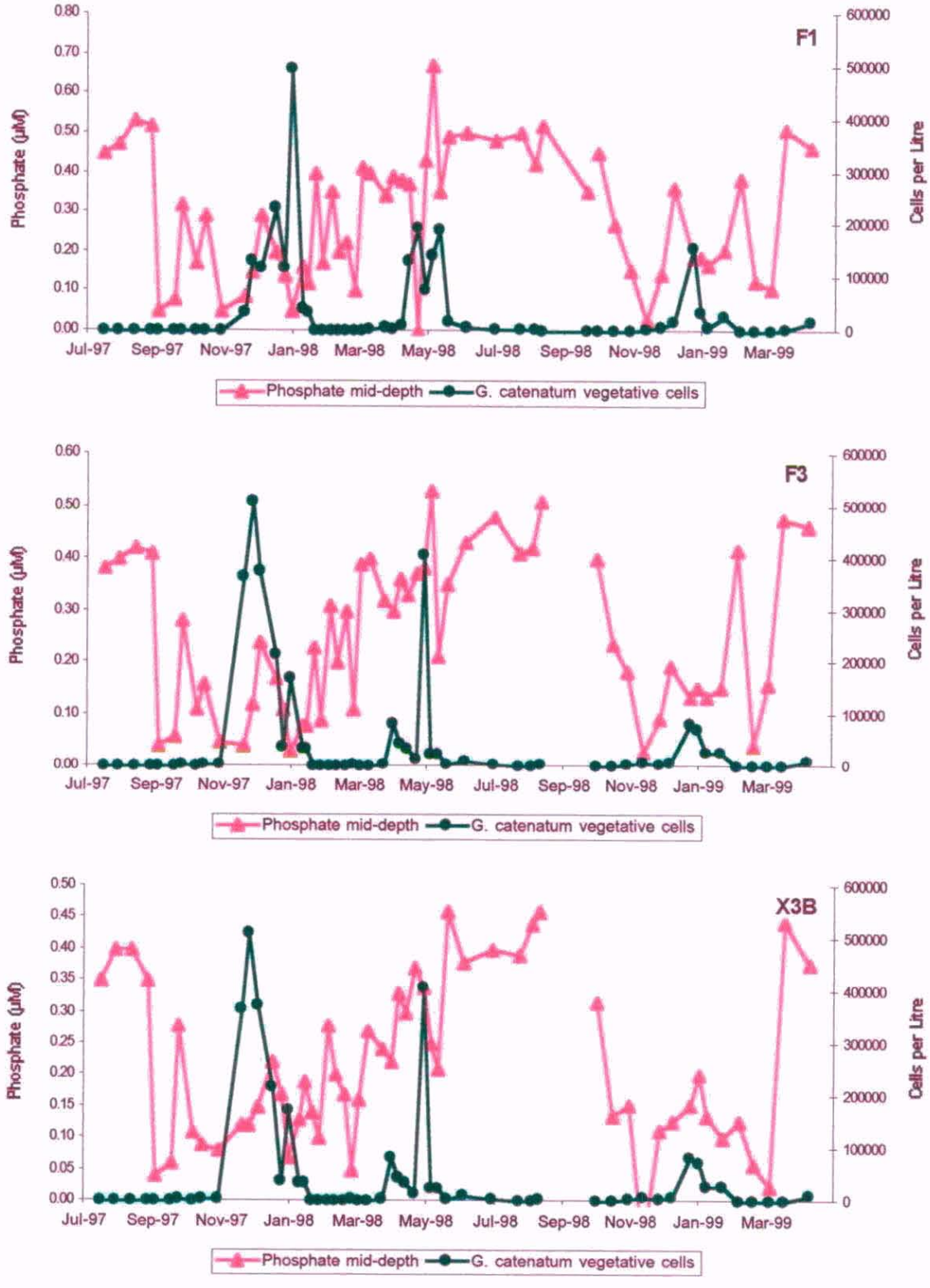


Figure 6.22: Vegetative cell densities of *Gymnodinium catenatum* compared with phosphate concentrations measured at mid-depth from F1 (Killala Bay), F3 (Wheatleys) and X3B (Pt. Cygnet) during the second two years of the study.

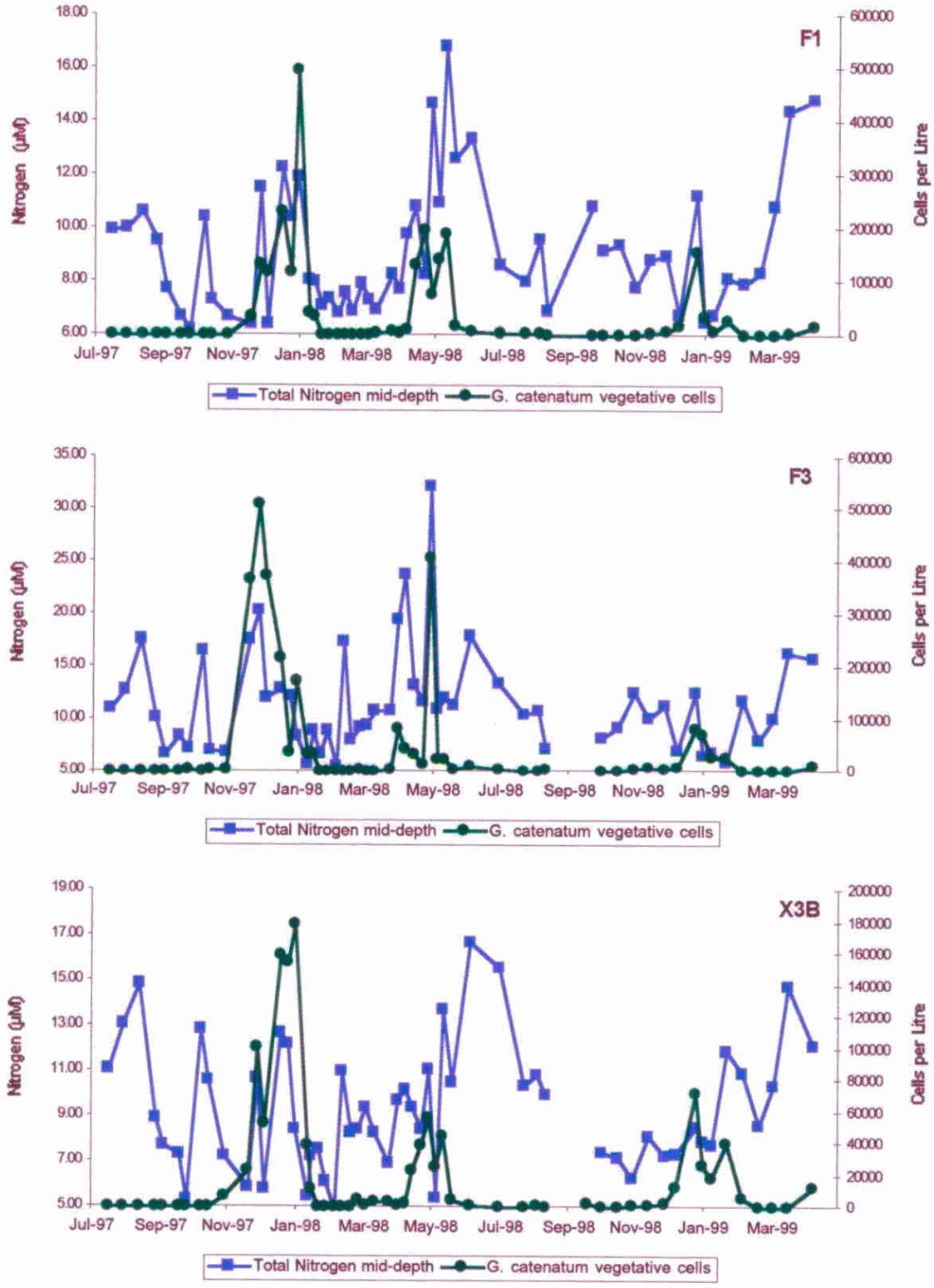


Figure 6.23: Vegetative cell densities of *Gymnodinium catenatum* compared with total nitrogen concentrations measured at mid-depth from F1 (Killala Bay), F3 (Wheatleys) and X3B (Pt. Cygnet) during the second two years of the study.

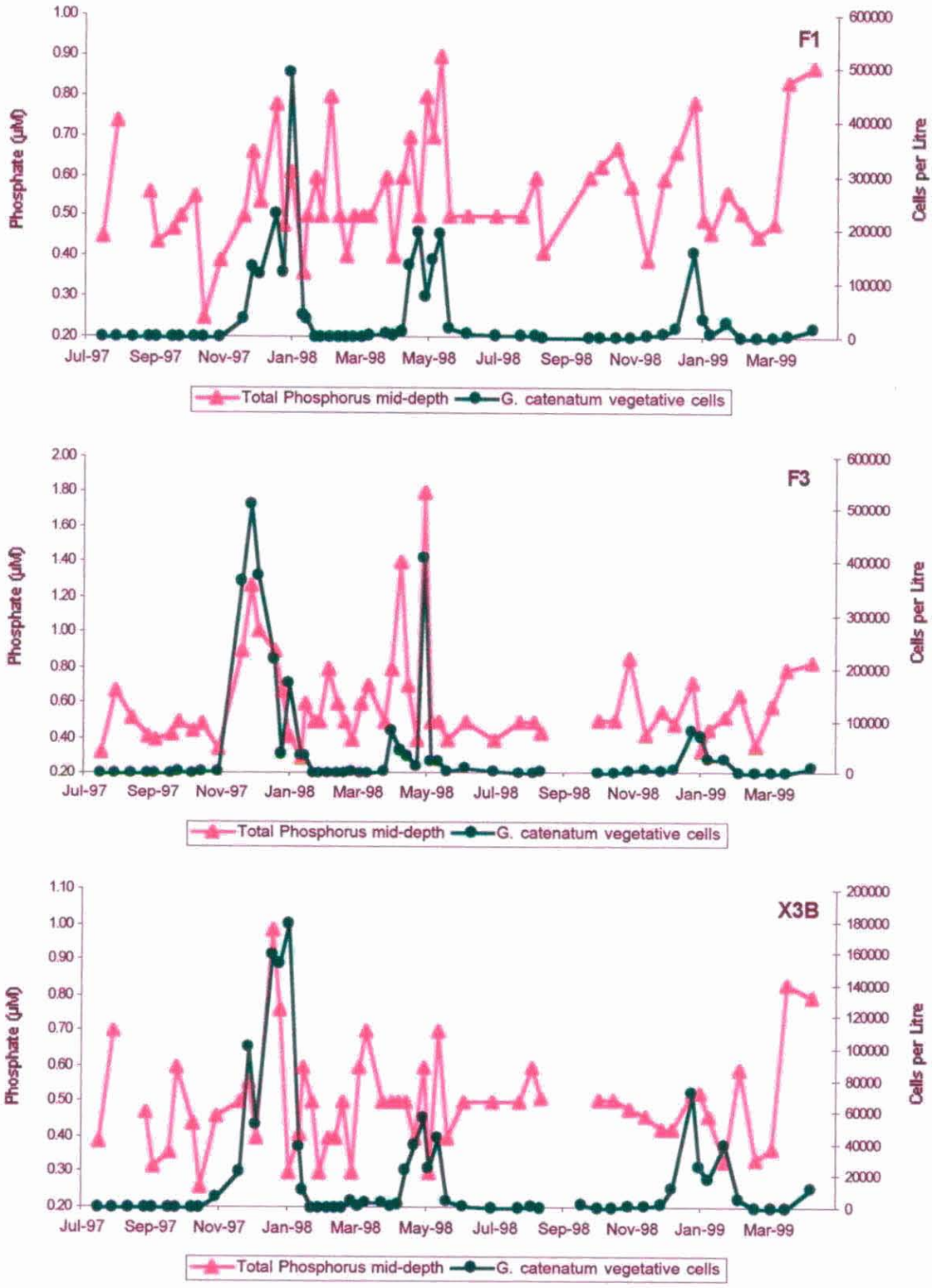


Figure 6.24: Vegetative cell densities of *Gymnodinium catenatum* compared with total phosphorus concentrations measured at mid-depth from F1 (Killala Bay), F3 (Wheatleys) and X3B (Pt. Cygnet) during the second two years of the study.

6.18 PHYSICAL CHARACTERISTICS OF THE HUON ESTUARY DURING *G. CATENATUM* BLOOMS

6.18.1 TEMPERATURE AND SALINITY

Surface temperature in the Huon Estuary undergoes strong seasonal cycles and was positively correlated with *G. catenatum* vegetative cell densities ($r=0.185$ $p<0.01$) (Figs 6.25a and 6.25c). Seasonal averages of surface temperatures were significantly different as would be expected in a temperate system ($F_{11,40}=111.26$, $p<0.01$) and were similar between sites (Figs 6.25a and 6.25c). For each season, temperature averages were statistically similar between years except for the spring of 1996 which was cooler than in 1997, and significantly cooler than in 1998 (Table 6.6). Spring and summer of 1996 also had low biomass, dinoflagellates were only present in small numbers, and *G. catenatum* blooms did not occur.

Surface salinity was highly variable and influenced by sporadic flow from the Huon River and was not significantly correlated with vegetative cell densities (Figs 6.25b and 6.25d). Salinities at Wheatleys (F3) on the north/northeast side of the estuary were more variable than at Killala Bay (F1), directly across the other side of the estuary (Figs 6.25b and 6.25d). There were no significant differences in surface salinity between seasons ($F_{11,40}=0.74$, $p=0.69$).

Table 6.6: Results of Post Hoc tests (Tukey Test, $p<0.05$) of seasonal averages of surface temperatures indicating homogenous subsets of seasons (i.e. seasons for which surface temperature was not significantly different).

SEASON	N	HOMOGENOUS SUBSETS			
		1	2	3	4
WIN 99	3	10.05			
WIN 97	5	10.56			
WIN 98	5	10.70			
AUT 98	5		12.72		
AUT 97	5		12.77		
AUT 99	3		12.89		
SPR 96	5		13.30		
SPR 97	5		13.80	13.80	
SPR 98	3			14.71	
SUM 97	5				16.80
SUM 96	5				17.35
SUM 98	3				17.69

6.18.2 HUON RIVER FLOW AND WIND SPEED

Huon River flow did not correlate significantly with *G. catenatum* vegetative cells and there were no apparent differences in flow between bloom years and non-bloom years (Fig. 6.26). However, cumulative flow from August through till December (i.e. the period of spring bloom initiation) differed between years (Fig. 6.27) with 1996 having greater and more

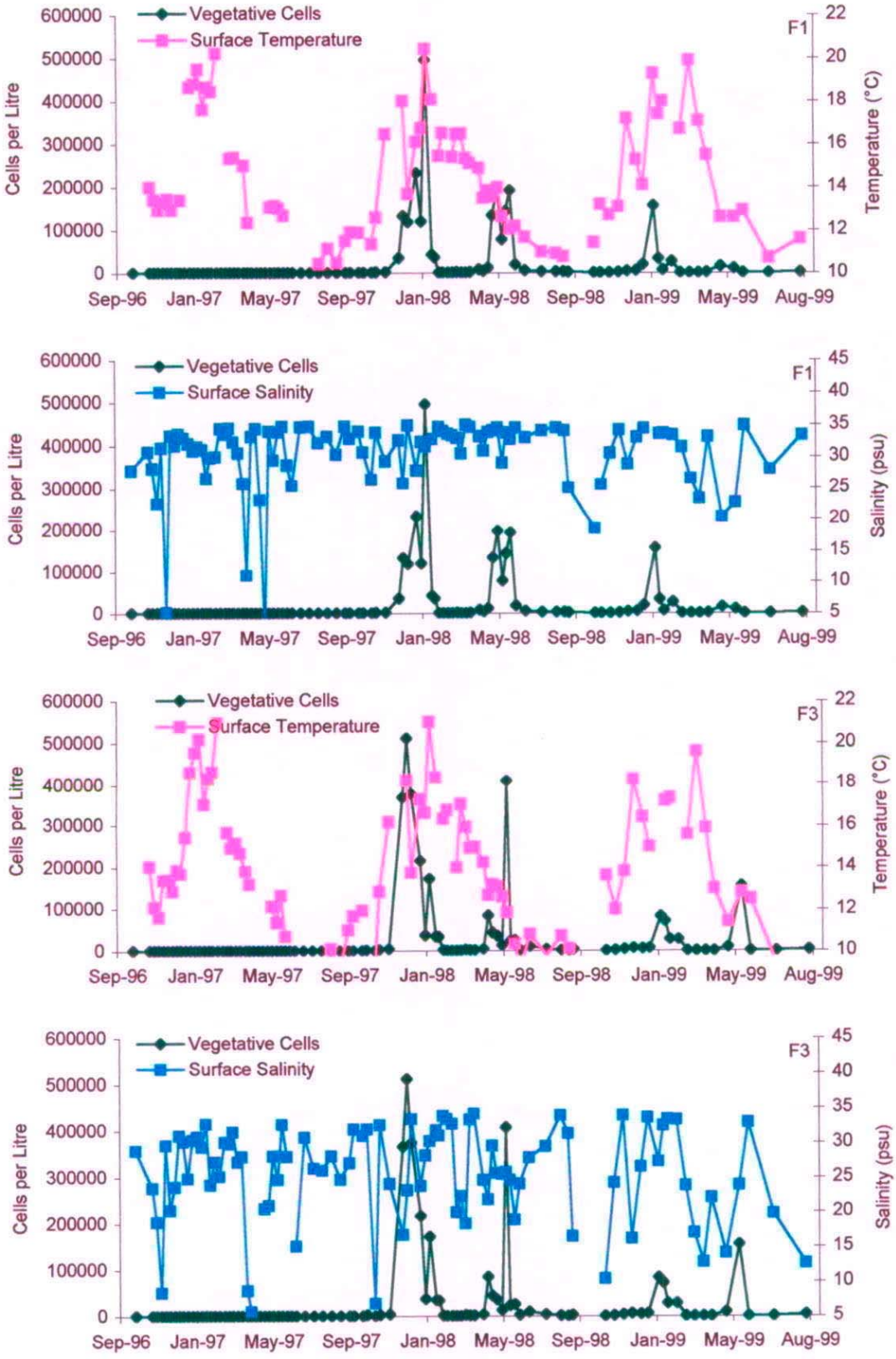


Figure 6.25: Surface temperature (a and c) and surface salinity (b and d) compared with vegetative cell concentrations of *Gymnodinium catenatum* at Killala Bay (F1) and Wheatleys (F3) during the three years of the study.

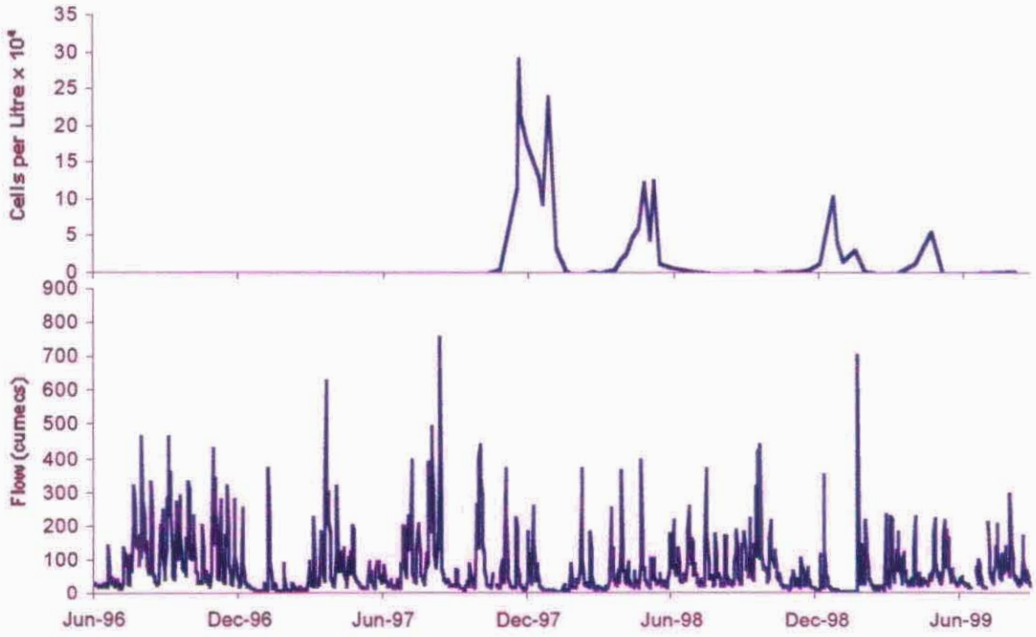


Figure 6.26: Mean *Gymnodinium catenatum* cells per litre for the five biological sites (upper plot) and Huon River flow in $m^3 s^{-1}$ (lower plot) over the three years of the study.

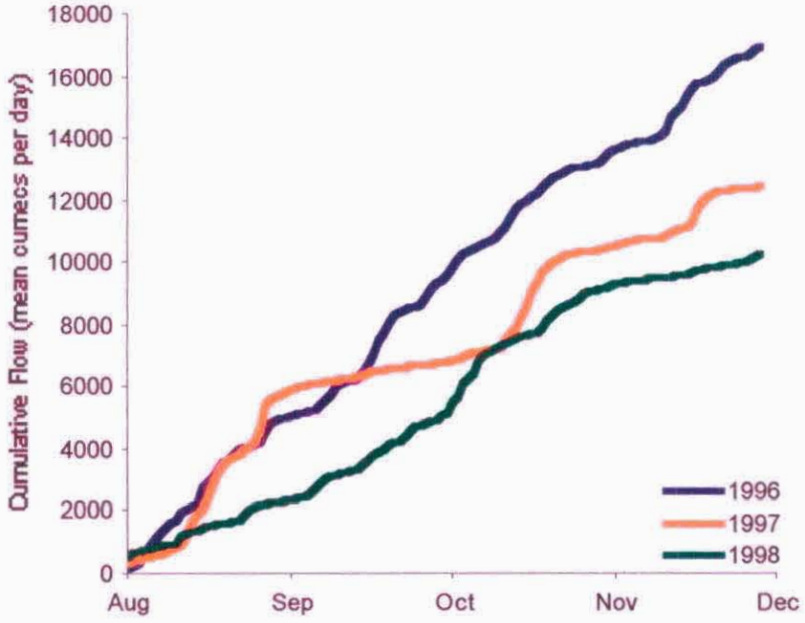


Figure 6.27: Cumulative mean daily flow of the Huon River measured at Frying Pan Creek for the period from August through till December for the three years of the study.

consistent flow while the bloom years, 1997 and 1998, had less cumulative flow and greater variability in flow rate over that time period.

Wind speeds measured from Cape Bruny were compared between 1996/97 (dinoflagellates including *G. catenatum* blooms absent) and 1997/98 (*G. catenatum* blooms observed) but there were no apparent differences between the two years (Fig. 6.28).

6.18.3 WATER COLUMN CHARACTERISTICS

Detailed water column characteristics of the estuary during the *G. catenatum* blooms of summer 1997/98 and autumn 1998 were measured using the continuous profiler. Figure 6.29 shows fluorescence, salinity and temperature midday profiles from the Killala Bay automatic profiler. Figure 6.29a indicates that, at the time of initiation of the first bloom in 1997 the water column at Killala Bay was weakly stratified, with a surface temperature of around 14 °C and surface salinity of 33.5 psu increasing to 35 psu at depth. This salinity pattern reflects the river runoff in October and November (Figs. 6.26 and 6.27). The fluorescence trace indicates an increase in algal biomass between 29/11/97 and 31/12/1997. There were no extended periods of low winds recorded at Cape Bruny during bloom initiation (Fig. 6.28). At the peak of the bloom in late December 1997 (Fig. 6.29b), the water column was strongly stratified, with surface water temperatures up to 18 °C and a low surface salinity of 28 psu. The fluorescence trace shows the subsurface biomass maximum (consisting almost entirely of *G. catenatum* based on microscopic counts and cell volume, Fig. 6.13a and 6.13b). The decline of this bloom in mid-January was associated with complete destratification of the water column and some surface cooling to 17 °C (Fig. 6.29c), which coincided with strong winds (Fig. 4.27) that may have caused localised upwelling. By late January a well-mixed water column was present, with low fluorescence levels throughout (Fig. 6.29d). These data indicate that a partially stratified water column was present during bloom formation and that temperatures of greater than 14°C were sufficient for bloom formation. The bloom persisted through a variety of salinity and temperature conditions although the complete destratification of the water-column that occurred in mid January coincided with bloom collapse.

