# Importance of selenium and humic substances from land runoff in the development of *Gymnodinium catenatum* toxic dinoflagellate blooms

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requirements for the degree of
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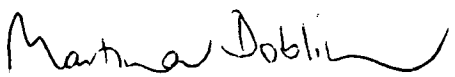

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# Abstract

Toxic dinoflagellate blooms of *Gymnodinium catenatum* Graham (causative organism of paralytic shellfish poisoning) can have major impacts on aquaculture, human health and the aquatic environment. In south-east Tasmanian estuaries, these blooms are preceeded by rainfall events and the associated input of freshwater and humic substances (from soil leachates). This study examined the potential nutritive role of terrestrially-derived humic substances / micronutrients and the importance of vertical water column stratification in the development of *G. catenatum* blooms.

Laboratory experiments showed that humic additives (standard aquatic humic acid purchased from the International Humic Substances Society, and dissolved organic matter isolated from the Huon Estuary by ultrafiltration) stimulated *G. catenatum* growth and biomass production. Under these conditions biomass was limited by the macro-nutrients nitrate and phosphate, but in seawater medium with no humic additives the micro-nutrient selenium was limiting. This suggests that humic substances change overall nutrient availability and / or uptake by *G. catenatum*, either by adding nutrients or by interaction with other nutrients.

Bioassays indicated that 1 - 100 nM selenium (IV) stimulated *G. catenatum* growth and biomass production. However not all strains of this dinoflagellate species tested (including isolates from Tasmania, Japan and Spain) had an obligate selenium requirement. Another PSP dinoflagellate *Alexandrium minutum* showed a selenium requirement similar to *G. catenatum*, but the bloom forming diatom, *Chaetoceros* cf. *tenuissimus* showed no reduction in growth or biomass production under selenium-deficient conditions. Inorganic selenium (selenite and selenate) concentrations in the Huon Estuary were commonly < 0.01 nM (below detection) and thus are potentially limiting for *G. catenatum*. Since selenium enters estuarine systems primarily through river run-off, rainfall may be a crucial trigger for dinoflagellate blooms in these waters by increasing selenium levels.

Water column stability and stratification, also often associated with rainfall, occurs in south-east Tasmanian coastal waters during summer, and this is also critical in the development of *G. catenatum* blooms. Laboratory experiments in stratified laboratory water columns (0.1 x 1m) examined the migration behaviour of *G. catenatum* and showed that nutrient-deficient cells migrate downwards to access nutrients from bottom layers at night, while nutrient-replete cells tend to remain at the surface. This migration

pattern was similar when using humic and non-humic surface waters, indicating that humic substances do not enhance cellular nutrient concentrations to such a degree as to preclude the need for deep nutrient uptake. Diel vertical migration to facilitate dark nutrient assimilation provides an ecological advantage for *G. catenatum* over other non-migratory species, particularly during late summer in Tasmanian estuaries when surface nutrients are depleted.

This study indicates that selenium and humic substances contained in river run-off are stimulatory to *G. catenatum* growth and biomass production, providing essential micro-nutrients during bloom initiation. Furthermore, water column stability and vertical stratification caused by river run-off are of paramount importance in maintaining *G. catenatum* blooms. During this latter stage, vertical migration by nutrient-deficient cells forms an integral part of the successful ecological strategy utilised by this red tide dinoflagellate.