Conditions during initiation of the autumn bloom were very different to those in the preceding summer bloom. There was some runoff due to rainfall events (Fig. 6.26), but these had little effect on the water column, which was weakly stratified (Figs. 6.29g and h). Water temperatures at the peak of the bloom were just over 12 °C (Fig. 6.29i), having decreased from 14 °C at the time of bloom initiation. Winds were relatively high during this period (ranging from 5-9 m s^{-1}). Bloom decline was not associated with any significant

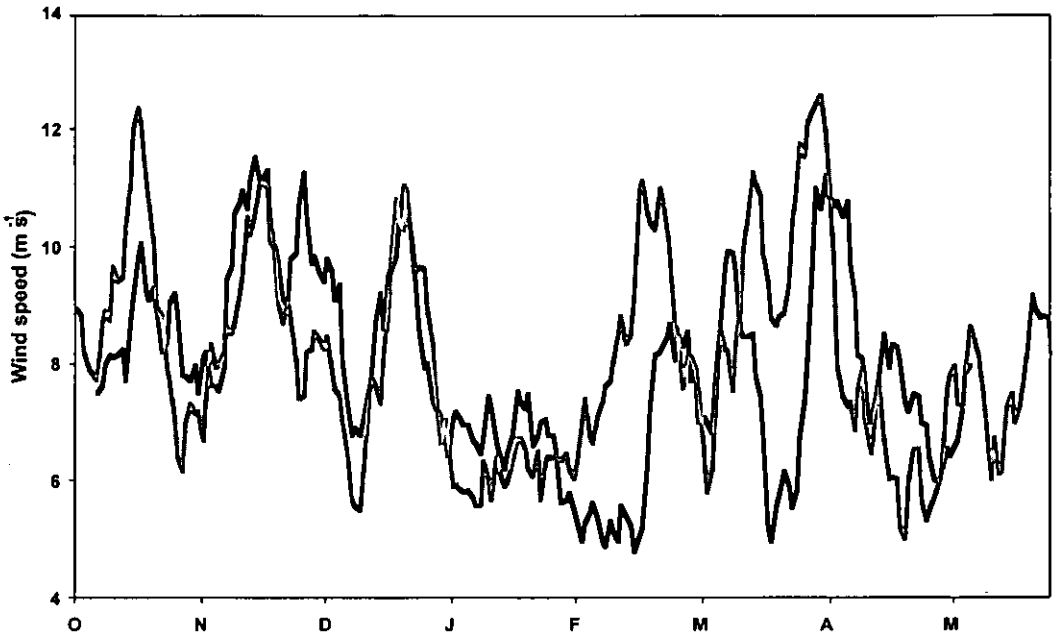


Figure 6.28: Wind speed at Cape Bruny versus month for the spring, summer and autumn period of 1996/97 (black line) and 1997/98 (grey line). Three-hourly wind speed has been smoothed using a 5-day running average (reproduced from HEST, 2000).

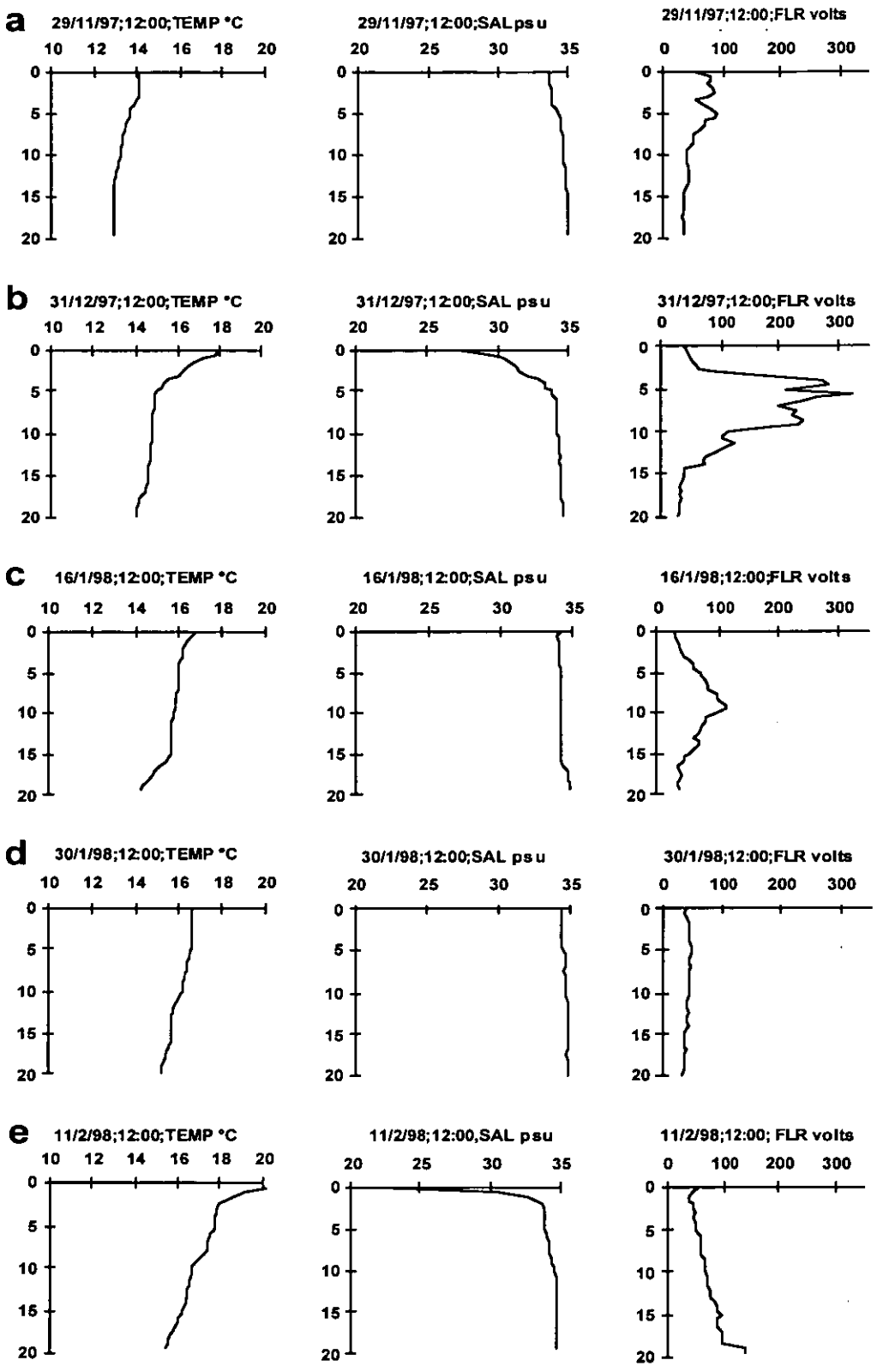


Figure 6.29a-e: Midday water column characteristics at Killala Bay during 1997/98. TEMP = temperature in °C; SAL = salinity in psu; FLR = fluorescence in volts. Y-axis is depth in metres. a – commencement of Summer 97/98 *Gymnodinium catenatum* bloom; b – peak of Summer 97/98 bloom; .c –decline of Summer 97/98 bloom; d – end of Summer 97/98 bloom; e – diatoms and *Ceratium* spp. dominant, February 1998.

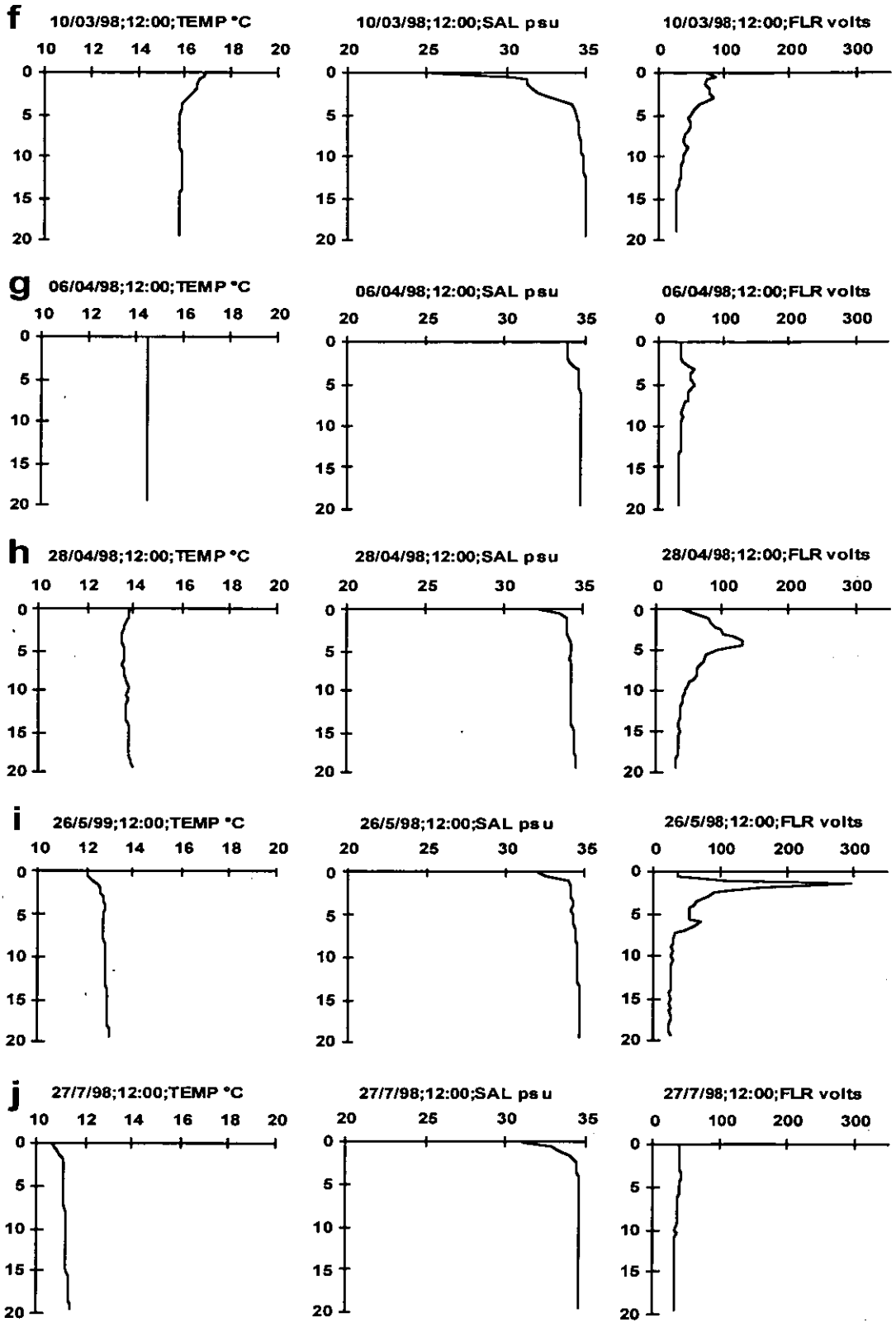


Figure 6.29f-j: Midday water column characteristics at Killala Bay during 1997/98. TEMP = temperature in °C; SAL = salinity in psu; FLR = fluorescence in volts. Y axis is depth in metres. f – diatoms dominant; g – mixed dinoflagellates with increasing *Gymnodinium catenatum* abundance; h – mixed dinoflagellates and initiation of Autumn *Gymnodinium catenatum* bloom; i – Peak of Autumn bloom; j – low biomass, mixture of dinoflagellates and diatoms.

changes in the water column (Fig. 6.29j), but was probably influenced by decreasing water temperatures coinciding with the seasonal decrease in light availability.

6.19 CANONICAL CORRESPONDENCE ANALYSIS

Canonical Correspondence Analysis (CCA) was used to assess the influence of environmental factors (chemical and physical variables) on the abundance of different taxa. Bloom species and pigment data were analysed. Three separate CCAs were calculated for physical variables plus nutrients at the three depths i.e. one CCA incorporated all physical variables plus either bottom nutrients, mid-depth nutrients or surface nutrients (due to high variance inflation factors if all nutrients were combined; see Section 6.6.3).

6.19.1 CCA OF PIGMENT CONCENTRATIONS AND ENVIRONMENTAL VARIABLES

The results of the three CCAs of pigment concentrations and environmental variables are displayed in Table 6.7 and Figure 6.30 (mid-depth nutrients only). In all cases the variance explained by the first two axes was low (maximum of 12 %) but the cumulative % variance in the algal-environment relationship was high (>80%) indicating a strong relationship between pigment concentrations and the 11 environmental variables.

Table 6.7: Results of CCA analysis of pigments and environmental variables. NO_2 = nitrite, NO_x = nitrate + nitrite, PO_4 = phosphate, TDN = total dissolved nitrogen, TDP = total dissolved phosphorus SAL = salinity and TEMP = temperature. B, M and S refer to bottom, mid-depth and surface nutrients respectively and F indicates that measurements were made on filtered samples.

Results of CCA Analysis on pigments and environmental variables	Bottom Nutrients + Physical Parameters		Mid-Depth Nutrients + Physical Parameters		Surface Nutrients + Physical Parameters	
	Axis 1	Axis 2	Axis 1	Axis 2	Axis 1	Axis 2
Eigenvalues	λ_1=0.09	λ_2=0.01	λ_1=0.09	λ_2=0.012	λ_1=0.097	λ_2=0.011
Algal-Environment Correlations	0.821	0.735	0.82	0.772	0.847	0.867
Variables significantly associated with axis 1 or 2 or both.	BTDN, BNO_2F	B NO_2F	MTDN, MNO_xF, MSAL,	MTDN, MTDP	STDN, SNO_xF, STDP, SPO_4F	STDN, SNO_xF, STDP, SSAL, MSAL, STEMP
Variance explained by the first two axes	12%		13%		13%	
Cumulative %variance in algal-environment relation for axes 1 and 2.	81.8%		81.6%		83.6%	
Minimal subset of significant forward selected variables in order of importance	BTDP, BPO_4F, BTDN, BNO_xF		MNO_xF, MTDN, MSAL, MTDP		SPF, SNO_xF, STDP	
Variance explained by forward selected variables	75%		85%		69%	

With forward selection and unrestricted Monte Carlo permutation tests (99 permutations, $p \leq 0.05$), CCA identified a minimal subset of three to four environmental variables that explained significant ($p \leq 0.05$) proportions of the variance in the algal taxa (Table 6.7). Results for the three CCA analyses (nutrients split between depths) indicated that of the

Figure 6.30: Canonical Correspondence Analysis biplot showing the relationship between phytoplankton pigments (green diamonds) and the four significant environmental factors (arrows to red circles). Pigment abbreviations are; ALLOX= alloxanthin, BECAR= β,ε-carotene, BBCAR= β,β carotene BUTFUCO= 19'butanoyloxyfucoxanthin, CHLA= chlorophyll *a*, CHLB= chlorophyll *b*, C1+C2= chlorophyll c_1+c_2, CHLC3= chlorophyll c_3, CHLAEP= chlorophyll *a* epimer, CHLAAL= chlorophyll *a* allomer, CHLIDEA= chlorophyllide *a*, DIADINO= diadinoxanthin, DIATOX= diatoxanthin, DINO= dinoxanthin, FUCOX= fucoxanthin, HEXFUCO= 19'hexanoyloxyfucoxanthin, LUTEIN= lutein, NEO= neoxanthin, PERID= peridinin, PHYTINA= phaeophytin a, PRASIN= prasinoxanthin, VIOLA= violaxanthin, ZEAX= zeaxanthin. Environmental variable abbreviations are; MNOxF= mid-depth filtered nitrate+nitrite, MTDN= mid-depth total dissolved nitrogen, MTDP= mid-depth total dissolved phosphorus, MSAL= mid-depth salinity.

physical variables only mid-depth salinity had a significant influence on pigments and was significantly associated with axis 1 and dinoflagellate pigments. Nitrate+nitrite at all depths had a significant influence on taxa and was associated with flagellate pigments. Other significant variables were surface and bottom-depth phosphate, total dissolved phosphorus at all depths, and total dissolved nitrogen in bottom and mid-depth waters. However, the low variance explained by the two axes (12 %) indicates that other variables, which were not included in the analysis contribute significantly to the distribution of algal taxa.

6.19.2 CCA OF BLOOM SPECIES AND ENVIRONMENTAL VARIABLES

The results of the three CCAs of bloom species and environmental variables are displayed in Table 6.8 and Figure 6.31 (surface nutrients only). In all cases the variance explained by the first two axes was low (maximum of 6 %) but the cumulative % variance in the algal-environment relationship was high (>90%) indicating a strong relationship between bloom species and the 11 environmental variables.

Table 6.8: Results of CCA analysis of bloom species and environmental variables. NO_2 = nitrite, NO_x = nitrate + nitrite, PO_4 = phosphate, Si = silicate, TN = total nitrogen, TP = total phosphorus, TDN =total dissolved nitrogen, TDP = total dissolved phosphorus SAL = salinity and TEMP = temperature. B, M and S refer to bottom, mid-depth and surface nutrients respectively and F indicates that measurements were made on filtered samples.

Results of CCA Analysis of bloom species and environmental variables	Bottom Nutrients + Physical Parameters		Mid-Depth Nutrients + Physical Parameters		Surface Nutrients + Physical Parameters	
	Axis 1	Axis 2	Axis 1	Axis 2	Axis 1	Axis 2
Eigenvalues	λ_1=0.043	λ_2=0.005	λ_1=0.05	λ_2=0.005	λ_1=0.048	λ_2=0.004
Algal-Environment Correlations	0.773	0.749	0.835	0.801	0.816	0.713
Variables significantly associated with axis 1 or 2 or both	BTN, BTP	BTP, BNO_2F, STEMP	MSAL, MTN	MTDP	STN, SSI, SNO_xF	SSi, SPO_4F STDP
Variance explained by the first two axes	6%		6%		5%	
Cumulative %variance in algal-environment relation for axes 1 and 2.	90.8%		92.4%		95.6%	
Minimal subset of significant forward selected variables	BTN, BNO_xF, STEMP		MTN, MTDP, MSAL		STN, SNO_xF, SSi, SPO_4F, STDP	
Variance explained by forward selected variables	80%		50%		83%	

With forward selection and unrestricted Monte Carlo permutation tests (99 permutations, $p \leq 0.05$), CCA identified a minimal subset of three to five environmental variables that explained significant ($p \leq 0.05$) proportions of the variance in the algal taxa (Table 6.8). Results for the three CCA analyses (nutrients split between depths) indicated that of the physical variables surface temperature and mid-depth salinity had a significant influence on bloom species. Total nitrogen at all depths had a significant influence on bloom species and was associated with dinoflagellate taxa. Other significant variables were nitrate+nitrite at surface and bottom-depths, TDP in bottom and mid-depth waters and surface silicate and phosphate. However, as was the case in the pigment CCA, the low variance explained by

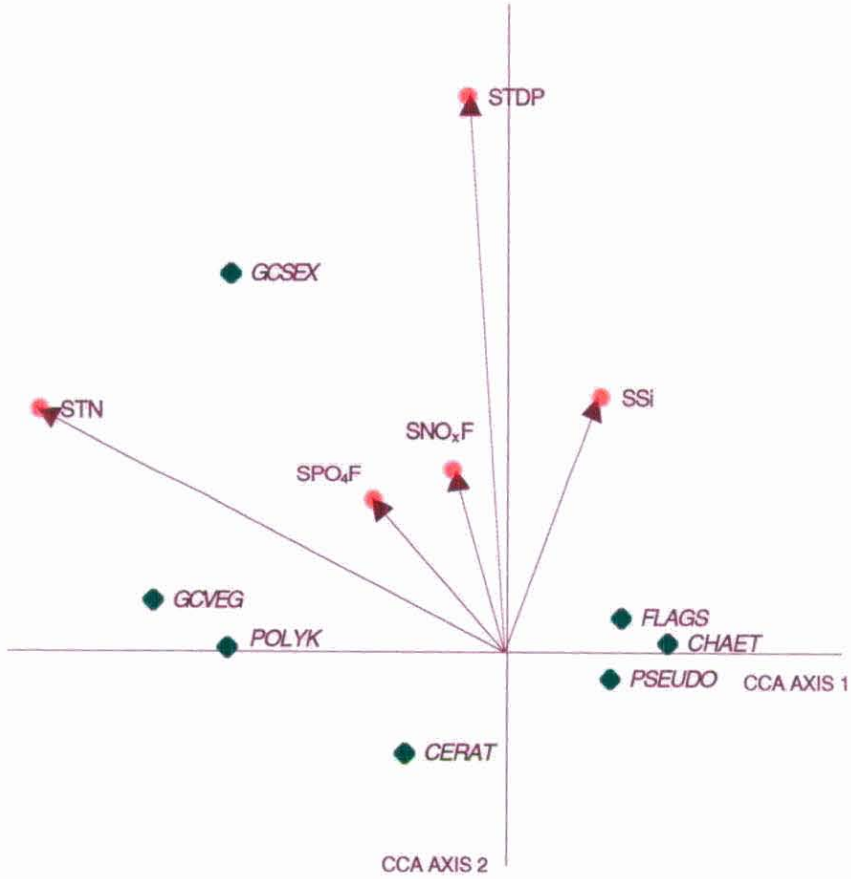


Figure 6.31: Canonical Correspondence Analysis biplot showing the relationship between bloom species (green diamonds) and the five significant environmental factors (arrows to red circles). Bloom species abbreviations are; GCVEG = *Gymnodinium catenatum* vegetative cells, GCSEX = *Gymnodinium catenatum* sexual cells (a passive variable in this anlaysis), CERAT = *Ceratium* spp., POLYK = *Polykrikos schwartzii*, CHAET = *Chaetoceros* spp., PSEUDO = *Psedonitzschia* spp. and FLAGS = small flagellates. Environmental variable abbreviations are; SNOxF = surface filtered nitrate+nitrite, STN = surface total nitrogen, SPO_4F = surface filtered phosphate, SSi = surface silicate.

the two axes (6 %) indicates that other variables, which were not included in the analysis contribute significantly to the distribution of algal taxa.

RESULTS SECTION 3: SEXUAL REPRODUCTION OF *G. CATENATUM*

6.20 *GYMNODINIUM CATENATUM* SEXUAL STAGE CONCENTRATIONS IN BLOOMS

6.20.1 SUMMER BLOOMS

The four *G. catenatum* blooms (summer and autumn 1997/98, summer and autumn 1998/99) had distinct differences in their sexual reproduction dynamics (Fig. 6.32). During the summer 1997/98 bloom, samples from Killala Bay (F1), Wheatleys (F3), Port Cygnet (X3B) and Brabazon Park (H3) contained sexual cells of *G. catenatum* (resting cysts, planozygotes or planomeiocytes and fusing gametes (see Chapter 1, Fig. 1.1). These sexual stages were observed in net samples at all biological sites from the time of first detection of the bloom in late November (personal observations) and quantified from fixed (Lugol's iodine) integrated water column samples at Killala Bay and Wheatleys (Fig. 6.32). It was not possible to discriminate planozygotes from planomeiocytes and therefore the large 'sexual cells' seen in samples may have been either the result of germination of resting cysts from the sediments (planomeiocytes) or due to sexual reproduction in the water column (planozygotes). However, the former is unlikely because the sampling strategy was inappropriate for detecting planomeiocytes (which would most likely be found near the sediment/water interface) and also because the large sexual cells co-occurred with fusing gametes and immature resting cysts. At both Killala Bay and Wheatleys (F1 and F3) the concentration of sexual stages fluctuated throughout the bloom with no apparent increase in sexual stages as the bloom progressed (Fig. 6.32). At site F3 the peak in concentration of sexual cell density occurred after the peak in vegetative cell density while at F1 this was observed before the peak in vegetative cell density.

A similar pattern was seen in the summer bloom of 1998/99 with low densities of sexual stages observed early in the bloom and continuing to form throughout the bloom period at the three sites (F1, F3, X3B) (Fig. 6.32). At all three sites the peak in concentration of sexual stages occurred after the peak in vegetative cells. Although vegetative cell concentrations were lower in the summer 1998/99 bloom, the concentrations of sexual stages were similar.

6.20.2 AUTUMN BLOOMS

In contrast to the summer blooms, very few sexual stages and no resting cysts were seen at any of the sites in the autumn 1998 bloom in net samples (personal observations) and sexual stages were at undetectable levels in water column integrated samples (Fig. 6.32).

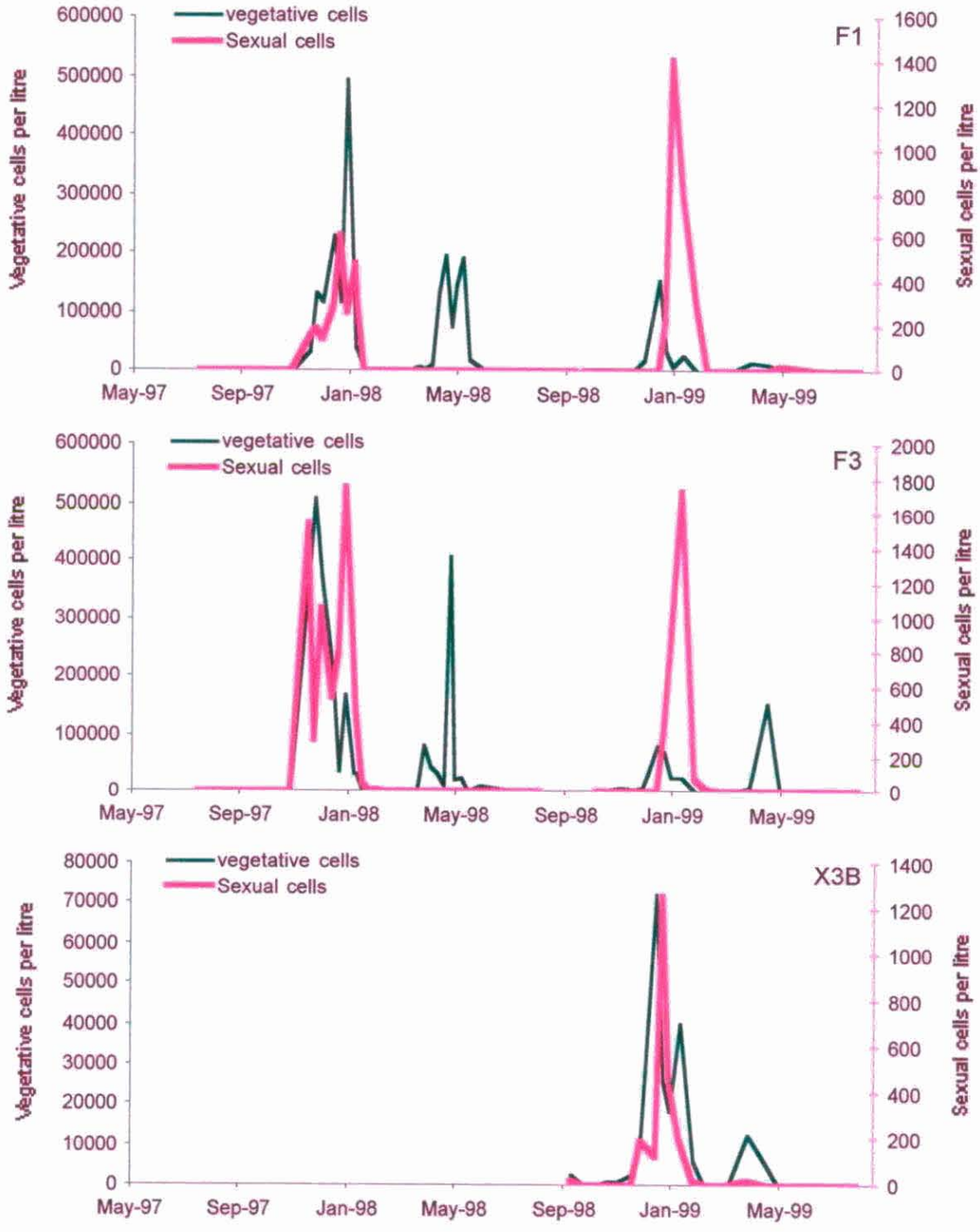


Figure 6.32: *Gymnodinium catenatum* cell densities (vegetative and sexual cells) during the three year study (sexual stages were only enumerated during 1998/99 for X3B – Pt Cygnet) quantified from fixed integrated water column samples.

In the autumn bloom of 1999 sexual stages were undetectable in both net samples and integrated samples.

6.20.3 RELATIONSHIP BETWEEN VEGETATIVE AND SEXUAL CELLS

In some cases (e.g. site F3 during blooms in 97/98 and 98/99) vegetative cell numbers in summer blooms were comparable with those in autumn yet, as stated above, sexual cells in autumn blooms were not observed. This suggests that greater sexual cell production (encystment) is not a simple function of increased vegetative cell density. However, there was a significantly positive relationship between sexual cells and vegetative cells calculated over the whole study (Fig. 6.33a, $r^2 = 0.216$, $p<0.01$) which was higher if only summer blooms were included in the analysis (Fig. 6.33b, $r^2 = 0.432$, $p<0.01$).

6.21 INFLUENCE OF CHEMICAL AND PHYSICAL FACTORS ON LIFE CYCLE TRANSITIONS

Correlations between chemical variables (nutrients) and the presence of sexual cells in the water column were calculated for the three year dataset. Significant and negative correlations ($p<0.01$) were found for several nutrients (Table 6.9). The strongest negative correlations were found for bottom nutrients (Table 6.9). These patterns were consistent for both sites at which sexual stages were quantified for the whole study (Killala Bay and Wheatleys) (Figs 6.34, 6.35 and 6.36).

Table 6.9: Correlation coefficients for numbers of *Gymnodinium catenatum* sexual stages and nutrient concentrations or physical variables. All correlations are significant ($p<0.01$).

Chemical Parameter	Pearson Correlation Coefficient (r)
Bottom-Depth	
Nitrate + Nitrite	-0.402
Nitrite	-0.316
Total Dissolved Nitrogen	-0.370
Phosphate	-0.416
Dissolved Oxygen	-0.307
Mid-Depth	
Nitrate + Nitrite	-0.293
Nitrite	-0.246
Phosphate	-0.304
Surface-Depth	
Nitrate + Nitrite	-0.287
Nitrite	-0.237
Total Dissolved Nitrogen	-0.242

Correlations between physical variables and the presence of sexual cells in the water column were calculated for the three-year dataset. Significant positive correlations ($p<0.01$) were found for temperature ($r=0.440$, $n=121$) and secchi depth ($r=0.230$, $n=133$) (Fig. 6.37). The

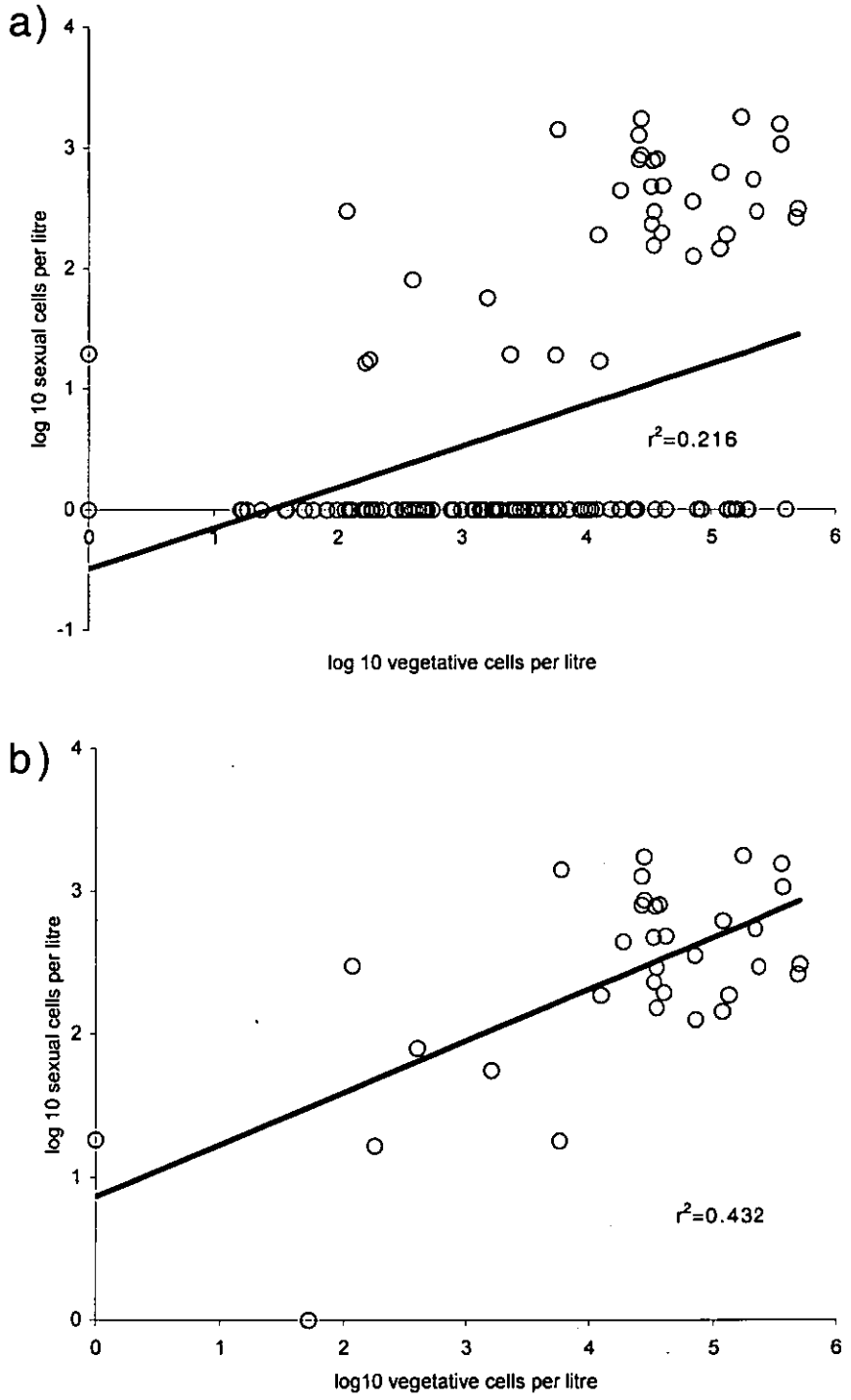


Figure 6.33: Relationship between *Gymnodinium catenatum* vegetative cells and *Gymnodinium catenatum* sexual cells a) the entire dataset and b) summer bloom samples only.

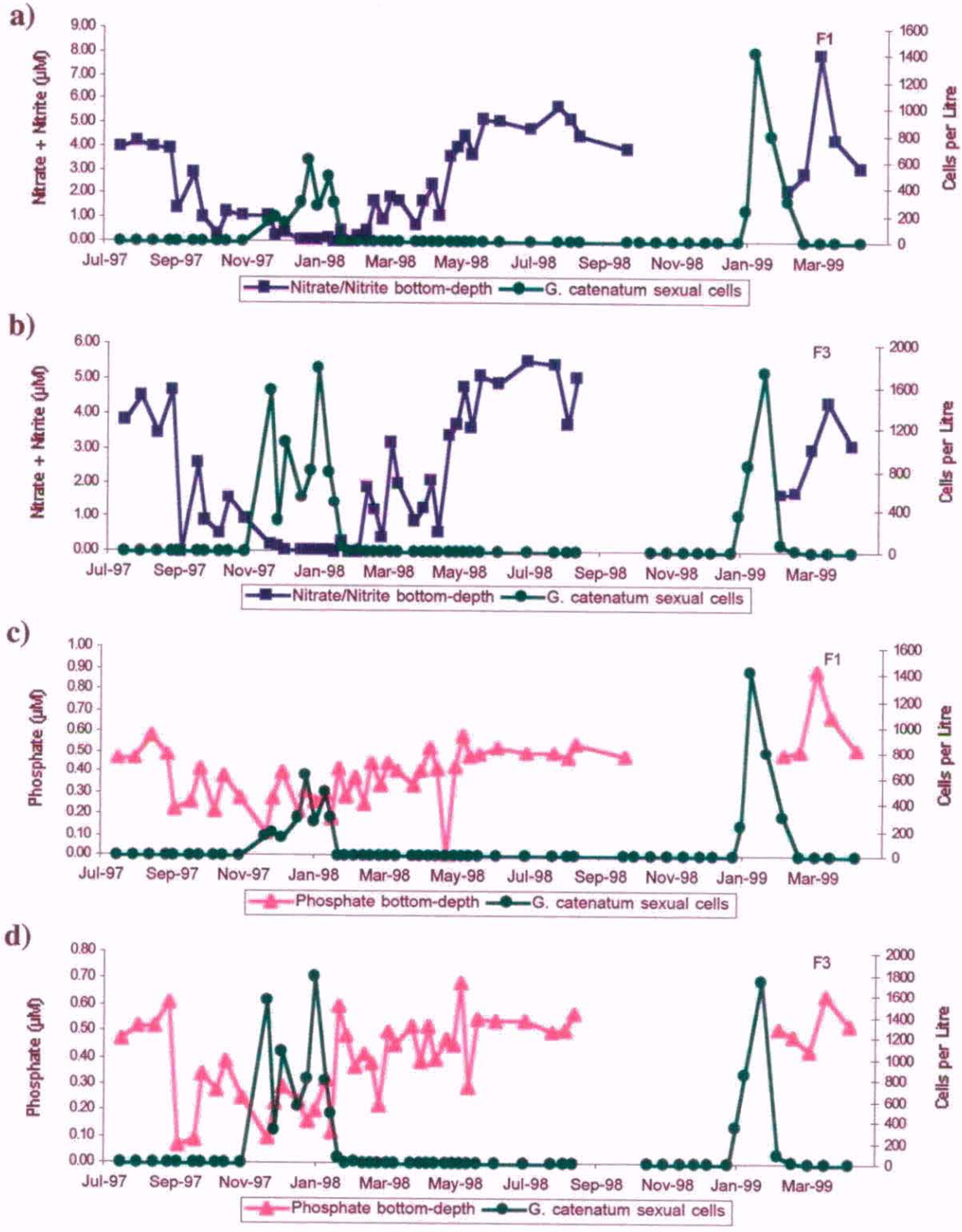


Figure 6.34: Sexual cell densities of *Gymnodinium catenatum* compared with nitrate/nitrite (a and b) and phosphate (c and d) concentrations measured at bottom depth from F1 (Killala Bay), and F3 (Wheatleys) during the second two years of the study.

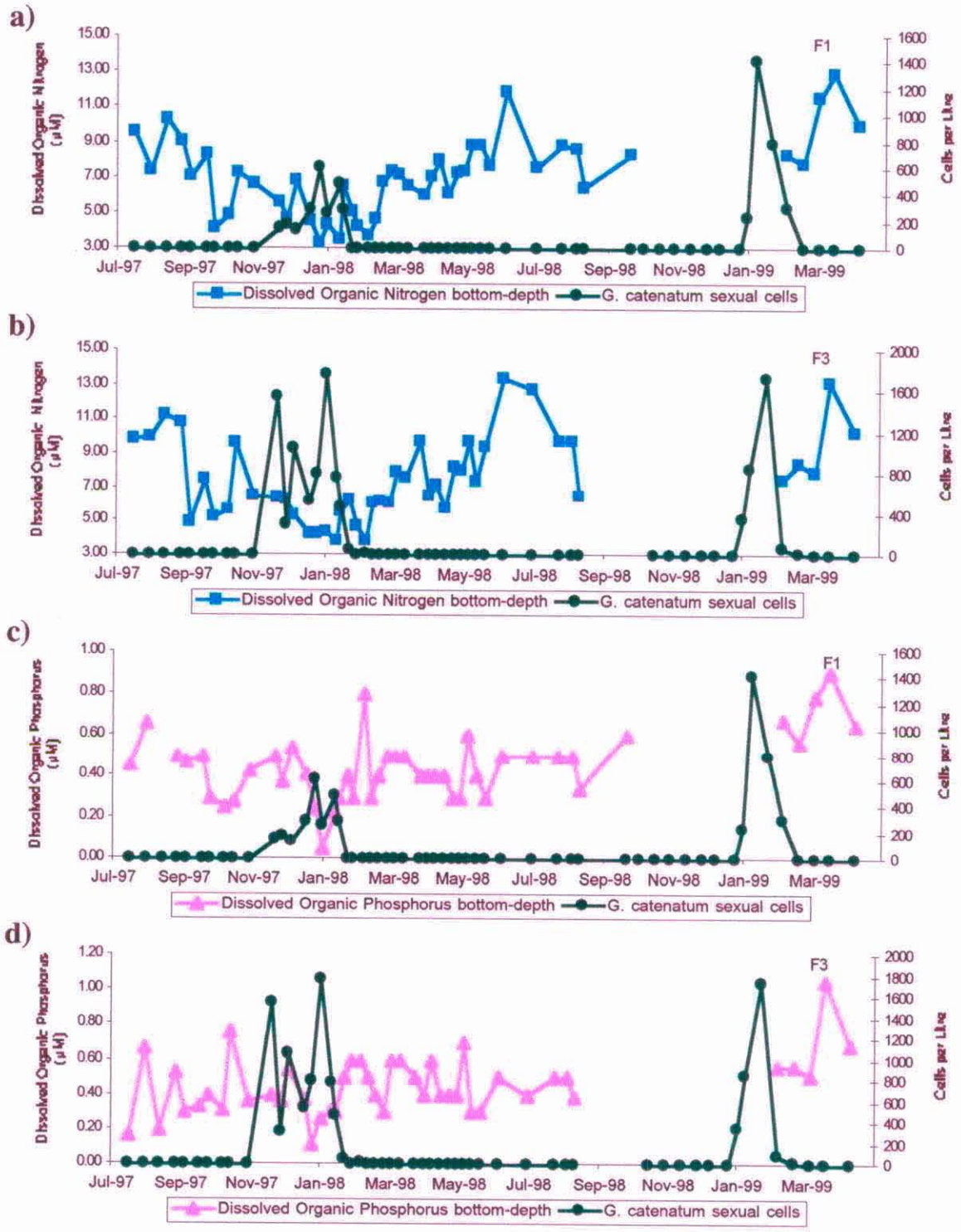


Figure 6.35: Sexual cell densities of *Gymnodinium catenatum* compared with dissolved organic nitrogen (a and b) and dissolved organic phosphorus (c and d) concentrations measured at bottom depth from F1 (Killala Bay), and F3 (Wheatleys) during the second two years of the study.

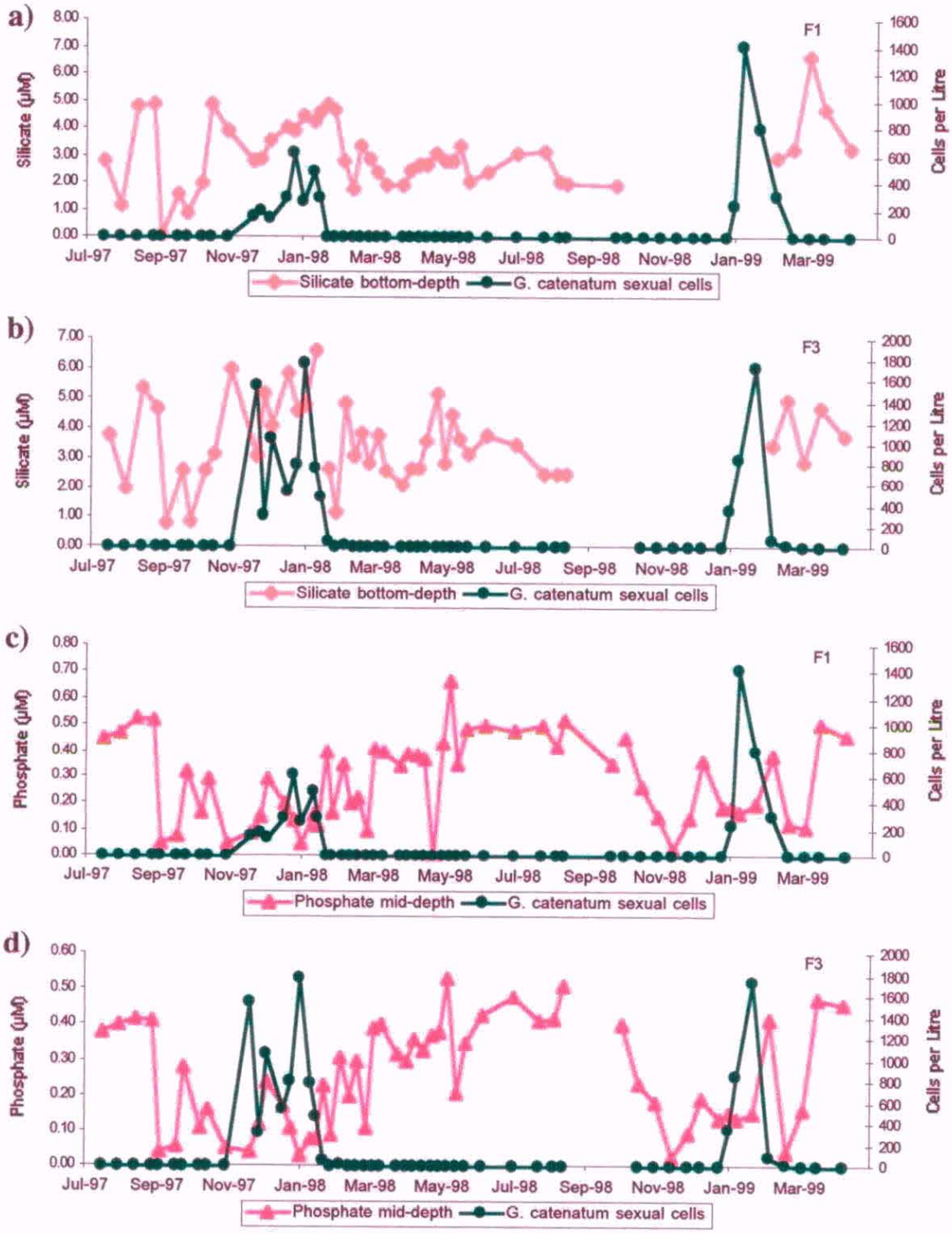


Figure 6.36: Sexual cell densities of *Gymnodinium catenatum* compared with bottom silicate (a and b) and mid-depth phosphate (c and d) concentrations from F1 (Killala Bay), and F3 (Wheatleys) during the second two years of the study.