# Acknowledgements

A large number of people have helped to make this Ph.D. project a success, all of whom I would like to thank. My university supervisor Gustaaf Hallegraeff, for allowing me to come to Tasmania and work on toxic algae and for providing valuable financial assistance, particularly during the final stages of writing my thesis. I am also thankful to Gustaaf for setting high standards to provide strong incentive to help me learn and complete this science apprenticeship. My CSIRO supervisor Sue Blackburn, who on a day-to-day level has provided the support and encouragement necessary to meet the challenges of scientific research and who has provided understanding and friendship during my Ph.D. Thanks are also extended to Ed Butler, CSIRO Division of Marine Research for his chemistry expertise, without which this project would not have been possible. Special thanks is also given to Peter Thompson, for helpful advice and discussion, rapid response emails and the skills to become a phytoplankton physiologist. Grant Douglas, Chris Bolch and Gary Jones have also provided useful discussion which helped shape the project and develop my analytical skills. The latter half of the project, which involved field work in the Huon Estuary was only possible through the logistic support of the CSIRO Huon Estuary Study and the help of Pru Bonham, Don MacEnzie, Matt Sherlock and Lesley Clementson, for which they are gratefully acknowledged. Ros Watson and Jeanette O'Sullivan are sincerely thanked for their practical chemistry advice, as are Val Latham, Kate Berry and Donna Roberts for their assistance with nutrient analyses. Acknowledgement is also given to Andy Revill and Steve Bell who provided assistance with PON/POC and TOC analyses, respectively, and subsequent data interpretation. The CSIRO Technical Services group (particularly Neal McQueen) are thanked for helping to set up the laboratory columns and practical problem-solving advice. I am also grateful to Greg and Lynda Cutter for assistance with selenium determinations and the opportunity to work in Virginia, U.S.A. Thanks are extended to Michael Bessell and Louise Bell for producing the maps shown in this thesis, and to Ian Jameson and Graham Knott for assistance with photomicrographs. The algal culture group are also thanked for providing a thorough training in phytoplankton cultivation and related pursuits, as well as much needed laughter and entertainment. Special thanks go to Lynda for many fantastic hours of distraction during the last three and a half years, the other mermaid, Naomi for the many stimulating and motivating work and personal discussions and for help with 24 hour experiments and to my babe sister Monika for help with formatting. This thesis is dedicated to all my friends and family who have always been supportive of my ventures into harmful algal bloom research and whom I all love dearly.

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# List of Abbreviations

| | |
|---|---|
| ANOVA | analysis of variance |
| BL | bottom layer |
| CSIRO | Commonwealth Scientific Industrial Research Organisation |
| DAPI | 4'6-diamidino-2-phenylindole |
| DIC | dissolved inorganic carbon |
| DIN | dissolved inorganic nitrogen |
| DIP | dissolved inorganic phosphorus |
| DO | dissolved oxygen |
| DOC | dissolved organic carbon |
| DOM | dissolved organic matter |
| DON | dissolved organic nitrogen |
| DOP | dissolved organic phosphorus |
| DVM | diel vertical migration |
| EDTA | ethylene diamine tetra acetic acid |
| ESAW | enriched artificial seawater medium |
| HAB | harmful algal blooms |
| HPLC | high performance liquid chromatography |
| HS | humic substances |
| $HSL_{-N}BL_r$ | nitrate-deplete humic enriched surface layer + nitrate-replete bottom layer |
| $HSL_rBL_r$ | nitrate-replete humic enriched surface layer + nitrate-replete bottom layer |
| IHSS | International Humic Substances Society |
| MQ | Milli-Q® |
| PAR | photosynthetically active radiation |
| POC | particulate organic carbon |
| PON | particulate organic nitrogen |
| PSP | paralytic shellfish poisoning |
| PST | paralytic shellfish toxins |
| RAPD-PCR | Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction |
| SHA | standard humic acid (Suwannee River) |
| SL | surface layer |
| $SL_dBL_r$ | nutrient- deplete surface layer + nutrient-replete bottom layer |
| $SL_rBL_r$ | nutrient- replete surface layer + nutrient-replete bottom layer |
| TDN | total dissolved nitrogen |
| TDP | total dissolved phosphorus |
| UV | ultraviolet |

# Chapter 1: *Gymnodinium catenatum* blooms in south-east Tasmania

## 1.1 Harmful algal blooms as a threat to aquaculture in south-east Tasmania

The incidence of harmful algal blooms (HABs) around the world appears to be increasing (Hallegraeff 1993, Smayda 1990), although reports of such events date from biblical times (Exodus 7:21). This increase is probably due to a greater scientific awareness of harmful algae and improved taxonomic knowledge, but may also be linked to coastal eutrophication, ballast water introductions or global warming (Fraga and Bakun 1993, Hallegraeff 1993, Nehring 1995). Furthermore, increased usage of the coastal zone for recreational, commercial, industrial and aquaculture activities has meant that HAB have a seemingly greater impact, resulting in an increased need for understanding these phenomena.

The Huon Estuary, south-east Tasmania, Australia, is a rapidly expanding site for aquaculture (Atlantic salmon, mussels and oysters). There are currently 21 marine farms with leases over 132 ha (0.6%) of the total estuary area, bringing considerable economic and employment benefits both to the region and the State (Gallagher 1996). Future development plans include establishment of four new shellfish sites and another finfish farm, along with the expansion of existing farm areas to allow more effective fallowing procedures (Department of Primary Industries and Fisheries 1994).

In previous years (1986 - 1993), Tasmanian shellfish farms have been closed for periods of up to 6 months because of toxic blooms of *Gymnodinium catenatum* Graham. *G. catenatum* is an unarmoured, chainforming dinoflagellate (Fig. 1.1), one of 11 species of phytoplankton currently known to produce paralytic shellfish toxins (Hallegraeff *et al.* 1995). These toxins accumulate in shellfish (through filter-feeding) and are harmful to human consumers, causing numbness, nausea, diarrhoea and in extreme cases, muscular paralysis and asphyxiation (Hallegraeff 1991). *G. catenatum* was originally described from the Gulf of California (Graham 1943) and was first linked with paralytic shellfish poisoning (PSP) in Spain in 1976 (Estrada *et al.* 1984). Cases of PSP by *G. catenatum* have been recorded from Mexico (where three children died; Morey-Gaines 1982, Mee *et al.* 1986), Portugal (Franca and Almeida 1989), Japan (Ikeda *et al.* 1989) and Tasmania, Australia (Hallegraeff and Sumner 1986).

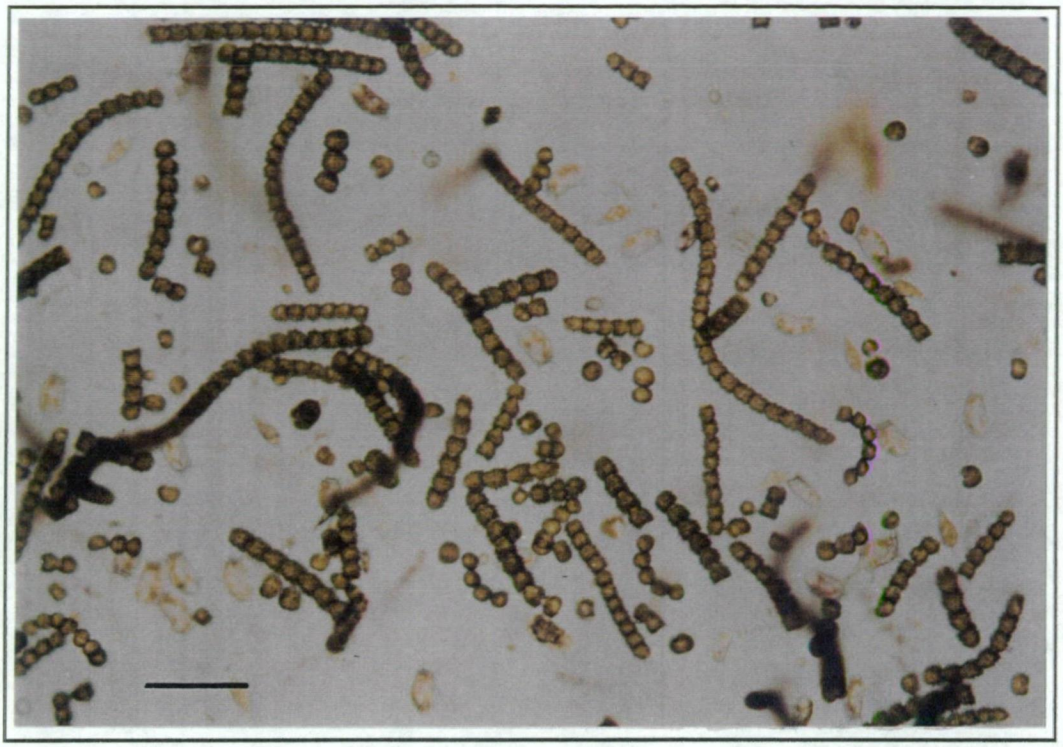

Fig. 1.1: LM. Vegetative chains and solitary cells of the toxic dinoflagellate *Gymnodinium catenatum* in a 20 µm net sample from the Derwent Estuary 1993. Photograph taken by C. Bolch; Scale bar = 200 µm.