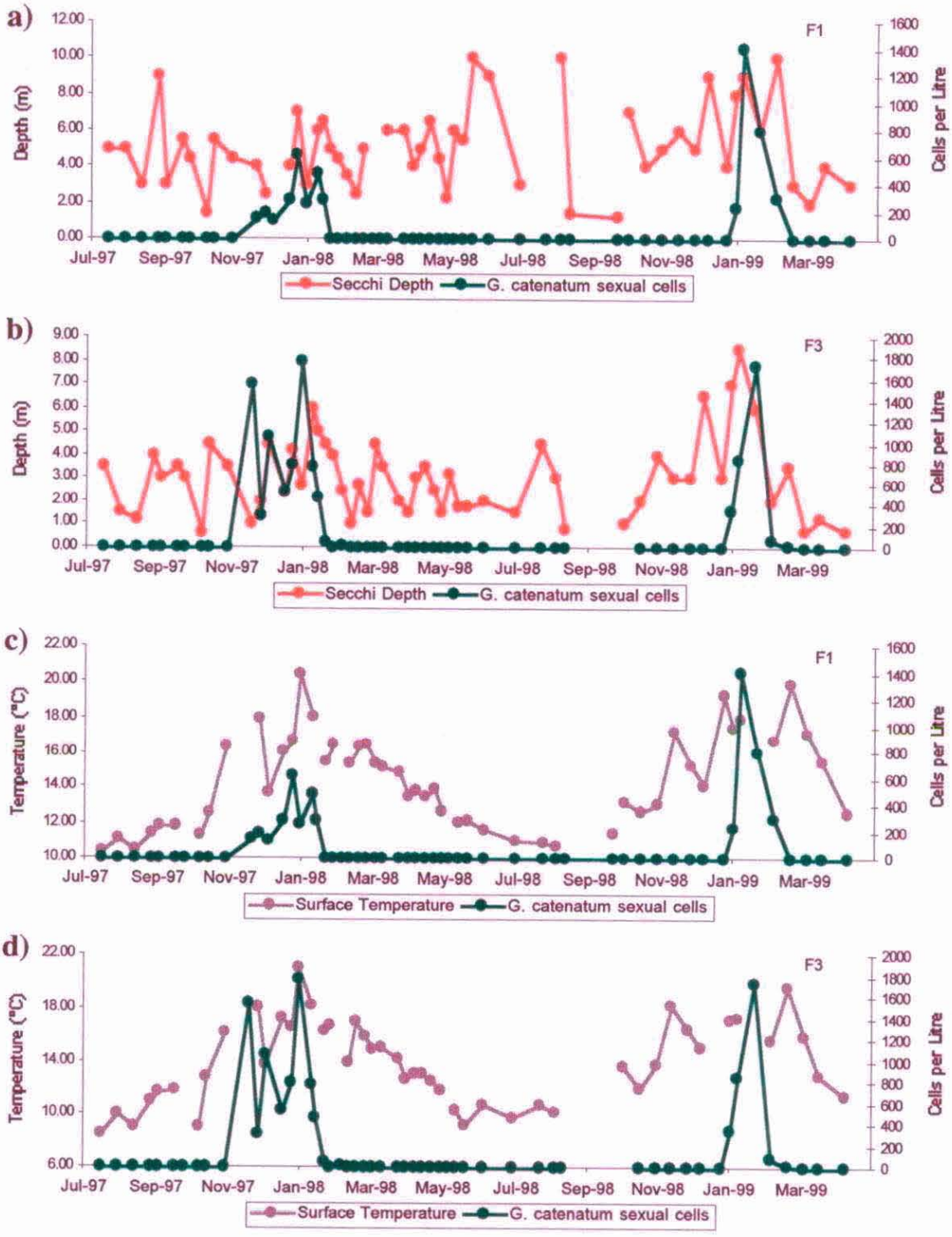


Figure 6.37: Sexual cell densities of *Gymnodinium catenatum* compared with secchi depth (a and b) and surface temperature (c and d) from F1 (Killala Bay), and F3 (Wheatleys) during the second two years of the study.

positive association between sexual stages of *G. catenatum* and temperature is particularly clear at both Killala Bay and Wheatleys (Fig. 6.37c and d).

6.22 CANONICAL CORRESPONDENCE ANALYSIS OF SEXUAL STAGES

Canonical Correspondence Analysis (CCA) was used to assess the influence of environmental factors (chemical and physical variables) on the abundance of different algal groups and in particular the influence of environmental factors on life cycle transitions of *G. catenatum*. For this analysis only the samples with observations of *G. catenatum* sexual stages were used (means of observations from Killala Bay – F1 and Wheatleys – F3) rather than seasonal averages for each site as were used in the CCAs described in Section 6.19. Three separate CCAs were calculated for physical variables plus nutrients at the three different depths e.g. one CCA incorporated all physical variables and bottom nutrients (due to high variance inflation factors if all nutrients were combined). The resulting three CCAs were similar, only the results for the bottom nutrients CCA, which contained the greatest number of significant variables are graphically presented (Fig. 6.38).

The first two CCA axes, with eigenvalues of λ_1=0.051 and λ_2=0.019 account for 8 % of the cumulative variance in the algal-environmental relationship. However, CCA axes 1 and 2 explain a large proportion of the variance in the algal-environmental relationship (88.4 %). The algal-environment correlations for CCA axis 1 (0.85) and axis 2 (0.67) are moderately high indicating a strong relationship between algal taxa and the 14 environmental variables.

With forward selection and unrestricted Monte Carlo permutation tests (99 permutations, $p \leq 0.05$), CCA identified a minimal subset of three environmental variables that explained significant ($p \leq 0.05$) proportions of the variance in the algal taxa. In descending order of importance these were surface temperature (STEMP), bottom filtered nitrite (BNO_2F), and bottom silicate (BSi). These three factors represent 75% of the variance described by the original 14 environmental variables. Surface and mid-depth CCAs also identified surface temperature as the most significant variable and in the mid-depth CCA mid-depth total nitrogen (MTN) was also significant. Based on the approximate t-tests both bottom total phosphorus (BTP), and bottom silicate (BSi) contributed significantly to axis 2.

The CCA indicated that *G. catenatum* sexual stages were associated with temperature and bottom silicate (both significant variables). Other dinoflagellates (*G. catenatum* vegetative cells (GCVEG), *Ceratium* spp. (CERAT), and *Polykrikos schwartzii* (POLYK)) were more closely associated with the river flow variables; rainfall (AVRAIN), flow (FLOW, FLOW3WK) and light extinction coefficient (LEXTINC). The CCA indicated that diatoms

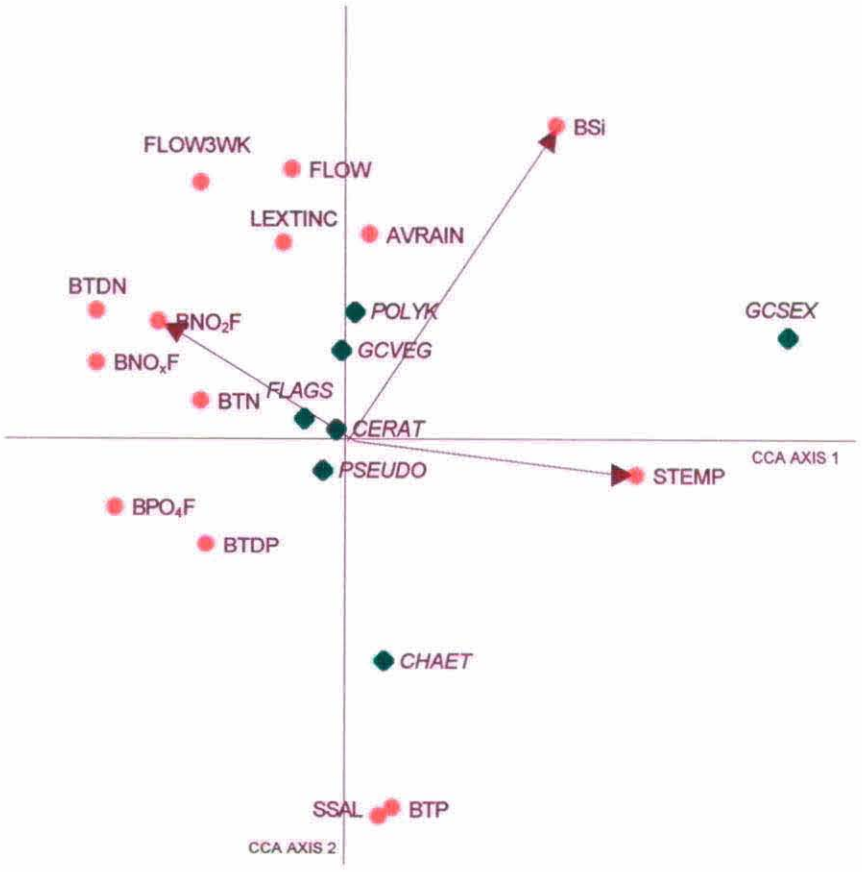


Figure 6.38: Canonical Correspondence Analysis biplot showing the relationship between bloom species (green diamonds) and the four significant environmental factors (arrows to red circles). Taxa abbreviations are GCVEG = *Gymnodinium catenatum* vegetative cells, GCSEX = *Gymnodinium catenatum* sexual cells, which is an active variable in this anlaysis, CERAT = *Ceratium* spp., POLYK = *Polykrikos schwartzii*, CHAET = *Chaetoceros* spp., PSEUDO = *Psedonitzschia* spp. and FLAGS = small flagellates. Environmental variable abbreviations are TEMP = temperature, SAL = salinity, LEXTINC = light extinction coefficient, AVRAIN = average rainfall from Geeveston and Franklin, FLOW = meann daily flow from frying pan creek and FLOW3WK = cumulative flow for the 3 weeks prior to sampling date, TN = total nitrogen, TDN = total dissolved nitrogen, NO_x = nitrate+nitrite, NO_2 = nitrite, TP = total phosphorus, TDP = total dissolved phosphorus, PO_4 = phosphate, Si = silicate. S and B indicate surface and bottom samples respectively and F indicates that the variable was measured on a filtered sample.

were positively associated with surface salinity and phosphorus species. Flagellates were associated with nitrogen species.

DISCUSSION

6.23 GENERAL PHYTOPLANKTON DYNAMICS

Phytoplankton dynamics in the Huon Estuary are affected by a number of key physical and chemical forcing factors. The estuary receives persistent runoff, maintaining a strong two layer estuarine circulation which results in short flushing times (HEST, 2000 and Table 6.1). Because of the estuary's temperate location (latitude 42° 45' S and 43° 45' S) it is also subject to seasonal variation in surface irradiance and temperature. The Huon River drains a relatively pristine catchment, and delivers low nutrient loads, but is very highly coloured by natural humic substances (HEST, 2000). The phytoplankton community composition and dynamics in the Huon Estuary during this study were similar horizontally, although the vertical distribution of phytoplankton was highly variable with subsurface chlorophyll maxima. The five biological sites – Hideaway Bay (B1), Port Cygnet (X3B), Killala Bay (F1), Wheatleys (F3) and Brabazon Park (H3) – provided a consistent picture of temporal changes in community composition.

6.23.1 LIGHT LIMITATION AND HUON RIVER FLOW

Flow of the Huon River does not have a strong seasonal signal but is variable throughout the year. This is very different to other Australian estuaries such as the Swan River estuary (Thompson, 1998), which has strong seasonal variation in flow, with high flow in winter and very low flow in summer. The year-round flow of the Huon River results in surface salinities in the Huon Estuary that do not differ significantly between seasons, and secchi depths that are also highly variable, increasing towards the mouth of the estuary. Huon River waters are high in coloured dissolved organic matter (CDOM) with absorption due to CDOM in the upper estuary (measured at a wavelength of 440 nm) ranging between 7 and 14 m^{-1} (HEST, 2000), these are among the highest values recorded in the literature for Australian water bodies (Kirk, 1994). The year-round flow of the optically shallow Huon River therefore results in a complex light field in the estuary; an extreme example of 'case 2' waters (Clementson et al., 1998).

The mean light extinction coefficient calculated for the estuary from the five biological sites during this three year study was 0.5 m^{-1}, ranging between 0.21 and 1.98 m^{-1}. Assuming a surface irradiance of 2000 and that the photic depth (Z_p) is the depth of 1% of surface irradiance (Cloern, 1987), this light extinction coefficient would result in average photic

depths for the estuary of 9.2 m, ranging between 2.3 and 21.9 m, calculated from equation 2 (Kirk, 1994):

$$I_d = I_0 e^{-k_d d} \qquad \text{Equation 2}$$

Where I_d = irradiance at depth d, I_0 = surface irradiance, and k_d = the extinction coefficient. The photic depth of large rivers and river-dominated estuaries is typically <5 m and often <1 m during peaks in river discharge (Cloern, 1987), therefore in comparison to other estuaries the photic depth of the Huon Estuary is not exceptionally shallow. The range of photic depths observed in the Huon Estuary are similar to other estuaries including the Port Hacking Estuary in New South Wales, Australia and the Lower Hudson River, USA (Scott, 1978; Malone, 1980) but significantly deeper than others including San Francisco Bay (Cloern, 1987).

In a well mixed water column phytoplankton growth becomes light limited when the ratio of photic depth to mixed depth is less than about 0.2, or alternatively when the mixed layer depth is more than 5 times the photic depth (Cloern, 1987). Assuming that the Huon Estuary is relatively well mixed to the bottom, bathymetric data from the Huon Estuary (HEST, 2000) indicates that the mid and lower estuary are therefore, on average, not light limited. However, these calculations are based on maximum surface light intensity. Cloud cover, sun angle and the complex light field described above due to year-round flow of the Huon River, would result in sporadic light limitation in the estuary throughout the year, particularly on the north/northeast side of the mid estuary where the influence of the Huon River is strongest.

These calculations highlight the impact of river flow on phytoplankton growth in the Huon Estuary. Such a temporally variable light field may favour slower growing phytoplankton species that are able to remain within the estuary and persist through periods of both low and high light penetration i.e. species which tend towards K selection (e.g. dinoflagellates) rather than the more opportunistic fast growing r selected species (e.g. diatoms) (MacArthur and Wilson, 1967).

6.23.2 BIOMASS VARIABILITY

Phytoplankton biomass in the Huon Estuary in winter during this three year study was uniformly low (mean of 0.6 mg Chl *a* m^{-3}), despite elevated nutrient concentrations, and was presumably light limited due to Huon River flow, coupled with the low angle of the sun in winter. Although overwintering biomass was consistently low, the interannual variability in the biomass of the Huon Estuary in other seasons was considerable. Phytoplankton biomass in 1996/97 continued to be low into spring and summer, while 1997/98 and 1998/99 were

characterised by a succession of phytoplankton blooms and elevated biomass throughout the spring, summer and autumn. These blooms followed a progression from diatom dominated blooms in the spring to dinoflagellate (especially *G. catenatum*) dominated summer blooms, mixed diatom and dinoflagellate late summer blooms and *G. catenatum* dominated blooms in the autumn. Cell counts and pigment analysis provided a consistent picture of these phytoplankton dynamics. The combination of the two methods provides an accurate picture of the phytoplankton, with pigment analysis identifying the presence of flagellate groups which are difficult to identify using light microscopy, and microscopy identifying important species, which is not possible through pigment analysis.

Patterns of phytoplankton productivity and biomass in temperate estuaries have often been noted to rise and fall with water temperature and day length (Day et al., 1989; Mallin, 1994) but superimposed on these important variables for phytoplankton growth are other physical, chemical and biological factors. Meteorology and hydrology have been identified as important for structuring estuarine phytoplankton communities in North Carolina, with the magnitude of winter rainfall influencing river flow and nutrient loads on the estuaries and subsequently phytoplankton productivity (Mallin, 1994). The link between runoff and phytoplankton biomass has been noted for other systems (Jordan et al., 1991; Gallegos, 1992). Turbidity and nutrients also influence biomass in estuarine systems (Cloern, 1987; Fisher et al., 1992). In the Huon Estuary the interannual variability in phytoplankton biomass could not be simply explained by a change in nutrients since the three years were not significantly different in their nutrient dynamics, although nutrients varied along estuarine and seasonal gradients. Physical characteristics of the estuary were also similar from year to year with strong seasonal patterns in temperature, and similar flow, surface salinity and rainfall. Chlorophyll data indicate that the presence of dinoflagellate blooms significantly increased phytoplankton biomass in the estuary and conversely their absence in 1996/97 may have been a major contributor to the low biomass observed in that year.

6.23.3 PHYTOPLANKTON SPECIES SUCCESSION

Species succession in phytoplankton communities is well documented for most aquatic systems from polar (e.g. Smith and Asper, 2001) to temperate (e.g. Mallin, 1994) and tropical (e.g. Devassy and Goes, 1988) regions. The environmental factors suggested as influencing species succession are many and varied but can be summarised into three broad categories which together contribute to defining the ecological niche of a species. The first of these categories is nutrient availability (e.g. Gallegos, 1992; Ault et al., 2000). Both absolute and relative nutrient concentrations, have been identified as important in structuring phytoplankton communities. In particular the ratio of different nutrients are considered to be

important (Cloern, 2001). In the Huon Estuary the ratio of N:P suggests that the ecosystem will become seasonally N limited (HEST, 2000).

Physical water column structure is also considered important in driving species succession, with certain algal groups preferring either stratified or well mixed environments (e.g., Margalef, 1978; Margalef et al., 1979; Tilstone et al., 2000). The Huon Estuary is a classic salt wedge estuary, with the fresh water of the Huon River overlying the saline oceanic waters. Fresh water from the Huon River, coupled with physical forcing from winds and tides, leads to variable water column characteristics. These characteristics can vary from one side of the estuary to the other with dominant winds pushing the freshwater plume towards the north-eastern shore as indicated by variation in secchi depths between sites on opposite sides of the estuary (Table 6.6) and by salinity patterns (HEST, 2000). When flow is high, the estuary becomes more stratified and in summer the dark fresh water is warm, setting up a strong temperature gradient in combination with the salinity gradient (HEST, 2000). A stratified water column is more likely to favour dinoflagellates while well-mixed conditions tend to favour diatoms (Margalef et al., 1979). The third category of factors which influence species succession is loss processes. Loss processes include sinking and grazing and can be highly selective due to variability in sinking rates of different species (Harrison et al., 1986) and selective grazing, (e.g. Graneli et al., 1999) which is influenced by phytoplankton size but may also be affected by toxicity of some algal species (Teegarden et al., 2001).

It is hypothesised here that the observed difference between 1996/97 and the other two years of the study may be partially due to a breakdown in the succession from the diatom to dinoflagellate dominated community. In 1996/97, consistently high Huon River flow throughout the spring (August to December) was observed (Fig. 6.27). This high flow, which was associated with slightly cooler surface temperatures (Table 6.5), high flushing rates (Table 6.1), and lower light penetration due to humic rich fresh water may have inhibited spring diatom growth, particularly in the upper estuary. An absent or reduced spring diatom bloom would in turn have reduced nutrient uptake and transport to the sediments, reducing short-term remineralisation and in particular the availability of ammonia (Cloern, 2001). It has been demonstrated that metabolic costs of ammonia uptake are less than the costs of nitrate uptake (Thompson et al., 1989) and also that dark uptake of ammonia by dinoflagellates is substantially higher than for nitrate (MacIsaac, 1978). Lower ammonia in bottom waters of the Huon Estuary were observed in the summer of 1996/97 than in the summer of 1997/98 (approximately $^2/_3$ of the concentrations; HEST, 2000). This observation of lower ammonium supports the hypothesis that remineralisation of diatom blooms may be important for dinoflagellate bloom development, but the number of observations was too low to test their statistical significance. A reduction in bottom

nutrients may decrease the competitive advantage of vertically migrating dinoflagellates, which are able to access these deep nutrients (Hamilton et al., 1998). In some respects this hypothesis is analogous to the terrestrial 'facilitation' model of species succession (i.e. species replacement occurs due to environmental modification by the previous species in the succession (Connell and Slatyer, 1977)), in that the senescent diatoms 'prepare the system' for dinoflagellate growth. The relevance of terrestrial models to planktonic systems has been questioned (Smayda, 1980) and planktonic systems are certainly less directional than terrestrial ones, but terrestrial models of succession are perhaps more applicable in estuarine systems with strong benthic-pelagic coupling. Species succession from diatoms to dinoflagellates has been observed in various other systems including the Gulf of Maine, (Gran and Braarud, 1935) and a general trend of succession from non-motile to motile species (except in polar regions where the reverse occurs) has been suggested (Smayda, 1980).

6.24 *GYMNODINIUM CATENATUM* DYNAMICS

Gymnodinium catenatum was not present in phytoplankton samples from 1996 and early 1997, but developed extensive blooms in summer and autumn of 1997/98 and 1998/99. This difference in the bloom dynamics is not simply explained by physical and chemical conditions in the estuary over the three years. The above discussion of a breakdown in species succession provides one possible explanation for this interannual variability but does not adequately explain the complete absence of *G. catenatum* in the estuary, this is discussed further in Section 6.25.

The *G. catenatum* blooms investigated during this study occurred under a variety of conditions. During the summer 1997/98 bloom, the water column was more stratified and warmer than in the autumn 1998 bloom. The winds were more variable, with some calm periods of a few days interspersed with strong wind events. During the autumn bloom the winds were relatively moderate and more persistent. River runoff occurred during both blooms, with larger pulses of fresh water during the summer bloom. Nutrient levels tended to be low during both blooms.

During the *Gymnodinium catenatum* blooms in the summer and autumn of 1997/98, chlorophyll *a* levels reached integrated water column values as high as 22 mg Chl *a* m^{-3}. The equivalent nitrogen content would be about 20 μM, more than twice the maximum observed concentrations of dissolved inorganic nitrogen and ten times the concentration available during bloom initiation (HEST, 2000). This implies a mechanism whereby *G. catenatum* blooms concentrate nitrogen. One possibility is that detrital organic nitrogen accumulates in sediments during winter and spring and is released into bottom waters in

summer and autumn. A second possibility is that the observed vertical migration between out-flowing surface and in-flowing bottom layers, enables *G. catenatum* to maintain its horizontal position in the estuary, accumulating nitrogen from the water circulating through the estuary although low nitrate in the water column suggests that either there is no influx of nitrate from oceanic waters or that any nitrate is taken up rapidly. Inverse and prognostic process models suggest that vertical migration plays a critical role in enabling *G. catenatum* to reach the high observed biomass concentrations (HEST, 2000).

Some river runoff appears to be essential for *G. catenatum* bloom formation. The major influences of flow from the Huon River are increasing water column stability through stratification and as a source of dissolved organic matter (DOM) and both of these factors are likely to contribute to *G. catenatum* bloom formation. Various studies have identified water column stability as an important prerequisite for dinoflagellate growth (Estrada and Berdalet, 1998) and dissolved organic matter has been found to stimulate growth of dinoflagellates (Doblin et al., 1999a). The micronutrient selenium, which is present in humic rich waters, has also been identified as important for growth of *G. catenatum* (Doblin, 1999b). The relationship between the growth of *G. catenatum* and DOM is complicated with evidence for a possible function of DOM in chelating trace metals (Doblin et al., 1999a, b, 2000).

The capacity for strong vertical migration is probably one key to the success of dinoflagellates in the Huon Estuary. In particular, the relatively high swimming velocity of *G. catenatum* compared with other dinoflagellates should be beneficial for access to deep nutrients. Observed swimming velocities in dinoflagellates range from 0.03 to 6.5 mh^{-1} with most velocities below 1.3 mh^{-1} (Levandowsky and Kaneta, 1987; Kamykowski et al., 1992). Estimates of swimming velocities of *G. catenatum* based on vertical migration detectable from profiler data are at the upper end of this range from 1.5 to 6.0 m h^{-1}. In addition to providing access to deep nutrients, vertical migration may also enable *G. catenatum* to avoid being flushed out of the estuary as the cells spend most of their time well below the rapid outflow of the surface layer. Through both avoidance of flushing and access to deep nutrients, vertical migration may be crucial for the development and persistence of monospecific *G. catenatum* blooms. *G. catenatum* is a potentially introduced species (Hallegraeff, 1993; Hallegraeff and Fraga 1998; McMinn et al 1997). Deep vertical migration may be one important factor which has enabled *G. catenatum* to outcompete other dinoflagellates for access to deep nutrients e.g. *Ceratium* spp. which have recorded swimming velocities of only 0.2 - 0.9 m h^{-1} (Levandowsky and Kaneta, 1987), and the non motile taxa such as diatoms. A third important function of vertical migration is that upward migration during the day provides *G. catenatum* with access to light. In the complex and

sporadically light limited environment of the Huon Estuary (see Section 6.23.1) this provides another competitive advantage.

Hallegraeff et al. (1995) identified key environmental variables that regulate *G. catenatum* blooms and associated shellfish toxicity in southern Tasmanian waters. They examined historical data of toxicity and hydrological and meteorological data from 1986 to 1994 in order to ascertain what stimulates *G. catenatum* bloom formation. Their hypothesis was that *G. catenatum* blooms can only develop within environmental constraints: a seasonal temperature window from January to June, with major blooms (as shown by high toxicity) only developing when water temperatures are greater than 14°C; a threshold runoff from the Huon River in the weeks preceding a bloom (greater than 100,000 megalitres flow over three weeks measured in the upper Huon River at Frying Pan Creek); and a calm stable water column for sustained bloom development as indicated by wind speeds of <5 m s^{-1} (measured at Cornelian Bay, Derwent Estuary) for five days or more.