As a result of their toxicity, *G. catenatum* blooms pose major environmental and public health problems and threaten aquaculture, fishing and tourism operations. Bloom management in Tasmania currently involves routine monitoring of shellfish for paralytic shellfish toxins (PSTs) (at the Department of Primary Industries and Fisheries using HPLC), and an early warning system for blooms using remote monitoring buoys is under development (Hallegraeff *et al.* unpublished). Nevertheless, we still have relatively little understanding about the factors involved in *G. catenatum* outbreaks.

This study examines the importance of river runoff on the development of *G. catenatum* blooms, with a view to expand the current conceptual model for these events (see Hallegraeff *et al.* 1995) and improve its predictive capability. Two aspects of river runoff are evaluated for their effect on *G. catenatum* bloom development: (i) the potential nutritive role of terrestrially-derived humic (or dissolved organic) substances and (ii) the vertical separation of light and nutrients in a vertically stratified water column.

## 1.2 Global and local distribution of *Gymnodinium catenatum*

*G. catenatum* has a disjunct global distribution, with vegetative cells being identified in coastal waters from temperate and tropical regions: e.g. north-west Spain, Mexico, Japan, Portugal, Venezuela, Thailand, Phillipines, Palau, Uruguay, Morocco, Malasia and Australia - see Table 1.1). Non-toxic, non-chain forming strains have also been isolated from northern Europe (Ellegaard *et al.* 1993), but these are now considered to represent a different species (Ellegaard *et al.* unpublished).

Table 1.1: Chronology of first plankton records of *Gymnodinium catenatum* in different parts of the world; from Hallegraeff and Fraga (1998).

| Year | Location | Reference |
|---|---|---|
| 1939 | Gulf of California | Graham 1943 |
| 1962 | Mar del Plata, Argentina | Balech 1964 |
| 1976 | NW Spain | Estrada *et al.* 1984 |
| 1979 | Pacific coast of Mexico | Mee *et al.* 1986 |
| 1985 | Tasmania, Australia | Hallegraeff and Sumner 1986 |
| 1986 | Southern Japan | Ikeda *et al.* 1989; Nishioka *et al.* 1993 |
| 1986 | Portugal | Franca and Almeida 1989 |
| 1988 | Venezuela | La Barbera-Sanchez *et al.* 1993 |
| 1989 | Gulf of Thailand | Matsuoka and Fukuyo 1994 |
| 1990 | Phillipines | Fukuyo *et al.* 1993; Corrales *et al.* 1996 |
| 1990 | Palau | Hallegraeff and Oshima, unpublished |
| 1992 | Uruguay | Mendez and Brazeiro 1993 |
| 1993 | Denmark | Ellegaard et al. 1993; however strain is to be reclassified as a different species |
| 1994 | Morocco | Tagmouti *et al.* 1995 |
| 1994 | Malaysia | Anton and Mohamad-Noor, 1998 |

Within Australia, vegetative cells of *G. catenatum* appear to be restricted to the Huon Estuary, D'Entrecasteux Channel and Derwent Estuary in south-east Tasmania, although during the most extensive bloom in 1993, toxicity was recorded in Adventure Bay (Bruny Island), Port Arthur and Triabunna (Hallegraeff *et al.* 1995; Fig. 1.2). Surveys of Recent sediments have also identified viable cysts in other areas: Port

Lincoln, South Australia since 1996 (Bolch unpublished data), various locations along the Victorian coastline, including Portland, Lorne, Port Phillip Bay and Port Welshpool since 1992 (Sonneman and Hill 1997), and Triabunna, Spring Bay and Georges Bay in Tasmania (Bolch and Hallegraeff 1990; Fig. 1.3). Empty cysts have also been recorded from St. Helens on Tasmania's east coast (Bolch and Hallegraeff 1990).

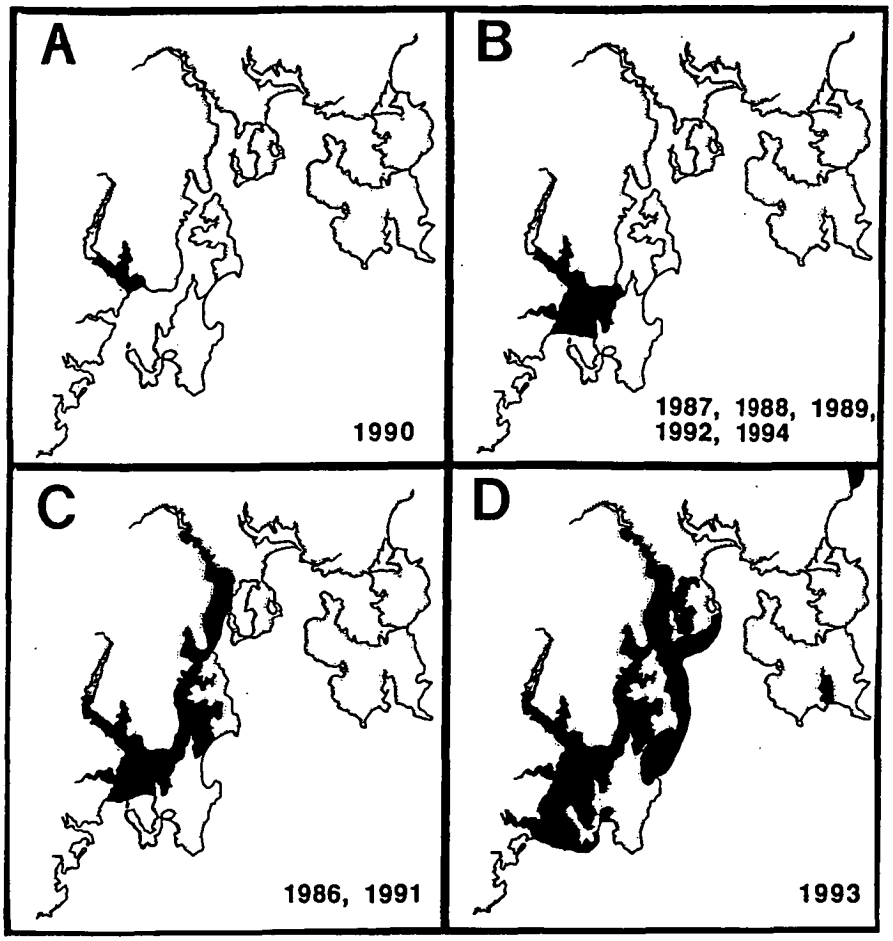


Fig. 1.2: Spatial distribution of shellfish toxicity outbreaks in southern Tasmania. a) Minor bloom event in 1990 confined to the Huon River only; b) Medium bloom events in 1987, 1988, 1989, 1992 and 1994 distributed across the Huon Estuary and Port Esperance; c) Large bloom events in 1986 and 1991 covering the Huon Estuary, Port Esperance, d'Entrecasteaux Channel and Derwent Estuary; d) Largest bloom event in 1993, for the first time stretching to Adventure Bay, Port Arthur and Triabunna; from Hallegraeff *et al.* 1995.

Analysis of historic plankton samples and cysts in sediment cores have indicated that *G. catenatum* is not indigenous to Tasmania and was introduced to the region after 1972, probably via transfer of ships' ballast water (Hallegraeff and Bolch 1992, McMinn *et al.* 1998).