The *G. catenatum* blooms reported herein developed at water temperatures of approximately 14°C but the autumn blooms were most dense at temperatures a little above 12°C. This is similar to blooms in 1987 and 1990 that Hallegraeff et al. (1995) described as exceptions. However, it is likely that decreasing growth rates associated with decreasing water temperatures contributed to the decline of these blooms (Blackburn et al. 1989). The wind data presented in this thesis is from Cape Bruny, a site Hallegraeff et al. (1995) found was not well correlated with *G. catenatum* bloom development in the Huon Estuary. Instead they used wind data from Cornelian Bay, Derwent Estuary as a proxy for water column stability. The potential influence of winds on bloom development is complex, as wind direction, duration and fetch all need to be considered. A single site – either Cape Bruny or Cornelian Bay – does not adequately describe the wind field in the Huon, nor the degree of stratification. Direct measurement of water column stability is preferable to either of these measures. The detailed data on water column characteristics from the automatic profiler showed that blooms can develop when water column stratification is weak, while strong stratification appears to enhance bloom intensity. The automated profiler data also identified that destratification caused by a combination of wind and oceanic influences was associated with bloom decline. The January to June window for bloom formation suggested by Hallegraeff et al. (1995) is clearly not exclusive since booms in 1997/98 and 1998/99 began in November/December. The data presented here also suggests that a range of flow regimes may support bloom formation since bloom formation takes approximately 3 months at net growth rates of 0.08 d^{-1}.

The processes leading to bloom decline are as complex as those influencing bloom initiation. Data from this study indicate that complete destratification and declining temperatures are two important factors. Grazing may also be important, with blooms of the heterotrophic dinoflagellate *Polykrikos schwartzii* coinciding with *G. catenatum* blooms and exerting grazing pressure on the population although the magnitude of this is unknown. Another potential loss factor is through the action of algicidal bacteria which can cause cell lysis. Six species of bacteria (*Pseudoalteromonas* sp. (ACEM1), *Pseudoalteromonas* sp. (ACEM4), *Cellulophaga* sp. (ACEM 20), *Cellulophaga lytica* (ACEM21), *Plonococcus* sp. (ACEM22) and *Bacillus mycoides* (ACEM32)) isolated from the Huon Estuary have been found to have an algicidal effect on *G. catenatum* (Lovejoy et al., 1998; Skerratt, 2001). Further study is required to determine the quantitative impact of these algicidal bacteria on *G. catenatum* bloom dynamics, but this group of organisms may contribute to *G. catenatum* bloom decline (Skerratt, 2001).

In summary observations of key environmental variables that support or enhance blooms were identified from the two years in which blooms occurred: water column stratification, temperatures of 12°C and greater, some river run-off and the availability of nutrients at depth. The capacity of *G. catenatum* to efficiently access both surface light and deep nutrients, particularly ammonia, while also avoiding being flushed from the estuary through deep vertical migration may be a crucial factor for bloom formation of this species.

6.25 *GYMNODINIUM CATENATUM* SEXUAL REPRODUCTION

Dinoflagellate resting cysts have been shown to form in response to nutrient depletion in culture suggesting that adverse conditions – notably nutrient depletion – stimulate sexual reproduction (Chapter 1, Table 1.1). However, there is increasing evidence to suggest that resting cysts form in response to a variety of factors (Anderson et al., 1983; Ellegaard et al., 1998; Kremp and Heiskanen, 1999; Sgrosso et al., 2001) rather than a simple response to nutrient depletion (see Chapter 5). Significant negative correlations were identified between sexual cell concentrations and bottom nutrients. However, based on CCA analysis, macronutrient concentrations were not significantly associated with sexual reproduction and resting cyst formation of *G. catenatum* in the Huon Estuary, and laboratory experiments also failed to show enhanced encystment of *G. catenatum* in response to nutrient depletion (see Chapter 5).

Resting cysts formed throughout summer blooms with the first sexual stages (gametes, planozygotes and resting cysts) being detected in the first bloom samples. Continuous encystment throughout blooms also occurs in *Peridinium bipes* (Park and Hayashi, 1993). In other dinoflagellate species mass encystment has been suggested as a major factor in bloom

decline (e.g. *Gymnodinium pseudopalustre*, Tyler and Heinbokel, 1985). In the four *G. catenatum* blooms of this study, there was no dramatic increase in resting cyst production towards the end of blooms. Encystment is therefore unlikely to play an important role in bloom decline for this species in the Huon Estuary. However, at times sexual cells represented greater than 30% of the *G. catenatum* cells observed in the field, and under some experimental conditions very high encystment has been observed (> 50% – see Chapter 5). Encystment can therefore not be discounted as a contributing factor to bloom decline.

Sexual reproduction was not observed in autumn blooms. The autumn bloom populations did contain compatible gametes (as determined by crossing experiments of strains isolated from these blooms - see Chapter 3) and the fact that encystment did not occur suggests the influence of environmental factors. CCA analysis indicated that surface temperature was significantly associated with sexual stages of *G. catenatum*. Experimental results also indicate that the lower water temperatures recorded in autumn would significantly reduce encystment (Chapter 5). Reduced encystment at lower temperatures has also been observed in the related species *Gymnodinium nolleri* (Ellegaard et al., 1998) and in other dinoflagellates (Anderson et al., 1984).

In this study, *G. catenatum* blooms occurred in both summer and autumn but toxin data and previous studies have shown that years in which only autumn blooms occur are more common (Hallegraeff et al., 1995, I. Jameson personal communication). This has major implications for sexual reproduction of *G. catenatum* since encystment is below detection in these blooms. Sexual reproduction, genetic recombination and consequent replenishment of resting cysts of *G. catenatum* in the sediments may therefore be minimal in most years in the Huon Estuary.

During this study, the seed source for the *G. catenatum* blooms was probably from both resting cysts and over-wintering vegetative cells. Although our sampling strategy precluded sediment / water interface studies for resting cyst germination, the presence of sexual stages during the summer blooms suggests a genetically heterogeneous origin which would be expected from either resting cyst germination or a diverse over-wintering population (Bolch et al. 1999). In the 1997/98 summer bloom, there were sufficient cells in the water column by early October to initiate a bloom through vegetative cell division given a net growth rate of 0.08 d^{-1} without the addition of new cells from resting cyst germination. However, the complete lack of vegetative cells in the water column prior to April 1997 suggests that resting cyst germination contributed new cells to the water column prior to bloom development. There is also the possibility that cells were advected into the estuary from adjacent water bodies but this is unlikely given that blooms were first detected in the upper

estuary and the highest biomass and 'healthiest' cells are also found in this area. Cells found at the mouth of the estuary and in the adjacent D'Entrecasteaux Channel are generally less 'healthy' (Hallegraeff et al., 1995; N. Parker, personal observations).

Earlier work has shown Port Cygnet to be one source of *G. catenatum* resting cysts (Bolch and Hallegraeff, 1990). In the present study blooms were first detected in the brackish water end of the estuary suggesting this as a potential 'seed source' for blooms. This area has not been sampled for resting cysts in previous sediment studies but should be investigated as a possible point of bloom initiation. Strong spring winds may resuspend sediments in shallower regions of the estuary, enhancing resting cyst germination by increasing the oxygen levels they are exposed to (anaerobic conditions have been found to prohibit germination of resting cysts; Chapter 5, Anderson et al., 1987). Detailed analysis of sediments in the shallow bays in the mid to upper estuary, along with water column turbulence at key points within the estuary are required to determine the role of resuspended resting cysts in bloom formation and possible seed sources.

Supply of germinating resting cysts and conditions suitable for germination may have contributed to the differences in phytoplankton dynamics between 1996/97 and the other two years of the study. *G. catenatum* resting cysts have a short obligate endogenous dormancy period (a minimum of 2 weeks; Blackburn et al., 1989). This short dormancy implies that resting cysts of these species might not provide a particularly effective long-term survival strategy. It is hypothesised here that *G. catenatum* resting cysts may function at two different levels. The first is that resting cysts in surface sediments, which are frequently resuspended, regularly germinate to seed the vegetative population, supporting bloom development by avoiding or bridging unsuitable periods for growth. The second layer of the strategy is that resting cysts which are buried relatively deeply, through bioturbation or high sedimentation rates, can remain viable in the sediments for much longer time periods (on the order of years) due to darkness and low oxygen conditions which inhibit excystment (Chapter 5 and Blackburn et al., 1989). These deeper cysts may be resuspended during relatively rare climatological events such as extremes of river flow or wind stress.

The observations of *G. catenatum* in this three year study can be put into the context of this two level strategy. In the first year of the study *G. catenatum* was not observed in the Huon Estuary, and toxin data suggest that it was also absent in the summer of 1994/95 and 1995/96. The reason for its absence is unclear, possibilities are some dramatic event such as high flushing rates, a lack of encystment due to several years of only autumn blooms, or alternatively population decline over prolonged periods unsuitable for growth or losses due to grazing, bacterial or viral attack. The lack of observations of *G. catenatum* toxins for

several years also indicates that the pool of the 'shallow resting cysts' of the two layer strategy may have been largely exhausted. The re-establishment of the *G. catenatum* population in the estuary in 1997 was most likely from the resuspension of deeply buried resting cysts. An anomalously high flow event that occurred in February of 1996 may have caused resuspension of deep resting cysts but may also have resulted in high flushing of cells from the estuary since *G. catenatum* growth was not observed in the estuary in that year. 1996 was also unusually wet and cool (Bureau of Meteorology, 1996) which may have prevented either germination of the resting cysts or growth of excysted cells. 1997 on the other hand was an anomalously warm year with relatively low winds (Bureau of Meteorology, 1997). A high Huon River flow event in March coupled with high local rainfall and high winds in early April (Bureau of Meteorology, 1997) could potentially have resuspended deep resting cysts which were then exposed to suitable growth conditions, germinated, and seeded the water column with new vegetative cells. The frequency of such events and the magnitude of physical forcing required are unknown but this is one explanation for interannual variability in the presence of *G. catenatum* in the estuary.

6.26 FUTURE RESEARCH

Although a number of key environmental variables that influence phytoplankton bloom dynamics in the Huon Estuary have been recognised, there are still many unanswered questions. This lack of answers is highlighted by the low amount of variance explained by environmental factors in the CCA analyses (maximum of 13 %). The low variance explained indicates that there are variables not included in the analysis, or not measured at the right time intervals, which contribute to phytoplankton dynamics and the development of toxic blooms. The use of time series analysis on the data presented in this thesis may provide further insight into factors influencing phytoplankton dynamics in the Huon Estuary and dynamics of *G. catenatum* in particular through the added power of being able to evaluate time lags in data series as well as simultaneous measurements.

For vegetative growth, primary production rates for the Huon Estuary are unknown and the capacity for *G. catenatum* to accumulate nitrogen also requires further study. In particular the determination of remineralisation rates is required to confirm or refute the hypothesis that vertical migration enables *G. catenatum* to thrive in the estuary. The importance of ammonia, particularly in bottom waters as a factor in phytoplankton dynamics could not be determined due to gaps in the dataset and this requires further experimentation and field observations. With regard to sexual reproduction, future research should endeavour to evaluate the two-level resting cyst survival strategy suggested here, and consequently quantify the importance of resting cysts in bloom initiation through studies of sediment

resuspension, resting cyst distribution and viability, resting cyst deposition rates and in situ germination studies.

6.27 CONCLUSION

The dynamic nature of sexual reproduction in *G. catenatum* blooms was identified in this study with encystment being observed throughout summer blooms. Based on approximate encystment rates in blooms it is unlikely that encystment is a major contributor to bloom decline although this can not be discounted. Low surface water temperature was identified as one key factor in the inhibition of resting cyst formation in autumn blooms. It seems likely that a benthic-pelagic transition is an important part of the ecological strategy of *G. catenatum*. Sexual reproduction and resting cyst formation provides a means to survive environmental change on short and long time scales as well as potentially contributing to bloom initiation and decline. It is hypothesised here that *G. catenatum* resting cysts may provide this species with a two-level survival strategy. In this strategy resting cysts present in surface sediments germinate relatively frequently to support either blooms or overwintering populations and deeply buried cysts provide a longer term survival strategy, remaining dormant until rare deep resuspension events occur.

This study represents a significant increase in the understanding of phytoplankton dynamics in the Huon Estuary and identifies key environmental factors that influence population dynamics of the toxic dinoflagellate *G. catenatum*. Huon River flow is a key element in phytoplankton dynamics in the estuary. It enhances water column stability, may be a key determining factor in interannual biomass variability through the effect of flow on light and flushing rates, and is also a source of micronutrients. Strong vertical migration of *G. catenatum* was observed in this study and is suggested as one major reason for the success of this species in the estuary, by reducing flushing from the estuary and providing *G. catenatum* with access to both light and deep nutrients.

REFERENCES

Anderson DM (1997) Bloom dynamics of toxic *Alexandrium* species in the northeastern U.S. Limnology and Oceanography 42: 1009–1022.

Anderson DM, Garrison DL (1997) Preface - The Ecology and Oceanography of Harmful Algal Blooms Limnology and Oceanography 42(5) part 2.

Anderson DM, Keafer BA (1985) Dinoflagellate cyst dynamics in coastal and estuarine waters. In Anderson DM, White AW, Baden DG (eds), Toxic Dinoflagellates. Elsevier Science Publishing Co, New York: 219–224.

Anderson DM, Keafer BA (1987) An endogenous annual clock in the toxic marine dinoflagellate *Gonyaulax tamarensis*. Nature 325: 616–617.

Anderson DM, Chisholm SW, Watras CJ (1983) Importance of life cycle events in the population dynamics of *Gonyaulax tamarensis*. Marine Biology 76: 179–189.

Anderson DM, Kulis DM, Binder BJ (1984) Sexuality and cyst formation in the dinoflagellate *Gonyaulax tamarensis*: Cyst yield in batch cultures. Journal of Phycology 20: 418–425.

Anderson DM, Taylor CD, Armbrust EV (1987) The effects of darkness and anaerobiosis on dinoflagellate cyst excystment. Limnology and Oceanography 32: 340–351.

Anderson DM, Jacobson DM, Bravo I, Wrenn JH (1988) The unique, microreticulate cyst of the naked dinoflagellate *Gymnodinium catenatum*. Journal of Phycology 24: 255–262.

Ault T, Velzeboer R, Zammit R (2000) Influence of nutrient availability on phytoplankton growth and community structure in the Port Adelaide River, Australia: bioassay assessment of potential nutrient limitation. Hydrobiologia 429: 89–103.

Blackburn SI, Hallegraeff GM, Bolch CJ (1989) Vegetative reproduction and sexual life cycle of the toxic dinoflagellate *Gymnodinium catenatum* from Tasmania, Australia. Journal of Phycology 25: 577–590.

Blackburn SI, Bolch CJ, Haskard KA, Hallegraeff GM (2001) Reproductive compatibility among four global populations of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). Phycologia 40: 78–87.

Bolch CJ, Hallegraeff GM (1990) Dinoflagellate cysts in recent marine sediments from Tasmania, Australia. Botanica Marina 33: 173–192.

Bolch CJS, Blackburn SI, Hallegraeff GM, Vaillancourt RE (1999). Genetic variation among strains of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). Journal of Phycology 35: 356–367.

Bravo I, Anderson DM (1994) The effects of temperature, growth medium and darkness on excystment and growth of the toxic dinoflagellate *Gymnodinium catenatum* from northwest Spain. Journal of Plankton Research 16: 513–525.

Bureau of Meteorology (1996) Monthly Weather Review – Tasmania.

Bureau of Meteorology (1997) Monthly Weather Review – Tasmania.

Clementson LA, Turnbull AR, Bonham PI, Parslow JS (1998) Changes in pigment and spectral characteristics of the toxic dinoflagellate *Gymnodinium catenatum* during bloom conditions. Proceedings Ocean Optics XIV, Hawaii, (CDROM).

Cloern JE (1987) Turbidity as a control on phytoplankton biomass and productivity in estuaries. Continental shelf research 7: 1367–1381.

Cloern JE (2001) Our evolving conceptual model of the coastal eutrophication problem. Marine Ecology Progress Series 210: 223–253.

Cole BE, Cloern JE (1987) An empirical model for estimating phytoplankton productivity in estuaries. Marine Ecology Progress Series 36: 299–305.

Connell JH, Slatyer RO (1977) Mechanisms of succession in natural communities and their role in community stability and organization. American Naturalist 111: 1119–1144.

Cowley R, Critchley G, Eriksen R, Latham V, Plaschke R, Rayner M, Terhell D (1999) Hydrochemistry Operations Manual. CSIRO Marine Laboratories Report 236.

CSIRO Huon Estuary Study Team (2000) Huon Estuary Study - Environmental Research for Integrated catchment management and aquaculture. Final report to Fisheries Research and Development Corporation. Project Number 96/284, June 2000. CSIRO Division of Marine Research. Marine Laboratories, Hobart.

Day JW, Hall CAS, Kemp WM, Yanez-Arancibia A (1989) Estuarine Ecology, John Wiley and Sons, New York.

Devassy VP, Goes JI (1988) Phytoplankton community structure and succession in a tropical estuarine complex (central west coast of India). Estuarine, Coastal and Shelf Science 27: 671–685.

Doblin MA, Blackburn SI, Hallegraeff GM (1999a) Growth and biomass stimulation of the toxic dinoflagellate *Gymnodinium catenatum* (Graham) by dissolved organic substances. Journal of Experimental Marine Biology and Ecology 236: 33–47.

Doblin MA, Blackburn SI, Hallegraeff GM (1999b) Comparative study of selenium requirements of three phytoplankton species: *Gymnodinium catenatum*, *Alexandrium minutum* (Dinophyta) and *Chaetoceros* cf. *tenuissimus* (Bacillariophyta). Journal of Plankton Research 21: 1153–1169.

Doblin MA, Blackburn SI, Hallegraeff GM (2000) Intraspecific variation in the selenium requirement of different geographic strains of the toxic dinoflagellate *Gymnodinium catenatum*. Journal of Plankton Research 22: 421–432.

Dyer KR (1997) Estuaries: A Physical Introduction, 2nd Edition. John Wiley and Sons, Chichester.

Ellegaard M, Kulis DM, Anderson DM (1998) Cysts of Danish *Gymnodinium nolleri* Ellegaard et Moestrup Sp. Ined. (Dinophyceae) – studies on encystment, excystment and toxicity. Journal of Plankton Research, 20: 1743–1755.

Estrada M, Berdalet E (1998) Effects of turbulence on phytoplankton. In Anderson DM, Cembella AD, Hallegraeff GM (eds), Physiological ecology of harmful algal blooms. Springer-Verlag, Berlin: 601–618.

Fisher TR, Peele ER, Ammerman JW, Harding LW Jr (1992) Nutrient limitation of phytoplankton in Chesapeake Bay. Marine Ecology Progress Series 82: 51–63.

Flynn KJ, Flynn K, John EH, Reguera B, Reyero MI, Franco JM (1996) Changes in toxins, intracellular and dissolved free amino acids of the toxic dinoflagellate *Gymnodinium catenatum* in response to changes in inorganic nutrients and salinity. Journal of Plankton Research 18: 2093–2111.

Fraga S (1996) Wintering of *Gymnodinium catenatum* Graham (Dinophyceae) in Iberian waters. In Yasumoto T, Oshima Y, Fukuyo Y (eds), Harmful and Toxic Algal Blooms. IOC, Paris: 211–214.

Gallegos CL (1992) Phytoplankton photosynthesis, productivity, and species composition in a eutrophic estuary: comparison of bloom and non-bloom assemblages. Marine Ecology Progress Series 81: 257–267.

Gallegos CL, Jordan TE (1997) Seasonal progression of factors limiting phytoplankton pigment biomass in the Rhode River estuary, Maryland (USA). 1. Controls on phytoplankton growth. Marine

Gomez Fermin E, Figueiras FG, Arbones B, Villarino ML (1996) Short-time scale development of a *Gymnodinium catenatum* population in the Ria de Vigo (NW Spain). Journal of Phycology 32: 212–221.

Gran HH, Braarud T (1935) A quantitative study of the phytoplankton in the Bay of Fundy and the Gulf of Maine (including observations on hydrography, chemistry and turbidity). Journal of the Biological Board Canada 1: 279–467.

Graneli E, Carlsson P, Turner JT, Tester PA, Bechemin C, Dawson R, Funari E (1999) Effects of N:P:Si ratios and zooplankton grazing on phytoplankton communities in the northern Adriatic Sea. I. Nutrients. Aquatic Microbial Ecology 18: 37–54.

Grasshof K (1976) Methods of Seawater Analysis. Verlag Chemie, New York.

Guillard RRL (1978) Counting slides. In: Phytoplankton Manual. Sournia A. (ed). UNESCO Paris. pp. 182–189.

Hallegraeff GM (1993) A review of harmful algal blooms and their apparent global increase. Phycologia 32: 79–99.

Hallegraeff GM, Fraga S (1998) Bloom dynamics of the toxic dinoflagellate *Gymnodinium catenatum*, with emphasis on Tasmanian and Spanish coastal waters. In Anderson DM, Cembella AD, Hallegraeff GM (eds) Physiological Ecology of Harmful Algal Blooms. NATO-ASI Vol. 41. Springer Verlag, Heidelberg: 59–80.

Hallegraeff G, Sumner C (1986) Toxic plankton blooms affect shellfish farms. Australian Fisheries, December 1986.

Hallegraeff GM, McCausland MA, Brown RK (1995) Early warning of toxic dinoflagellate blooms of *Gymnodinium catenatum* in southern Tasmanian waters. Journal of Plankton Research 17: 1163–1176.

Hallegraeff GM, Nichols PD, Volkman JK, Blackburn SI, Everitt DA (1991) Pigments, fatty acids, and sterols of the toxic dinoflagellate *Gymnodinium catenatum*. Journal of Phycology 27: 591–599.

Hamilton DP, Thompson PA, Kurup R, Horner-Rosser SMJ (1998) Dynamics of dinoflagellate blooms in the Swan River Estuary. In McComb AJ, Davis JA (eds), Wetlands for the future. Gleneagles Publishing, Adelaide: 273–286

Harrison PJ, Turpin DH, Bienfang PK, Davis CO (1986) Sinking as a factor affecting phytoplankton species succession: The use of selective loss semi-continuous cultures. Journal of Experimental Marine Biology and Ecology 99: 19–30.

Holmes RW (1970) The secchi disk in turbid coastal waters. Limnology and Oceanography 15: 688–694.

Ishikawa A, Tanuguchi A (1996) Contribution of benthic cysts to the population dynamics of *Scrippsiella* spp. (Dinophyceae) in Onagawa Bay, northeast Japan. Marine Ecology Progress Series 140: 169–178.

Jeffrey SW, Humphrey GF (1975) New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. Biochem. Physiol. Pflanzen 167: 191–194.

Jeffrey SW, Mantoura RFC, Wright SW (1997) Phytoplankton Pigments in Oceanography. United Nations Educational, Scientific and Cultural Organisation, Paris, France: 661pp.

Jeffrey SW, Vesk M (1997) Introduction to marine phytoplankton and their pigment signatures. In: Jeffrey SW, Mantoura RFC, Wright SW (eds), Phytoplankton Pigments in Oceanography. United Nations Educational, Scientific and Cultural Organisation, Paris, France: 37–84.

Jones R (1991) An improved fluorescence method for the determination of nanomolar concentrations of ammonium in natural waters. Limnology and Oceanography 36: 814–819.

Jordan TE, Correll DL, Miklas J, Weller DE (1991) Long term trends in estuarine nutrients and chlorophyll, and short-term effects of variation in watershed discharge. Marine Ecology Progress Series 75: 121–132

Kamykowski D, Reed RE, Kirkpatrick GJ, (1992) Comparison of sinking velocity, swimming velocity, rotation and path characteristics among six marine dinoflagellate species. Marine Biology 113: 319–328.

Kirk JTO (1994) Light and Photosynthesis in Aquatic Systems. Cambridge University Press, Cambridge: 509pp.

Kremp A, Heiskanen A-S (1999) Sexuality and cyst formation of the spring-bloom dinoflagellate *Scrippsiella hangoei* in the coastal northern Baltic Sea. Marine Biology 134: 771–777.

Latham V (2001) Hydrochemistry methods used for Huon Estuary Survey. CSIRO Marine Research Hydrochemistry report 1: 30pp.

Levandowsky M, Kaneta PJ (1987) Behaviour in Dinoflagellates. In Taylor FJR (ed), The Biology of Dinoflagellates. Blackwell Scientific Publications, London: 360–397.

Lewitus AJ, Koepfler ET, Morris JT (1998) Seasonal variation in the regulation of phytoplankton by nitrogen and grazing in a salt-marsh estuary. Limnology and Oceanography 43: 636–646.

Lovejoy C, Bowman JP, Hallegraeff GM (1998) Algicidal effects of a novel marine *Pseudoalteromonas* isolate (Class Proteobacteria, Gamma subdivision) on harmful algal bloom species of the genera *Chattonella, Gymnodinium,* and *Heterosigma.* Applied and Environmental Microbiology 64: 2806–2813.