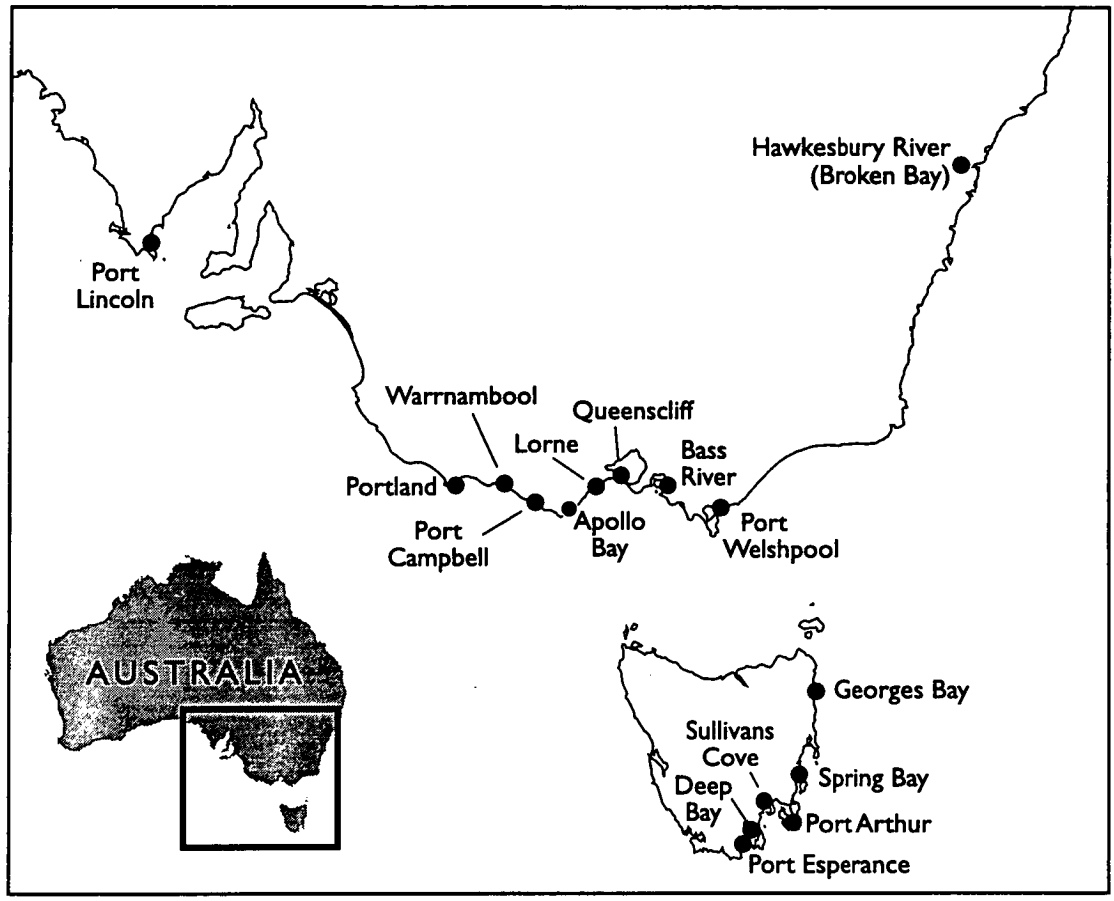


Fig. 1.3: Distribution of *Gymnodinium catenatum* resting cysts in Recent sediments of southern Australia; from Bolch and Hallegraeff 1990, Sonnenman and Hill 1997 and C. Bolch (unpublished data)

## 1.3 *Gymnodinium catenatum* blooms in south-east Tasmania

The first recorded bloom of *G. catenatum* in Tasmanian waters (defined as cell density $> 10^4$ cells $l^{-1}$), occurred in the Derwent Estuary in 1980 (Hallegraeff and Sumner 1986). From 1986-1993, blooms were annually recurrent events, developing during a seasonal temperature window between January - June. There was high interannual variability in spatial extent and toxicity of blooms, with highest shellfish toxicity occuring in 1993 (Hallegraeff *et al.* 1995). Blooms developed in the Huon Estuary and Port Esperance almost every year, in the D'Entrecasteux Channel and Derwent Estuary only in some years and in 1993, extended to Adventure Bay, Port Arthur and Triabunna (Fig. 1.2). Since then, there has been no *G. catenatum* bloom, although regular phytoplankton monitoring has shown that there is a small resident population in the Huon Estuary throughout the year (CSIRO Huon Estuary Study unpublished data).

## 1.4 The Huon Estuary - site of *Gymnodinium catenatum* bloom formation in Tasmania

The Huon Estuary is a typical "drowned river valley estuary", with the estuarine zone extending approximately 38 km, from Ranelagh to Huon Island (Fig. 1.4). The Huon River and its tributaries drain an area of approximately 3900 $km^2$, with monthly average flows (recorded upstream of Frying Pan Creek) ranging from a low of 30-40 cumecs between January and March to peak flows of 125-130 cumecs in July and August (Deparment of Primary Industries and Fisheries 1995). Most of the catchment is comprised of State Forest (125,400 ha including cool temperate rainforest and dry sclerophyll forest), with a further 35,000 ha being used for agriculture (crops, pastures and fruit production). River usage is restricted to recreational activities and marine farming operations (132 ha). Licensed discharges include effluent from four secondary treated sewage sites, two fish processing plants, and a pulp processing mill, with diffuse inputs from unsewered properties and municipal refuse tips (Gallagher 1996).

A distinguishing feature of the Huon Estuary (and many other estuaries in Tasmania), is the incidence of tea-coloured river water, a result of its high content of humic substances. The phytoplankton flora is highly diverse (Jameson 1995), and is often characteristically dominated by dinoflagellates (e.g. *Ceratium furca*, *C. tripos*, *Dinophysis acuminata*, *Protoperidinium* spp., *Prorocentrum gracile*). *G. catenatum* is a conspicuous member of the plankton (Fig. 1.1), present in relatively low abundance throughout July-December, and normally forming dense blooms during the warmer months (January - June).