MacArthur RH, Wilson EO (1967) The theory of island biogeography. Princeton University Press, Princeton.

Macdonald RW, McLaughlin FA, Wong CS (1986) The storage of reactive silicate samples by freezing. .Limnology and Oceanography 31: 1139–1142.

MacIsaac IJ (1978) Diel cycles of nitrogen uptake in a natural phytoplankton population dominated by *Gonyaulax polyedra.* Limnology and Oceanography 23: 1–9.

Mallin MA (1994) Phytoplankton ecology of North Carolina estuaries. Estuaries 17: 561–574.

Malone TC (1980) Size-fractionated primary productivity of marine phytoplankton. In Falkowski PG (ed), Primary Productivity in the Sea. Plenum Press, New York: 301–319.

Margalef R (1978) Life-forms of phytoplankton as survival alternatives in an unstable environment. Oceanologica Acta 1: 493–509.

Margalef R, Estrada M, Blasco D (1979) Functional morphology of organisms involved in red tides, as adapted to decaying turbulence. In Taylor DL, Seliger HH (eds), Toxic Dinoflagellate Blooms. Elsevier/North-Holland, New York: 89–94.

McMinn A, Hallegraeff GM, Thomson P, Short S, Heijnis H (1997). Microfossil evidence for the recent introduction of the toxic dinoflagellate *Gymnodinium catenatum* into Tasmanian waters. Marine Ecology Progress Series 161: 165–172.

Oshima Y, Blackburn SI, Hallegraeff GM (1993) Comparative study on paralytic shellfish toxin profiles of the dinoflagellate *Gymnodinium catenatum* from three different countries. Marine Biology 116: 471–476.

Park H-D, Hayashi H (1993) Role of encystment and excystment of *Peridinium bipes* f. *occulatum* (Dinophyceae) in freshwater red tides in Lake Kizaki, Japan. Journal of Phycology 29: 435–441.

Pienitz R, Smol JP, Birks HJB (1995) Assessment of freshwater diatoms as quantitative indicators of past climatic change in the Yukon and Northwest Territories, Canada. Journal of Paleolimnology 13: 21–49.

Pinckney JL, Paerl HW, Harrington MB, Howe KE (1998) Annual cycles of phytoplankton community-structure and bloom dynamics in the Neuse River Estuary, North Carolina. Marine Biology 131: 371–381.

Plaschke R (1999) Shipboard analysis of seawater for salinity, dissolved oxygen and nutrients. CSIRO Marine Laboratories, Report 237. CSIRO Marine Laboratories, Hobart, Tasmania.

Sampayo MA (1985) Encystment and excystment of a Portuguese isolate of *Amphidinium carterae* in cultures. In Anderson DM, White AW, Baden DG (eds), Toxic Dinoflagellates. Elsevier Science Publishing Co., New York: 125–130.

Scott BD (1978) Phytoplankton distribution and light attenuation in Port Hacking Estuary. Australian Journal of Marine and Freshwater Research 29: 31–44.

Sgrosso S, Esposito F, Montresor M (2001) Temperature and daylength regulate encystment in calcareous cyst-forming dinoflagellates. Marine Ecology Progress Series 211:77–87.

Sin Y, Wetzel RL, Anderson IC (1999) Spatial and temporal characteristics of nutrient and phytoplankton dynamics in the York River Estuary, Virginia: analyses of long-term data. Estuaries 22: 260–275

Skerratt JH (2001) Marine microbial interactions in a Tasmanian estuary. PhD Thesis 2001, University of Tasmania.

Smayda TJ (1980) Phytoplankton species succession. In Morris I (ed), The physiological ecology of phytoplankton, Studies in Ecology Vol. 7. Blackwell Scientific Publications, Oxford: 493–571.

Smayda TJ (1990) Novel and nuisance phytoplankton blooms in the sea: evidence for a global epidemic In Graneli E, Sundstrom B, Edler L, Anderson DM (eds) Toxic marine phytoplankton. Elsevier, New York: 29–40.

Smith WO, Asper V (2001) The influence of phytoplankton assemblage composition on biogeochemical characteristics and cycles in the southern Ross Sea, Antarctica. Deep-Sea Research 48: 137–161.

Swadling KM, Pienitz R, Nogrady T (2000) Zooplankton community composition of lakes in the Yukon and Northwest Territories (Canada): relationship to physical and chemical limnology. Hydrobiologia 431: 221–224.

Teegarden GJ, Campbell RG, Durbin EG (2001) Zooplankton feeding behavior and particle selection in natural plankton assemblages containing toxic *Alexandrium* spp. Marine Ecology Progress Series 218: 213–226.

Ter Braak CJF (1986) Canonical correspondence analysis: a new eigenvector technique for multivariate direct gradient analysis. Ecology 67: 1167–1178.

Ter Braak CJF (1990) Update notes: CANOCO version 3.10. Agricultural Mathematics Group, Wageningen: 35pp.

Thompson PA (1998) Spatial and temporal patterns of factors influencing phytoplankton in a salt wedge estuary, the Swan River, Western Australia. Estuaries 21: 801–817.

Thompson PA, Levasseur ME, Harrison PJ (1989) Light-limited growth on ammonium vs. nitrate: what is the advantage for marine phytoplankton? Limnology and Oceanography 34: 1014–1024.

Throndsen J (1978) Preservation and storage. In Sournia A (ed) Phytoplankton Manual. UNESCO Paris: 69–74.

Tilstone GH, Miguez BM, Figueiras FG, Fermin EG (2000) Diatom dynamics in a coastal ecosystem affected by upwelling: Coupling between species succession, circulation and biogeochemical processes. Marine Ecology Progress Series 205: 23–41.

Tyler MA, Heinbokel JF (1985) Cycles of red water and encystment of *Gymnodinium pseudopalustre* in the Chesapeake Bay: Effects of hydrography and grazing. In Anderson DM, White AW, Baden DG (eds), Toxic Dinoflagellates. Elsevier Science Publishing Co., New York: 213–218.

Voltolina D (1993) The origin of recurrent blooms of *Gymnodinium sanguineum* Hirasaka in a shallow coastal region. Journal of Experimental Marine Biology and Ecology 168: 217–222.

Woodward IO, Gallagher JB, Rushton MJ, Machin PJ, Mihalenko S (1992) Salmon farming and the environment of the Huon Estuary, Tasmania. Division of Sea Fisheries, Tasmania, Technical Report 45.

Wright SW, Jeffrey SW, Mantoura RFC, Llewellyn CA, Bjoernland T, Repeta D, Welschmeyer N (1991) Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. Marine Ecology Progress Series 77: 183–196.

Wyatt T, Jenkinson IR (1997) Notes on *Alexandrium* dynamics. Journal of Plankton Research 19: 551–575.

Yentsch CM (1984) Paralytic Shellfish Poisoning: An emerging perspective. In Ragelis EP (ed), Seafood Toxins. American Chemical Society Washington DC: 9–23.

CHAPTER 7

SEXUAL REPRODUCTION AND BLOOM DYNAMICS OF TOXIC DINOFLAGELLATES FROM AUSTRALIAN ESTUARINE WATERS - SYNTHESIS AND SUMMARY

SYNTHESIS OF FINDINGS FOR THE DINOFLAGELLATES *GYMNODINIUM CATENATUM* AND *ALEXANDRIUM MINUTUM*

This thesis provides a significant increase in the knowledge of sexual reproduction of *Alexandrium minutum* and sexual reproduction and bloom dynamics of *Gymnodinium catenatum*. Based on the research results presented in this thesis and previous work by other authors it is now possible to draw some conclusions and construct some hypotheses as to the autecological role of resting cysts in these species in Australian estuaries.

7.1 *ALEXANDRIUM MINUTUM*

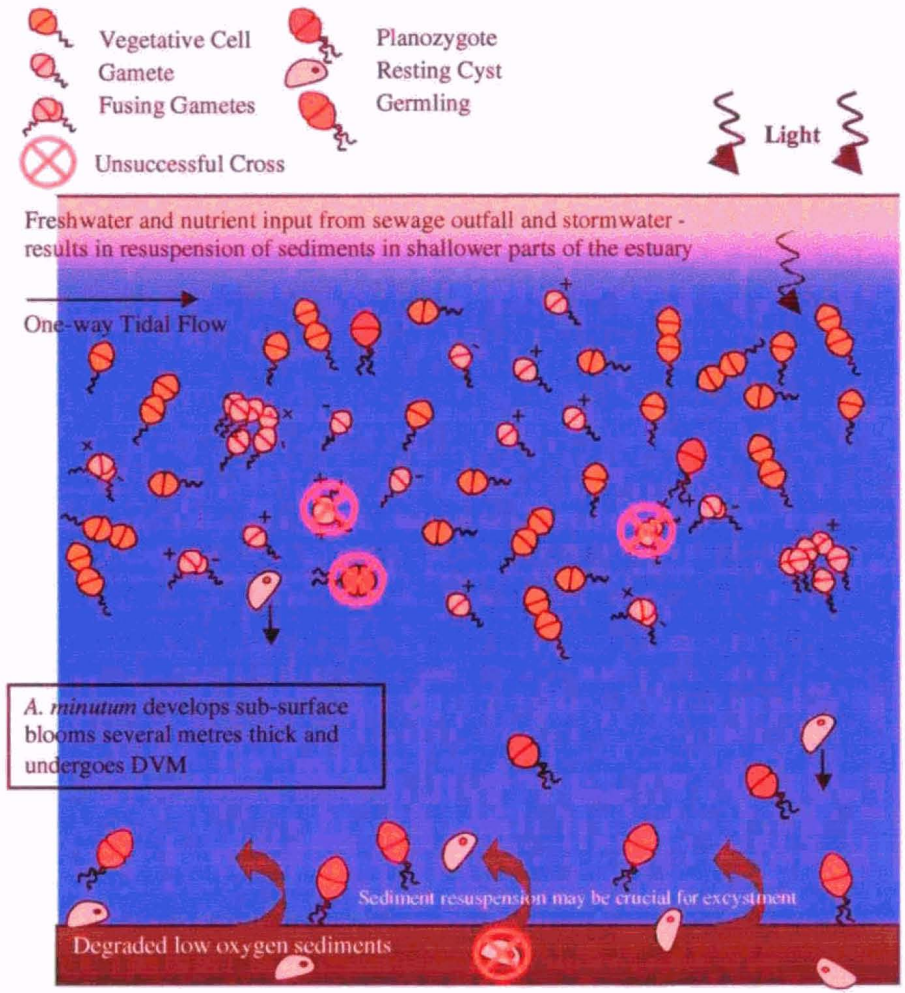
Alexandrium minutum strains used in this study were isolated from the Port River Estuary. The Port River Estuary is a shallow (5-15 m depth) estuarine waterway situated in metropolitan Adelaide, South Australia. The estuary is approximately 20 km in length and mostly less than 1 km wide, although the mouth of the estuary is approximately 3 km wide. Current flow in the estuary is restricted due to the action of a 1-way water-gate that requires significant tidal action to allow the influx of seawater from an artificial marine lake into the upper reaches of the estuary (Ault, 2000; Ault et al., 2000). In the upper reaches of the estuary, an outfall discharges 30 - 40 megalitres per day of secondarily treated effluent from a waste water treatment plant resulting in total phosphorus exceeding 1 mg L^{-1}, total nitrogen exceeding 3 mg L^{-1} and stratification due to the influx of fresh water. The estuary also receives much of Adelaide's stormwater (Cannon, 1993b). Neap tides (during which the solar and lunar components of tidal action result in minimal tidal movement) and a stable water column are considered important conditions for bloom formation (Cannon, 1990; Ault 2000). Most blooms in the estuary are phytoflagellates, although diatom blooms are also observed (Ault et al., 2000). Temperatures in the estuary range from 10 – 25 °C and salinities from 20 – 35 psu (Cannon, 1993b).

Alexandrium minutum blooms in the Port River Estuary develop in the upper estuary in sub-surface waters (3 – 4 m), gradually moving to the surface further down the estuary in response to the one way tidal flow (Cannon, 1990). Blooms form in dense horizontal bands, several metres thick, are patchy, most dense along the sides of the river and have been observed to undergo diel vertical migration (Cannon, 1990). *Alexandrium minutum* blooms tend to occur under stable water column conditions with calm winds and low tidal amplitude in this system. Blooms of both *A. minutum* and *A. catenella* have resulted in closures to wild shellfish harvesting in the estuary. Interestingly, although *A. minutum* blooms were annually recurrent events in the late 1980s and early 1990s (Cannon, 1993b) there have been few *A. minutum* blooms in the Port River Estuary in recent years. *Alexandrium catenella* was

identified in the region in 1997 (Appendix B) and has formed annually recurrent blooms since, possibly outcompeting the closely related *A. minutum*.

Sexual reproduction of *A. minutum* has been observed in field populations (Cannon, 1993a) and low densities of cysts have been found in the sediments; approximately 10 % of all dinoflagellate resting cysts found in Port River Estuary sediments (Bolch et al., 1991). Induction of encystment in the dense sub-surface blooms of the Port River Estuary is likely to be subject to various factors including light, temperature, nutrients, and the presence of compatible gametes (Fig. 7.1). Because *A. minutum* is mostly heterothallic, successful encystment can only occur if compatible gametes are present and able to undergo gamete fusion. The mating system of *A. minutum* is complex (Chapter 3) and this implies that a genetically diverse population is optimal for encystment. Blooms of *A. minutum* in the Port River Estuary are believed to arise from resting cysts (Cannon, 1993a) and are therefore likely to be genetically diverse (Bolch et al., 1999). It is hypothesised here that cells must reach some threshold cell density for encystment to occur and that this density may be a function of the distance over which pheromone-like substances, (for which evidence is presented in Chapter 5) are able to function. *Alexandrium minutum* blooms in the Port River Estuary reach high cell densities (up to 3×10^8 L^{-1}). Such cell densities are similar to or greater than cell densities used in experiments in which encystment was observed (Chapter 5), suggesting that cell densities required for encystment are probably reached in most blooms. Under experimental conditions, unsuccessful encystment has been observed, with the fusion of gametes resulting in non-viable resting cysts (Chapter 3). This may also occur in the natural ecosystem but this is unknown and would be difficult to quantify.

Low light was identified as one cue for encystment of *A. minutum* (Chapter 5). The highly turbid waters and consequently low light environment of the Port River Estuary suggests that low light may be a primary cue for encystment in this ecosystem. Temperatures ranging from 14 to 28 °C support encystment although the optimal temperature is 20 °C (Chapter 5). Consequently encystment is more likely to occur in blooms in the warmer months (summer and autumn), which are the seasons in which blooms are commonly observed in the Port River Estuary (Cannon, 1990). In this thesis, low nutrients were identified as enhancing encystment of *A. minutum* under high light conditions (Chapter 5). However the high nutrient loads on the Port River Estuary and the turbidity of the waterway suggests that low nutrient and high irradiance conditions are rare and would not be a positive influence on encystment in this ecosystem. Percentage encystment of *A. minutum* in the laboratory exceeded 7% under some conditions, suggesting that encystment may significantly contribute to bloom decline under some conditions.



FACTORS INFLUENCING *ALEXANDRIUM MINUTUM* SEXUAL REPRODUCTION

ENCYSTMENT

- Compatible gametes required within the complex mating system
- Cell:cell interactions (possibly pheromones) probably combined with some cell density threshold
- Low light enhances encystment
- Encystment temperature window (14–28 °C) optimal at 20 °C
- Low nutrients under higher light enhance encystment - probably not important in Port River Estuary
- High % encystment (>7 % in laboratory) may contribute to bloom decline

EXCYSTMENT

- Minimum dormancy period (approximately 4 weeks but may be longer)
- Oxygen levels (anaerobic conditions probably inhibit excystment)
- Light (low light inhibits excystment)
- Temperature window of 8–31 °C (i.e. any time in this system) high from 18 to 28 °C (spring to autumn)

Figure 7.1: Annotated diagram of factors influencing sexual reproduction of *Alexandrium minutum* in the Port River Estuary (factors are listed in order of their suggested importance in this ecosystem).

Cannon (1993a) identified some of the optimal conditions for excystment of *A. minutum* resting cysts obtained from the field. These were 16 °C, 14 – 21 psu and 14 – 25 µmol PAR $m^{-2} s^{-1}$. Resting cysts studied in this thesis were able to excyst at temperatures from 8 – 31 °C, with the optimal range being between 18 and 28 °C. This optimal range corresponds to temperatures in the estuary from spring to autumn although germination is possible year round, particularly given the short minimum dormancy period (approximately 4 weeks) identified for resting cysts in the laboratory (Chapter 2). Sediments of the upper Port River Estuary are largely anaerobic (N. Parker, unpublished data). Tidal and stormwater flow coupled with effluent discharge and wind mixing results in sediment resuspension in the shallow waters of the upper estuary (Cannon 1990). Light is necessary for excystment of *A. minutum* (Cannon, 1993a; Chapter 5) and in all dinoflagellates studied to date, anaerobic conditions inhibit excystment (see Chapter 5). Consequently sediment resuspension in the shallower parts of the estuary is probably a prerequisite for excystment.

In summary, germination of resting cysts of *A. minutum* occurs under temperature and salinity conditions corresponding to the time of year when blooms are usually observed (spring to autumn), and it is probable that they contribute to bloom initiation. The dense sub-surface blooms occur in an environment likely to promote resting cyst formation with low light, an appropriate temperature range and at densities which should promote cell:cell communication (Fig. 7.1). Thus the evidence suggests that resting cysts of *A. minutum* contribute to both bloom initiation and decline in the Port River Estuary and are therefore very important in the overall bloom dynamics.

7.2 *GYMNODINIUM CATENATUM*

7.2.1 Factors influencing the spatial distribution of *Gymnodinium catenatum* blooms in the Huon Estuary

The Huon Estuary is a classic salt wedge estuary, with the salt wedge extending from the mouth of the estuary to above Huonville (Fig. 7.2). Flow of the Huon River is hypothesised to be an important factor in bloom formation, providing micronutrients (Doblin, 1999a, b) and water column stability which appears to be important for dinoflagellate blooms (Chapter 6). Huon River flow tends towards the east /northeast side of the estuary (Fig. 7.2). This flow may be important for the spread of *G. catenatum* cells throughout the mid and lower estuary although the combination of downstream surface flow and upstream tidal flow with diel vertical migration enables much biomass to remain relatively stationary (HEST, 2000; Chapter 6). It is here suggested that Huon River flow also limits the upriver extent of the bloom (Fig. 7.2) as increased pycnocline depths apparently lead to osmotic stress of *G. catenatum* cells (Chapter 6). Shallow embayments in the mid estuary may be an important source of resting cysts for the establishment of initial *G. catenatum* populations. In some of

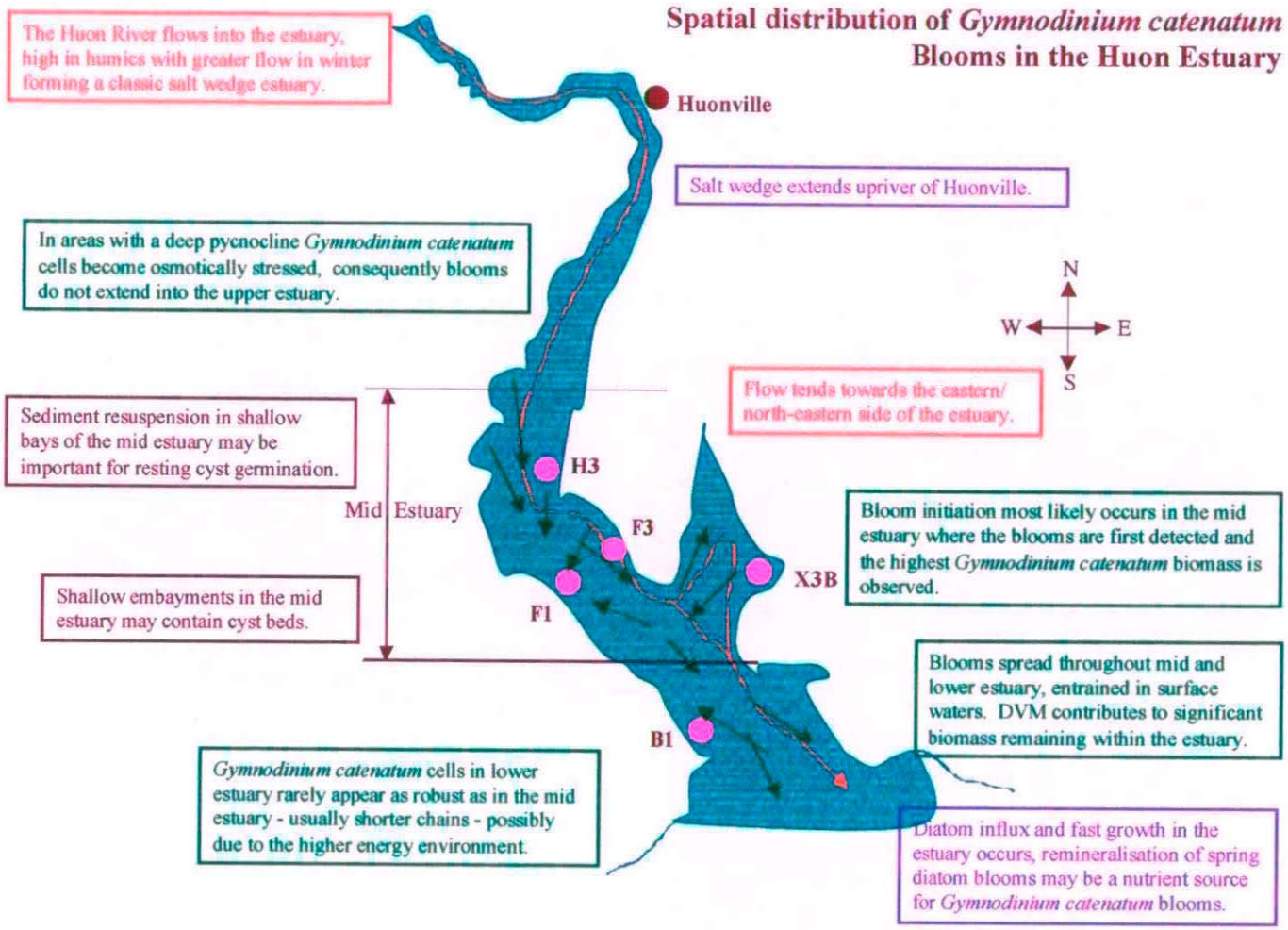


Figure 7.2: Annotated diagram of factors influencing the spatial distribution of *Gymnodinium catenatum* in the Huon Estuary.

these embayments anoxic surface sediments can be found (personal observations) which could result in resting cysts remaining dormant for extended periods (Chapter 5), and sediments in these shallower regions are more likely to be resuspended by strong wind events. *Gymnodinium catenatum* bloom initiation probably occurs in the mid estuary as the greatest biomass and earliest observations of blooms are from this area (Fig. 7.2). *Gymnodinium catenatum* cells at the mouth of the estuary usually form shorter chains and appear less 'healthy' than in the mid estuary (personal observations), possibly due to the higher energy environment of the more exposed waters.

7.2.2 Factors influencing vegetative growth of *Gymnodinium catenatum* in the Huon Estuary

Various factors influence vegetative growth and biomass production of *Gymnodinium catenatum* in the Huon Estuary (Fig. 7.3). As discussed in Chapter 6, the primary requirement is the presence of a source population whether as a result of resting cyst germination or low numbers of vegetative cells. The presence of a macronutrient source is also essential for vegetative growth. However, *G. catenatum* blooms in the Huon Estuary are observed during periods of low water column nutrient levels, often after diatom blooms have stripped the water column of nutrients. The ability to undergo deep vertical migration gives *G. catenatum* access to deep nutrients, which represent a constant source due to the release of nutrients via remineralisation of phytoplankton. The remains of spring and late summer diatom blooms which have sedimented to the bottom may provide an important source of nutrients for these dinoflagellate blooms. Light is also essential for growth and the Huon Estuary is periodically light limited during periods of high flow from the humic rich Huon River (Chapter 6). Diel vertical migration of *G. catenatum* enables this species to move upwards to access light in this complex light field. The fourth primary factor that appears to be required for *G. catenatum* biomass production or vegetative growth, is a stratified water column, with destratification of the summer bloom of 1997/98 coinciding with bloom collapse.