## 1.5 *Gymnodinium catenatum* bloom development

Several hypotheses have been put forward to explain initiation, maintenance and decline of harmful algal blooms (HAB) in other regions. These have involved: (i) the germination of resting cysts following environmental cues (Steidinger 1975, Anderson and Morel 1978); (ii) transport of viable cells from other regions (Tyler and Seliger 1978, Franks and Anderson 1992, Fraga 1988); (iii) competitive nutrient uptake strategies for bloom species involving vertical migration (Provasoli 1979); (iv) favourable hydrological conditions which support growth and biomass accumulation of bloom species (Slobodkin 1953, Margalef *et al.* 1979, Therriault *et al.* 1985) and (v) biological interactions involving excretion of inhibitors, grazing deterrents or growth promoting substances (Provasoli 1979). The importance of these factors in *G. catenatum* bloom development in south-east Tasmania is discussed below.

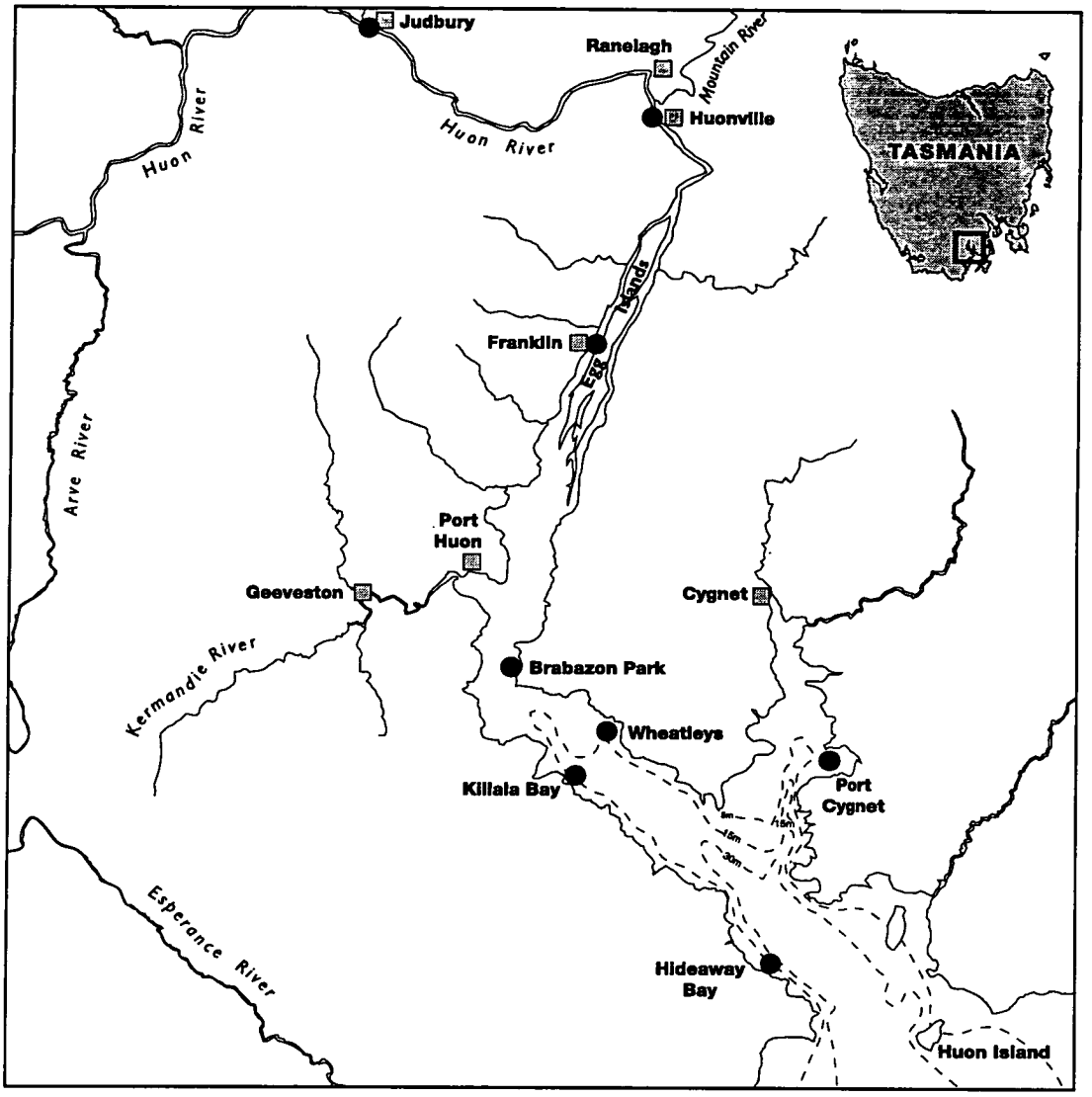


Fig. 1.4: The Huon Estuary showing water sampling locations, with the estuarine zone extending approximately 38 km from Ranelagh to Huon Island. Figure designed by Michael Bessell and reproduced with permission from the CSIRO Division of Marine Research.

### *1.5.1 Cyst germination*

*G. catenatum*, like many other dinoflagellates, produces resting cysts (hypnozygotes), formed from the pairing and fusion of sexually-compatible gametes (heterothallism; Blackburn *et al.* 1989). There are extensive cyst beds in the Huon and Derwent Estuaries (Bolch and Hallegraeff 1990), but given the (i) short dormancy period of cultivated cysts (several weeks - months); (ii) wide range of conditions under which they germinate (Blackburn *et al.* 1989, Bravo and Anderson 1994); and (iii) rare occurrence of planomeiocytes (recently germinated cysts) in field samples (Bolch and

Blackburn, unpublished data), it is considered unlikely that *G. catenatum* blooms arise from a synchronised mass germination event (Hallegraeff *et al.* 1995). Instead, cysts probably provide an inoculum to maintain a population of motile vegetative cells throughout the year (Hallegraeff *et al.* 1995).