A temperature range for growth of 12 - 20 °C (Chapter 5) limits *G. catenatum* growth to the warmer months (spring to autumn) although cells may survive with very slow growth rates at winter temperatures. The salinity range for growth (23-34 psu; Blackburn et al., 1989) limits *G. catenatum* distribution to the mid and lower estuary. Growth is also limited by the maximum growth rate of *G. catenatum* (0.33 day^{-1}; Blackburn et al., 1989) with observed in situ growth during bloom development being even lower (0.08 – 0.1 day^{-1}). Other factors such as physical concentration and advection also appear to influence biomass accumulation, with apparent growth rates as high as 0.9 day^{-1}at some sites over short time periods. Of the loss processes influencing *G. catenatum* biomass surface outflow probably has the greatest

FACTORS INFLUENCING *GYMNODINIUM CATENATUM* VEGETATIVE BIOMASS

GROWTH FACTORS

- Presence of a source population - either vegetative cells or excystment
- Macronutrients remineralised from sediments - diatom bloom as potential major source
- Light (limited by fresh water but partially counteracted by DVM)
- Water column stratification
- Temperature range (12 -20 °C)
- Salinity range (23 - 34 psu; Blackburn et al., 1989) partially counteracted by DVM
- Humics, selenium and other micronutrients from fresh water (Doblin 1998)
- Maximum growth rate (0.33 day^{-1}, Blackburn et al., 1989; 0.08-0.1 d^{-1} observed for field populations)

LOSS FACTORS

- Flushing of the estuary
- Grazing
- Cell death
- Encystment (Fig. 7.4)

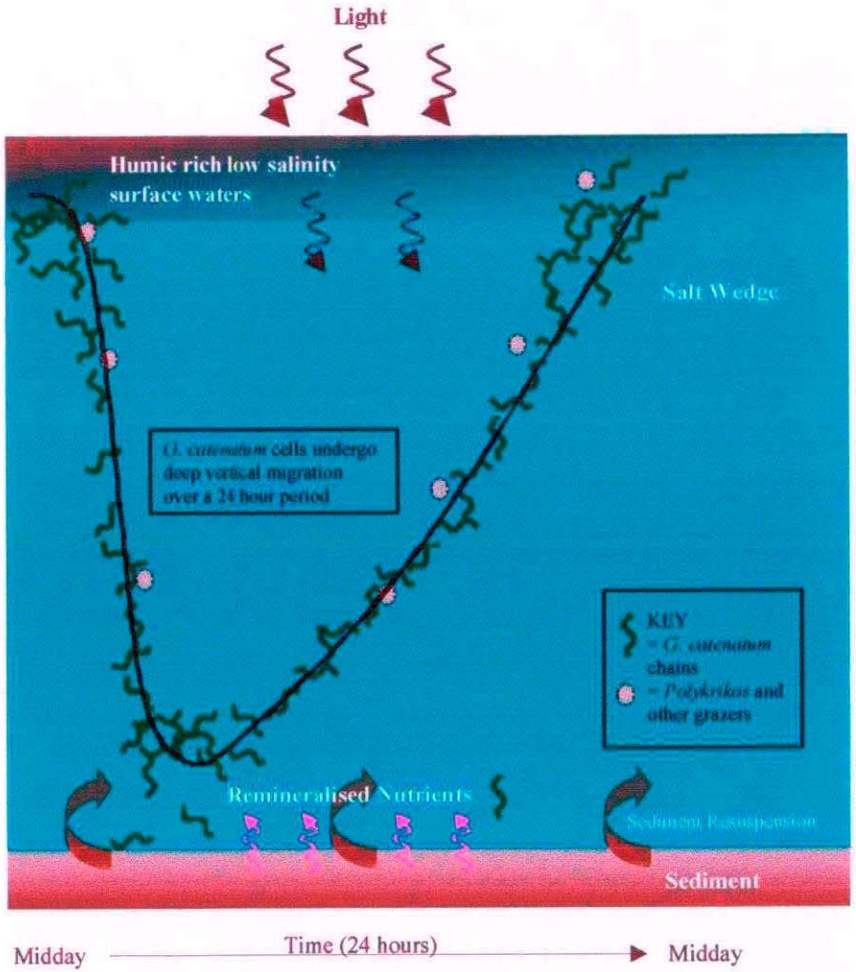


Figure 7.3: Annotated diagram of factors influencing vegetative biomass of *Gymnodinium catenatum* in the Huon Estuary (factors are listed in order of their suggested importance in this ecosystem).

influence although vertical migration ensures the cells spend only short periods in this upper layer (Chapter 6). Grazing may also have some influence on *G. catenatum* biomass with various grazers identified (Chapter 6) including *Polykrikos schwartzii* which shows concurrent high biomass with *G. catenatum* (Chapter 6). Encystment may be important under some conditions as discussed below (Section 7.2.3). Cell death, algicidal bacteria and parasites may all have some role in the regulation of *G. catenatum* biomass but these factors have not been quantified.

7.2.3 Factors influencing sexual reproduction of *Gymnodinium catenatum* in the Huon Estuary

Various factors are likely to influence sexual reproduction of *Gymnodinium catenatum* in the Huon Estuary (Fig. 7.4). Encystment requires a seasonal temperature window of 14 - 27 °C, and is optimal at 27 °C (Chapter 5). Temperature is suggested here to be the primary factor influencing encystment of *G. catenatum* in this ecosystem since encystment occurs throughout summer blooms but not in late autumn blooms of similar cell densities when water temperatures are cooler. The mating system of *G. catenatum* is heterothallic and complex with gradients of affinity between strains rather than clearly defined mating types (Chapter 3). A population with diverse mating types and compatible gametes is therefore required for sexual reproduction; the summer 1997/98 and autumn 1998 blooms were such populations with high variability in mating type affinity expressed in the 30 strains isolated (Chapter 3). There is evidence that pheromone-like substances contribute to sexual reproduction of *G. catenatum*, probably by triggering gametogenesis and/or through gamete attraction (Chapter 5). Such pheromone-like substances would probably require a cell density threshold so that cells are in close enough proximity for cell:cell communication to occur. Whether sexual reproduction is initiated by pheromones or cell:cell contact, the accumulation of biomass at the top and bottom of the diel vertical migration cycle (Chapter 6) could help promote encystment. A decrease in nutrients in bottom waters may also enhance encystment (Chapter 6).

Although encystment observed in the field was generally low, at times sexual cells represented greater than 30 % of *G. catenatum* cells observed, and under some laboratory conditions percentage encystment was greater than 50 %. Thus encystment may contribute significantly to the loss of cells from the water column and possibly to bloom decline under some conditions, although increased encystment was not observed during bloom decline (Chapter 6).

The key factors influencing excystment in the Huon Estuary are likely to be the endogenous dormancy period, which is short (minimum of 2 weeks, Blackburn et al., 1989), and resuspension of sediments. Newly formed resting cysts should be able to germinate after the

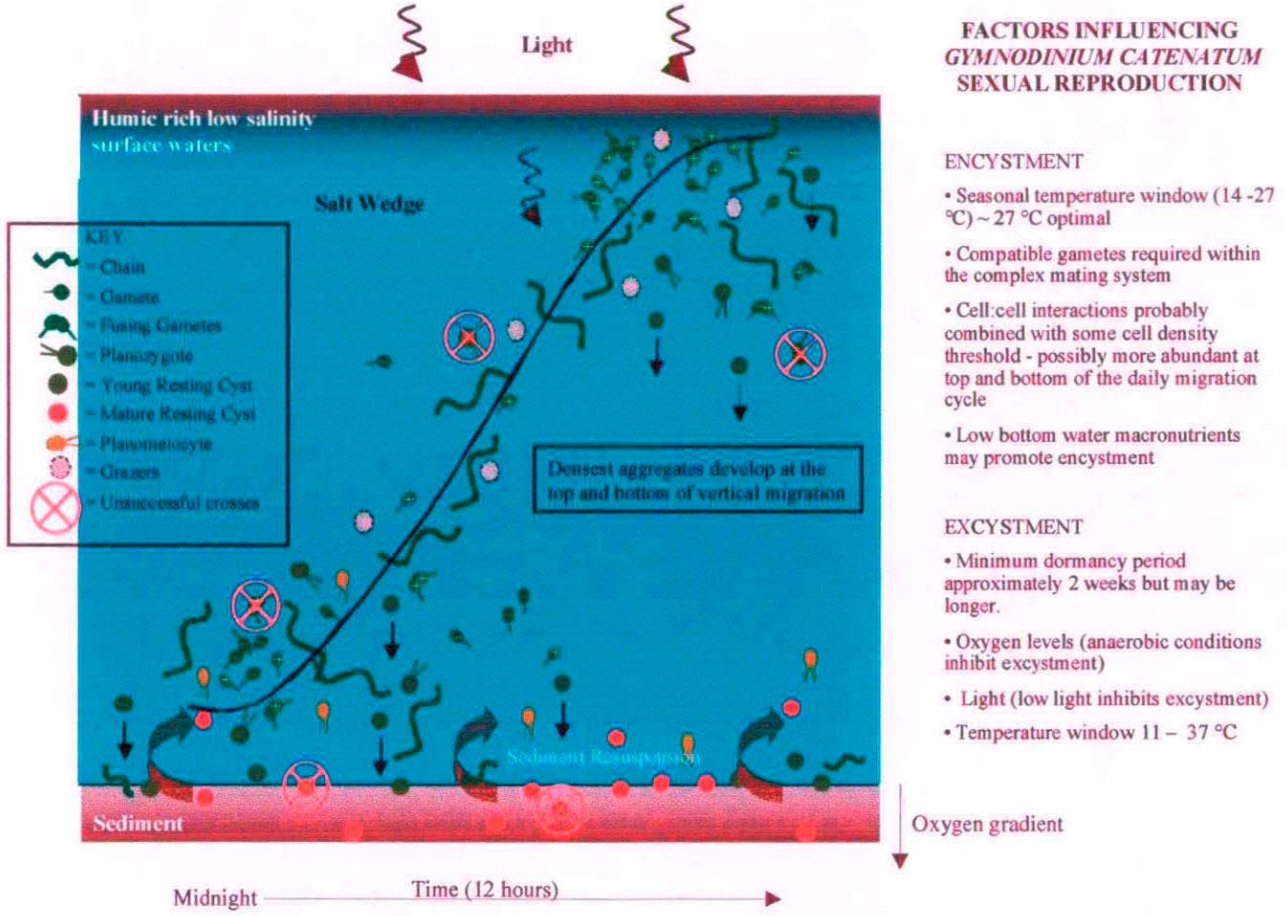


Figure 7.4: Annotated diagram of factors influencing sexual reproduction of *Gymnodinium catenatum* in the Huon Estuary (factors are listed in order of their suggested importance in this ecosystem).

short dormancy period unless they are buried in anaerobic dark sediments, which would inhibit excystment (Chapter 5). Sediment resuspension would be required to return buried cysts to an aerobic environment with some light, suitable for excystment. A period in anaerobic conditions may also increase the proportion of successfully germinating cysts (Chapter 5). Resting cyst germination below 11 °C was very low and resting cysts died above 37 °C; the range of temperature conditions in the Huon Estuary is usually well within these limits and it is therefore unlikely that temperature is a major determinant of excystment. It is suggested that resuspension of deeply buried cysts may be important for re-inoculating *G. catenatum* populations in the Huon Estuary while cysts in surface sediments provide a shorter term survival strategy (Chapter 6).

7.2.4 Conceptual framework for *Gymnodinium catenatum* blooms

To bring together the findings and hypotheses presented in this thesis on *G. catenatum*, a conceptual framework for *G. catenatum* bloom dynamics in the Huon Estuary is proposed (Fig. 7.5). The primary requirement for bloom formation is a seed source to form the initial population; this source may by either over-wintering vegetative cells or cells arising from resting cyst germination, probably in response to sediment resuspension. If macronutrients (particularly bottom nutrients) are available, light is adequate, the water column is partially stratified, temperature conditions are suitable, flushing rate is low (i.e. not 'too much' river flow), micronutrients (from river water) are available and grazing pressure is low then a bloom can develop. If these criteria are not met cells either die or remain at low concentrations. Once a bloom develops, continued warm stable conditions with some river flow, and release of remineralised nutrients from the sediments can lead to a large extended bloom. Factors which may prevent blooms from either developing at all or forming large extended blooms, in order of suggested importance include; water column destratification, decreasing water temperatures, insufficient macronutrients, low light, increased flushing and/or osmotic stress due to high river flow, encystment, insufficient micronutrients, and cell lysis/cell death due to algicidal bacteria, viruses or parasites. Any remaining cells may form a new 'initial population' or alternatively resting cyst germination may be required to seed a new population and reinitiate the bloom formation process (Fig. 7.5).

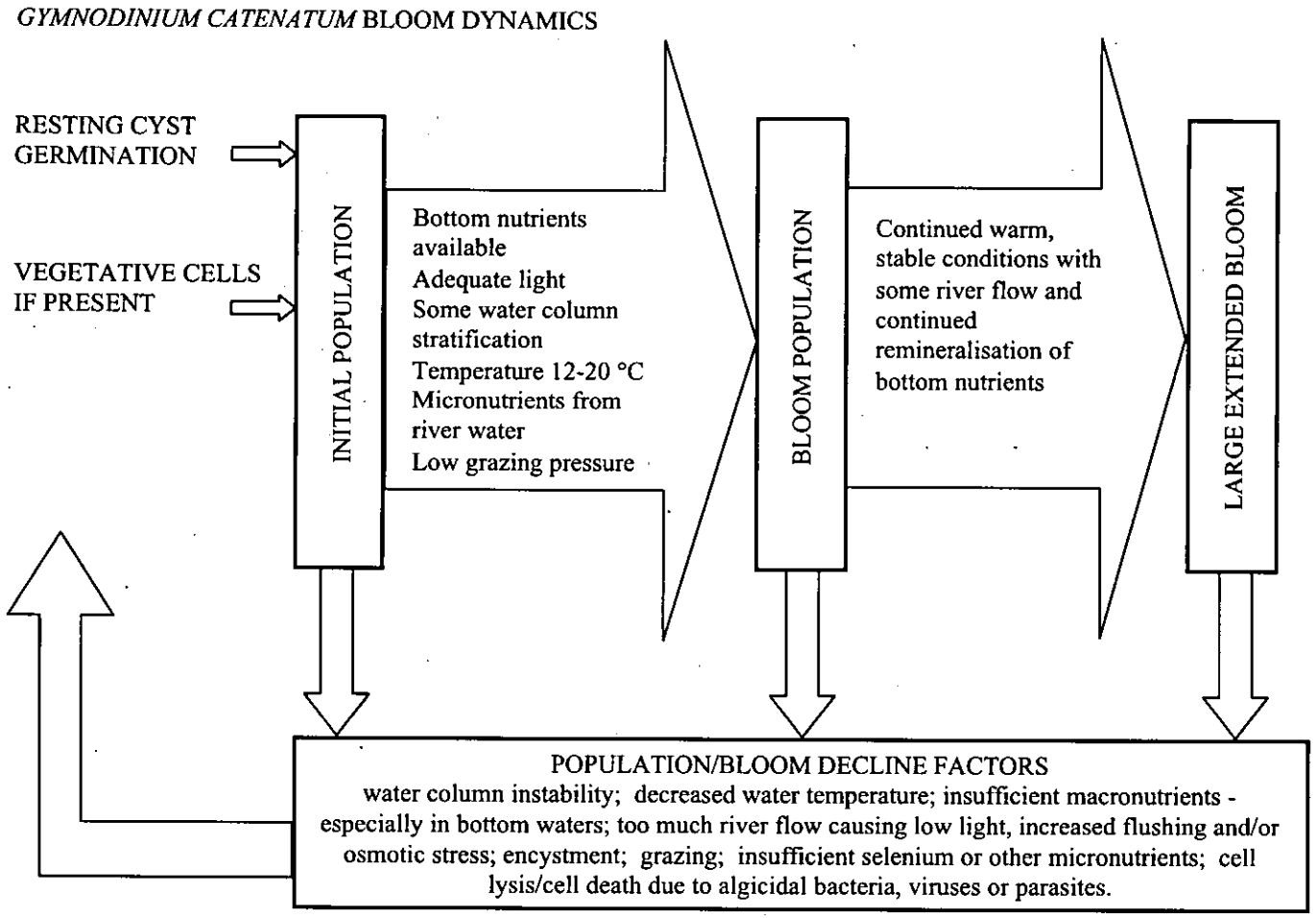


Figure 7.5: Conceptual framework for *Gymnodinium catenatum* bloom dynamics in the Huon Estuary.

SUMMARY

Toxic dinoflagellate blooms have been known from Australian waters since the 1980s. Four dinoflagellate species known to form toxic or potentially toxic blooms in Australian estuaries were studied in this thesis: *Gymnodinium catenatum*, *Alexandrium minutum*, *Alexandrium catenella*, and the potentially toxic *Protoceratium reticulatum*. These four species reproduce by vegetative cell division as well as through sexual reproduction. In all four species sexual reproduction results in the formation of a resting cyst, a resting stage which can sink to the sediments and can remain viable when conditions are unsuitable for vegetative growth.

The complete description of the life cycle of *Alexandrium minutum* and the development of reliable methods for resting cyst production providing a new set of tools for studying the autecology of this species were key outcomes of this thesis. These findings enabled the study of the mating system and cues for encystment and excystment of *A. minutum*. The life cycle of *A. minutum* includes the production of a colourless resting cyst containing a red accumulation body, similar to other species of the *Alexandrium* genus. *Alexandrium minutum* was also found to produce temporary cysts in response to cold shock and to have a short (approximately 4 weeks) minimum requisite dormancy period.

Of the four dinoflagellates for which mating systems were studied (*A. minutum* and *P. reticulatum* for the first time), only one, *Alexandrium catenella*, had a simple heterothallic mating system with essentially two mating types. The *A. catenella* mating system was consistent for strains from three geographically separated populations (Port Phillip Bay, the Port River Estuary and Sydney Harbour). The other three species studied had complex mating systems with gradients of affinity between strains rather than clearly defined mating types. Cluster analysis was determined to be a useful technique for identifying these gradients and determining the mating affinity of strains in these complex mating systems. The mating system of *G. catenatum* was similar between strains from two temporally separated bloom populations that had very different sexual reproduction dynamics (extensive encystment in a summer bloom and undetectable encystment an autumn bloom). This highlighted the influence of both mating systems and the environment on sexual reproduction as encystment in the autumn bloom was probably limited by temperature. Mating type affinities of *Alexandrium minutum* and *Gymnodinium catenatum* strains were found to change over time spans of years.

Cultivation of *A. minutum* in high biomass culture systems was found to be feasible. Aeration of *A. minutum* cultures increased growth rates and biomass production in contrast to observations by other authors that turbulent environments are not suitable for

dinoflagellate growth. The presence of sexually compatible strains in these high biomass culture systems did not affect growth and biomass production.

The processes of encystment and excystment involve a complex interplay of biological and environmental factors, which vary in their importance from species to species. In *A. minutum*, encystment was increased by nutrient depletion under higher irradiance (75 µmol PAR m^{-2} s^{-1}) but lower irradiance (25 µmol PAR m^{-2} s^{-1}) also enhanced encystment irrespective of nutrient concentrations. Encystment of *A. minutum* occurred within a temperature range of 14 to 28 °C and was optimal at approximately 20 °C. There is some evidence that pheromone-like substances may have an important function in sexual reproduction. Some light was required for excystment of *A. minutum*, which was only possible between temperatures of 8 and 31 °C. Excystment of *A. minutum* was also subject to a short (approximately 4 weeks) minimum requisite dormancy period.

Encystment of *G. catenatum* occurred within a temperature window of 14 to 27 °C with optimal encystment at approximately 27 °C. Nutrient depletion was not a primary cue for encystment although field data indicated that decreased nutrient levels in bottom waters may stimulate encystment. There was evidence that pheromone-like substances may also be involved in sexual reproduction of *G. catenatum*. Excystment of *G. catenatum* was inhibited by anaerobic conditions, low light and low temperatures and was unaffected by nutrient concentrations.

Algicidal bacteria did not affect either encystment rates or proportions of excystment of *G. catenatum* and *A. minutum*, and resting cysts remained viable at concentrations of algicidal compounds which would kill vegetative cells, indicating that encystment provides an excellent defense strategy against algicidal bacteria.

Sexual reproduction of dinoflagellates represents an important coupling between benthic and pelagic systems brought about through the interaction between the sexual life cycle and mating system of a species with the physical, chemical and biological environment.

There is considerable interannual variability in phytoplankton bloom dynamics of the Huon Estuary. A successional pattern of diatom blooms in the spring followed by dinoflagellate blooms in the summer was found in two of three years, while the third year had persistently low biomass with no bloom development throughout the spring, summer and autumn.

In the three year field study of the Huon Estuary, four *G. catenatum* blooms were observed, two in summer and two in autumn. Sexual reproduction occurred throughout summer blooms but was not detected in autumn blooms. Low temperatures probably prevented encystment of autumn blooms. River flow was identified as important for dinoflagellate

bloom formation in the Huon Estuary for water column stability, and potentially for micronutrients, however 'too much' flow may lead to greater flushing and osmotic stress of *G. catenatum* cells. Dinoflagellate blooms were absent in the first year of the study which may be attributed to increased flushing of the estuary due to high and persistent flow of the Huon River in that year. *Gymnodinium catenatum* was not detected in the first year of the study. A dual strategy for the contribution of resting cysts to the ecology of *G. catenatum* in the Huon Estuary is hypothesised whereby frequent germination of resting cysts from shallow sediments contributes to bloom populations and overwintering biomass while deeply buried and relatively rarely resuspended resting cysts contribute to long-term survival of the population in the estuary. Vertical migration, facilitating efficient nutrient uptake from bottom waters, access to higher light and avoidance of flushing is considered to be one of the primary factors leading to successful bloom development of *G. catenatum* in the estuary.

The resting cysts produced during sexual reproduction enable persistence over both short and long time scales when vegetative reproduction in the water column is either reduced or impossible, or vegetative cells are flushed from the ecosystem. Sexually produced resting cysts also have an important function in genetic recombination and in some cases may contribute to bloom development and bloom decline.

REFERENCES

Ault TR (2000) Vertical migration by the marine dinoflagellate *Prorocentrum triestinum* maximises photosynthetic yield. Oecologia 125: 466–475.

Ault T, Velzeboer R, Zammit R (2000) Influence of nutrient availability on phytoplankton growth and community structure in the Port Adelaide River, Australia: bioassay assessment of potential nutrient limitation. Hydrobiologia 429: 89–103.

Blackburn SI, Hallegraeff GM, Bolch CJ (1989) Vegetative reproduction and sexual life cycle of the toxic dinoflagellate *Gymnodinium catenatum* from Tasmania, Australia. Journal of Phycology 25: 577–590.

Bolch CJ, Blackburn SI, Cannon JA, Hallegraeff GM, (1991) The resting cyst of the red-tide dinoflagellate *Alexandrium minutum* (Dinophyceae). Phycologia 30: 215–219.

Cannon JA (1993a) Germination of the toxic dinoflagellate *Alexandrium minutum*, from sediments in the Port River, South Australia. In Smayda TJ, Shimizu Y (eds), Toxic Phytoplankton Blooms in the Sea. Elsevier, New York: 103–107.

Cannon JA (1993b) Growth in culture of the toxic dinoflagellate *Alexandrium minutum* from the Port River, South Australia. In Smayda TJ, Shimizu Y (eds), Toxic Phytoplankton Blooms in the Sea. Elsevier, New York: 741–745.

Cannon JA (1990) Development and dispersal of red tides in the Port River, South Australia. In Graneli E, Sundstrom B, Edler L, Anderson DM (eds.), Toxic Marine Phytoplankton. Elsevier Science Publishing, New York: 110–115.

Doblin MA, Blackburn SI, Hallegraeff GM (1999a) Growth and biomass stimulation of the toxic dinoflagellate *Gymnodinium catenatum* (Graham) by dissolved organic substances. Journal of Experimental Marine Biology and Ecology 236: 33–47.

Doblin MA, Blackburn SI, Hallegraeff GM (1999b) Comparative study of selenium requirements of three phytoplankton species: *Gymnodinium catenatum*, *Alexandrium minutum* (Dinophyta) and *Chaetoceros* cf. *tenuissimus* (Bacillariophyta). Journal of Plankton Research 21: 1153–1169.

APPENDIX A

GSe MEDIUM COMPONENTS

GSe MEDIUM - (MODIFICATION OF G.P. MEDIUM)

1. SEAWATER

Seawater collected from the east coast of Tasmania, east of Maria Island was purified (activated charcoal treatment) and filtered, and then autoclaved in 1 litre screw-cap teflon bottles.

2. STOCK SOLUTIONS

(1)	KNO_3		100 gL^{-1} H_2O
(2)	K_2HPO_4		34.8 gL^{-1} H_2O
(3)	Vitamins	B_{12}	0.1 mg 100 mL^{-1} H_2O
		Thiamine HCl	100 mg 100 mL^{-1} H_2O
		Biotin	0.2 mg 100 mL^{-1} H_2O
(4)	PII Metal Mix	Na_2EDTA	6.0 gL^{-1} H_2O
		$FeCl_3.6H_2O$	0.29 gL^{-1} H_2O
		H_3BO_3	6.85 gL^{-1} H_2O
		$MnCl_2.4H_20$	0.86 gL^{-1} H_2O
		$ZnCl_2$	0.06 gL^{-1} H_2O
		$CoCl_2.6H_2O$	0.026 gL^{-1} H_2O
(5)	Selenium	H_2SeO_3	1.29 mgL^{-1} H_2O
(6)	Soil Extract		

3. NUTRIENT SOLUTION (EXCLUDING SOIL EXTRACT)

A working solution of nutrients was made up in distilled water

For 100 mL mix:

10 mL nitrate stock

5 mL phosphate stock

5 mL vitamin stock

25 mL PII metal mix

5 mL selenium stock

50 mL distilled water

Nutrient solution was filter sterilised under aseptic conditions using a 0.22 μm disposable filter unit and used a concentration of 2 mL per 100 mL of final medium.

4. SOIL EXTRACT SOLUTION

Full strength soil extract (see below for preparation) was used at a concentration of 5 mL per 1000 mL medium.

5. DISTILLED WATER

Distilled water was autoclaved in schott bottles.

6. TO PREPARE FINAL MEDIUM

In a sterile 1 litre Schott bottle the following were added aseptically

800 mL sterile seawater (1)

200 mL sterile distilled water (5)

20 mL working nutrient solution (3)

5 mL working soil extract solution (4)

SOIL EXTRACT PREPARATION

Soil was collected from a natural uncultivated environment – topsoil from a local sandy bushland environment (no fungicides, insecticides or garden fertilizers were present).

1. Soil sifted once through a coarse sieve and twice through a fine (1 mm mesh) sieve.
2. 1 kg of soil mixed into 2 litres of distilled water.
3. Soil mix then autoclaved for 60 minutes at 121°C and cooled overnight.
4. Filtered through absorbent cotton wool packed into the stem of a glass filter funnel.
5. Centrifuged at 5000 rpm for 20 minutes in 250 mL polyethylene centrifuge tubes and deep brown supernatant collected.
6. Filtered again through absorbent cotton wool.
7. Supernatant dispensed into 100 mL glass bottles (50 mL aliquots)
8. Autoclaved for 15 minutes at 121°C.
9. After cooling, caps wrapped with Parafilm to prevent airborne contamination from fungal spores or bacteria.
10. Soil extract stored at 4°C.

MODIFICATIONS OF GSE MEDIUM

Modifications of GSe medium were used in experimental studies. These included various dilutions of GSe – e.g. $GSe/_{10}$ – which refers to GSe with $^{1}/_{10}^{th}$ of the concentrated nutrient solution added and $^{1}/_{10}^{th}$ of the soil extract. GSe^{-N-P} was also used which contained the same amount of other nutrients but without nitrate and phosphate added.

APPENDIX B

FIRST DETECTION OF *ALEXANDRIUM CATENELLA* FROM PORT RIVER ESTUARY, SOUTH AUSTRALIA: TOXIN CONCENTRATIONS AND PROFILES

ALEXANDRIUM CATENELLA FROM SOUTH AUSTRALIA

Prior to July 1997 *Alexandrium catenella* was unknown from South Australian waters. Blooms of the related species *Alexandrium minutum* had been regularly observed in this region (see Chapter 2) but *A. catenella* had never been detected as either vegetative or resting stages in water column or sediments. In July 1997 sediment grab samples were taken in the upper Port River Estuary to investigate the abundance of *A. minutum* cysts. In these samples, resting cysts of both *A. catenella* and *A. minutum* were found. *A. catenella* resting cysts were isolated into 55 mm presterilised polystyrene petri dishes with GSe medium and readily germinated under the standard growth conditions for *A. catenella* (18°C ± 0.5 °C at 80µmol photons PAR m-2 s-1 (cool white fluorescent lamps) on a 12:12 light:dark cycle). Six strains of *A. catenella* were isolated from the germinated cysts (two non-clonal and four clonal strains).

All strains were grown to 300 mL culture and tested for toxicity (by A. Negri, Australian Institute of Marine Science; methods for toxin analysis are described in Chapter 4). All six strains were found to be toxic with the toxin profile being dominated by the C toxins C1 and C2 along with C3, C4, GTX4, GTX1 and GTX5, trace amounts of other toxins were also present (Figure B.1 and Table B.1). All strains had similar toxin (Fig. B.1) although toxin concentration per cell varied (Table B.2).

It is probable that *A. catenella* has been recently introduced to the Port River Estuary. The phytoplankton composition in this area is regularly monitored and there have been previous investigations of sediments for resting cysts in the past ten years (C. Bolch and G. Hallegraeff, pers. comm.) without *A. catenella* being detected. The Port River Estuary is a highly modified system with significant waste- water input and forms part of the busy Port of Adelaide. It is therefore possible that resting cysts may have been introduced into the system through ballast water operations. In 1998 blooms of *A. catenella* were first recorded from this region (Ault pers. comm.). The initial detection of this species in the sediments points to the benefit of regular monitoring of both sediment and water column for potential introductions of toxic species.

Table B.1: Relative toxin Concentrations (Mol%) of six strains of *Alexandrium catenella* from the Port River Estuary, South Australia.

Strain	C1	C2	C3	C4	GTX4	GTX1	dcGTX3	GTX5	dcGTX2	GTX3	GTX2	NEO	dcSTX	STX
ACAD01	23.84	40.16	3.05	12.70	9.94	7.79	0.07	1.25	0.23	0.05	0.01	0.91	0.00	0.00
ACAD02	26.14	44.06	2.90	13.65	5.90	4.83	0.09	1.72	0.22	0.12	0.05	0.28	0.00	0.04
ACAD03	24.11	47.22	1.89	11.40	7.27	4.89	0.13	2.38	0.35	0.23	0.10	0.00	0.00	0.02
ACAD04	22.75	41.78	2.60	13.82	9.15	7.06	0.14	2.52	0.12	0.05	0.02	0.00	0.00	0.00
ACAD05	26.12	51.17	1.25	7.69	6.39	4.06	0.13	2.48	0.28	0.32	0.14	0.00	0.00	0.00
ACAD06	25.09	36.96	6.32	20.15	4.27	4.47	0.13	2.42	0.08	0.09	0.03	0.00	0.00	0.00

Table B.2: Toxin per cell Concentrations (fg per cell) of six strains of *Alexandrium catenella* from the Port River Estuary, South Australia.

Strain	Cells/ml	Total toxin conc. per cell
ACAD01	4111	87108
ACAD02	9680	58274
ACAD03	7524	55479
ACAD04	7284	40330
ACAD05	11564	72707
ACAD06	11924	47855

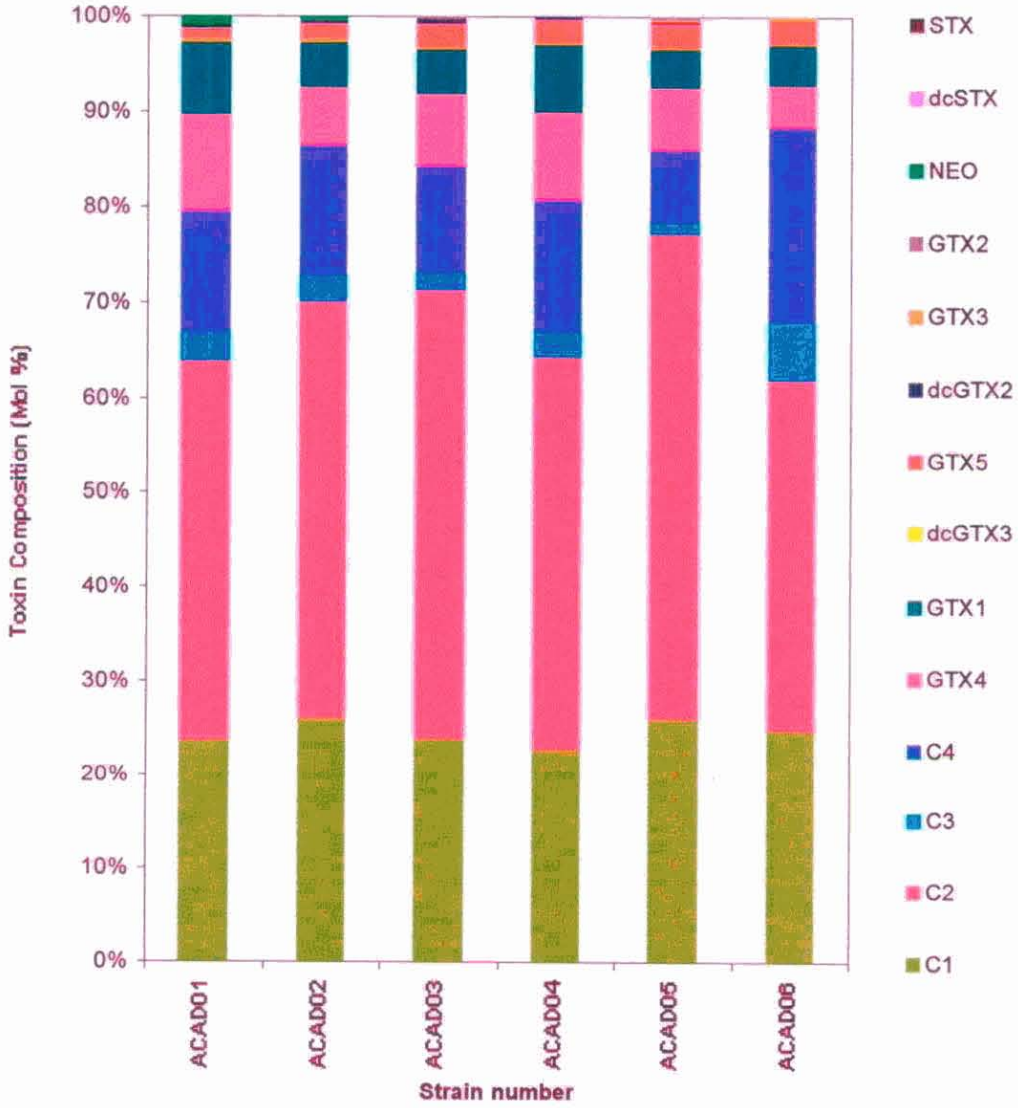


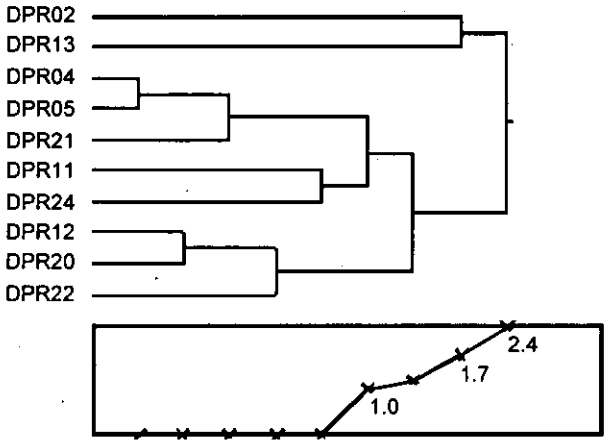
Figure B.1: Toxin composition (Mol %) of six strains of *Alexandrium catenella* isolated from the Port River Estuary, in South Australia.

APPENDIX C

MATING SYSTEM DETERMINATION: STRAIN DISTANCE MEASURES AND DENDROGRAMS

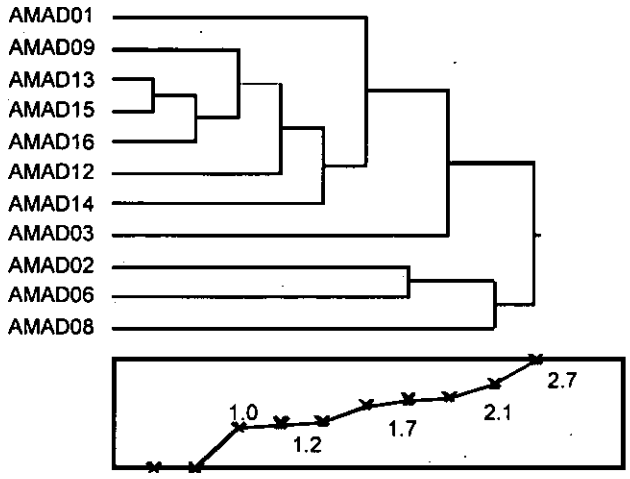
Protoceratium reticulatum

Number of Clusters	Distance	Leader	Joiner
9	0.000000000	04	05
8	0.000000000	12	20
7	0.000000000	04	21
6	0.000000000	12	22
5	0.000000000	11	24
4	1.000000000	04	11
3	1.183215957	04	12
2	1.732050808	02	13
1	2.371708245	02	04



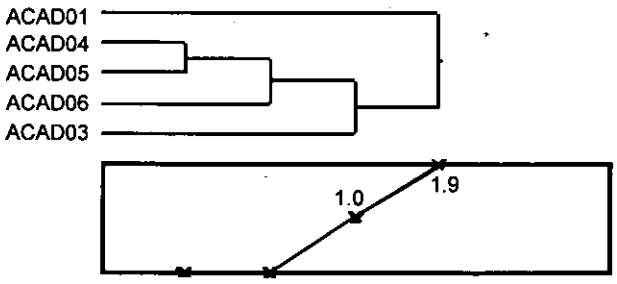
Alexandrium Minutum

Number of Clusters	Distance	Leader	Joiner
10	0.000000000	13	15
9	0.000000000	13	16
8	1.000000000	09	13
7	1.118033989	09	12
6	1.183215957	09	14
5	1.581138830	01	09
4	1.732050808	02	06
3	1.772810521	01	03
2	2.121320344	02	08
1	2.783882181	01	02



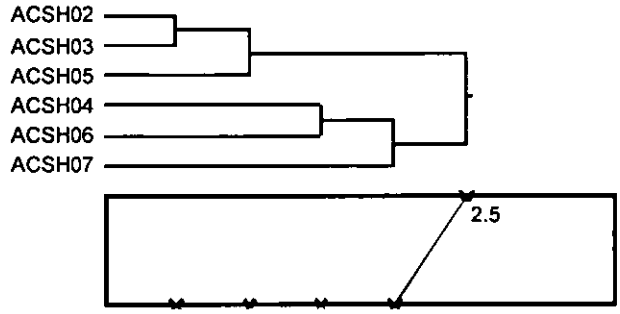
Alexandrium catenella
Port River, South Australia

Number of Clusters	Distance	Leader	Joiner
4	0.000000000	04	05
3	0.000000000	04	06
2	1.000000000	04	03
1	1.936491673	01	04



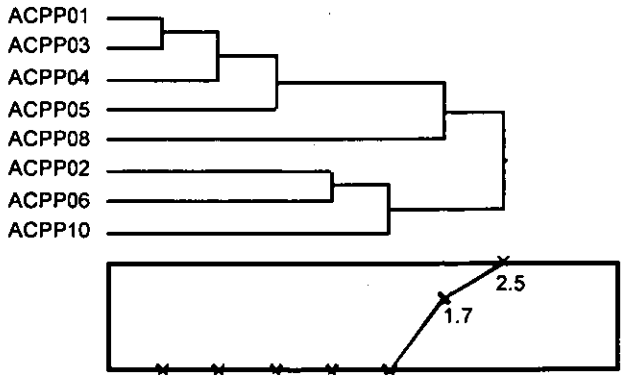
Alexandrium catenella
Sydney Harbour, New South Wales

Number of Clusters	Distance	Leader	Joiner
5	0.000000000	02	03
4	0.000000000	02	05
3	0.000000000	04	06
2	0.000000000	04	07
1	2.449489743	02	04



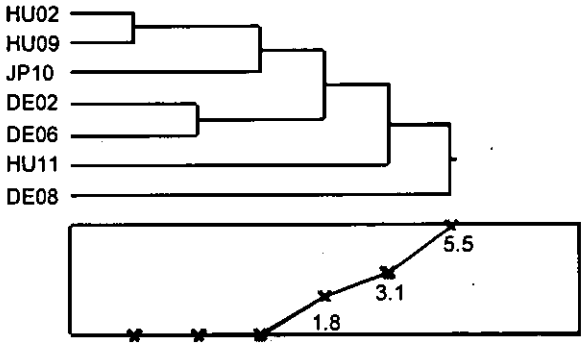
Alexandrium catenella
Port Phillip Bay, Victoria

Number of Clusters	Distance	Leader	Joiner
7	0.000000000	01	03
6	0.000000000	01	04
5	0.000000000	01	05
4	0.000000000	02	06
3	0.000000000	02	10
2	1.732050808	01	08
1	2.529822128	01	02



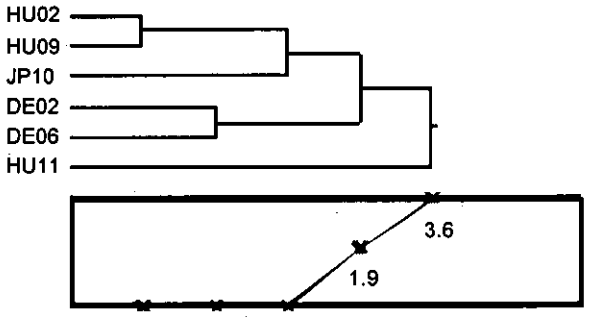
Long-term cultured *Gymnodinium catenatum* strains
This study - analysis includes selfing strain GCDE08

Number of Clusters	Distance	Leader	Joiner
6	0.000000000	HU02	HU09
5	0.000000000	DE02	DE06
4	0.000000000	HU02	JP10
3	1.870828693	HU02	DE02
2	3.130495168	HU02	HU11
1	5.507570547	HU02	DE08



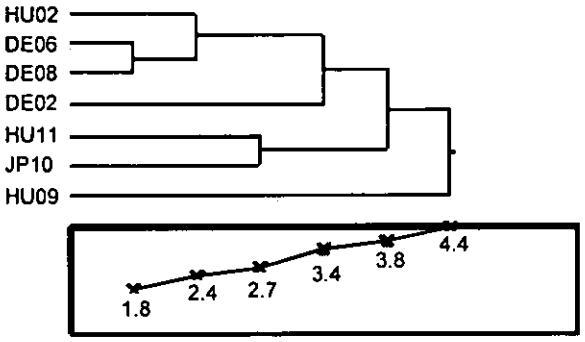
Long-term cultured *Gymnodinium catenatum* strains
This study - analysis does not include selfing strain GCDE08

Number of Clusters	Distance	Leader	Joiner
5	0.000000000	HU02	HU09
4	0.000000000	DE02	DE06
3	0.000000000	HU02	JP10
2	1.936491673	HU02	DE02
1	3.674234614	HU02	HU11



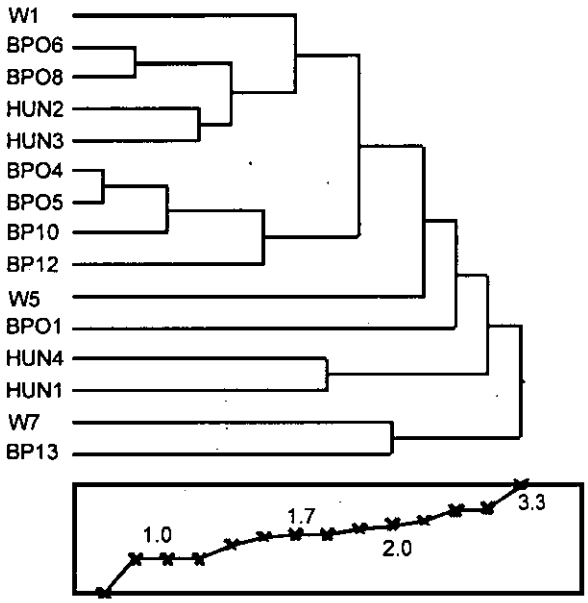
Long-term cultured *Gymnodinium catenatum* strains
Blackburn et al., 2001 results

Number of Clusters	Distance	Leader	Joiner
6	1.870828693	DE06	DE08
5	2.439262184	HU02	DE06
4	2.774887385	HU11	JP10
3	3.483293461	HU02	DE02
2	3.811167800	HU02	HU11
1	4.440345332	HU02	HU09



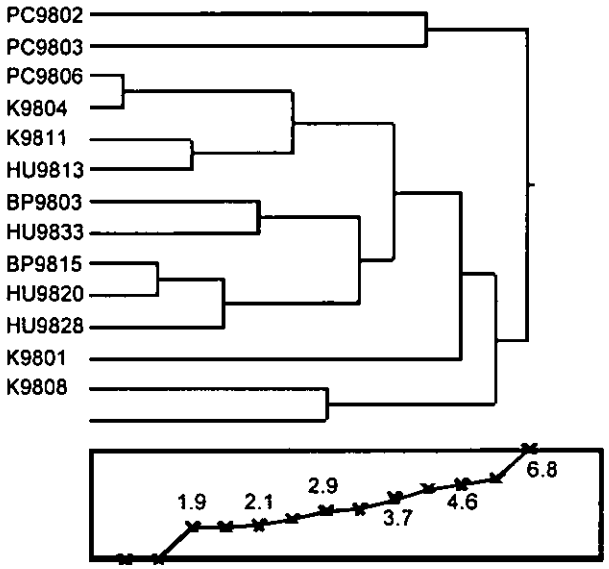
Gymnodinium catenatum
Summer bloom strains

Number of Clusters	Distance	Leader	Joiner
14	0.000000000	BPO4	BPO5
13	1.000000000	BPO6	BPO8
12	1.000000000	BPO4	BP10
11	1.000000000	HUN2	HUN3
10	1.414213562	BPO6	HUN2
9	1.632993162	BPO4	BP12
8	1.732050808	W1	BPO6
7	1.732050808	HUN4	HUN1
6	1.870828693	W1	BPO4
5	2.000000000	W7	BP13
4	2.134374746	W1	W5
3	2.469817807	W1	BPO1
2	2.531618239	W1	HUN4
1	3.258125936	W1	W7



Gymnodinium catenatum
Autumn bloom strains

Number of Clusters	Distance	Leader	Joiner
13	0.000000000	PC9806	K9804
12	0.000000000	BP9815	HU9820
11	1.927248223	K9811	HU9813
10	2.011080417	BP9815	HU9828
9	2.133072901	BP9803	HU9833
8	2.429318279	PC9806	K9811
7	2.931628292	K9808	K9815
6	3.085659783	BP9803	BP9815
5	3.688441836	PC9806	BP9803
4	4.306971093	PC9802	PC9803
3	4.597117974	PC9806	K9801
2	4.958535137	PC9806	K9808
1	6.787652188	PC9802	PC9806



Gymnodinium catenatum
Summer X Autumn strains

Number of Clusters	Distance	Leader	Joiner
14	0.000000000	PC9806	BP10
13	1.000000000	PC9806	K9811
12	1.000000000	BP06	BP08
11	1.290994449	PC9806	HUN4
10	1.414213562	HUN2	W1
9	1.414213562	HU9828	HUN3
8	1.581138830	BP06	HU9820
7	1.732050808	PC9802	K9804
6	1.870828693	PC9806	HU9828
5	1.986062548	PC9806	BP06
4	2.121320344	PC9802	HUN1
3	2.236067977	PC9802	BP9803
2	2.285218200	PC9806	HUN2
1	2.658605239	PC9802	PC9806