### *1.5.2 Transport of viable cells from other regions*

The shoreward transport of oceanic seed populations has been suggested by Mee *et al.* (1986) and Fraga *et al.* (1988) to be important in the formation of *G. catenatum* blooms in Mexico and Spain (respectively). Observations in south-east Tasmanian waters do not support such an hypothesis, since cells collected outside the Huon and Derwent Estuaries appear moribund and do not survive well in coastal waters (Hallegraeff *et al.* 1995). Furthermore, laboratory studies of salinity tolerance in *G. catenatum* precludes a freshwater origin for blooms of this species (Blackburn *et al.* 1989).

### *1.5.3 Competitive nutrient uptake strategies involving vertical migration*

Vertical migration has been identified as a competitive strategy for phytoplankton under conditions where light and nutrients are spatially separated (Ganf and Oliver 1982). Field observations of *G. catenatum* in Ria de Vigo, northwest Spain, and in the Huon Estuary, Tasmania, have confirmed that this species undergoes such behaviour, sinking in the late afternoon and rising in the early hours of the morning to form subsurface maxima during the day (Figueras and Fraga 1990, Hallegraeff and Fraga 1998). Vertical migration could be important in phytoplankton dynamics in south-east Tasmania, where dinoflagellate blooms commonly occur during summer when surface nutrients are depleted (I. Jameson, unpublished data) and high concentrations of humic substances in river water cause rapid light attenuation (CSIRO Huon Estuary Study). Diel vertical migration may therefore be a significant factor in the ecological success of *G. catenatum* during blooms.

### *1.5.4 Favourable hydrological conditions*

*G. catenatum* blooms in south-east Tasmania are preceded by large rainfall events (and the associated influx of humic substances through land runoff), followed by prolonged periods of low wind stress and subsequent vertical stratification (Hallegraeff *et al.* 1995). The sensitivity of this species to disturbance in culture (Flynn *et al.* 1996, and this study) and relatively slow growth rate ($k = 0.33$ day$^{-1}$ Blackburn *et al.* 1989 and this study), support the hypothesis that blooms could only develop after several weeks of calm weather (Hallegraeff *et al.* 1995). Furthermore, double peaks of shellfish

toxicity during most previous *G. catenatum* blooms (see Fig. 1.5), suggests that the persistence of this species in the water column is strongly affected by the degree of turbulence or horizontal advection (Hallegraeff *et al.* 1995).

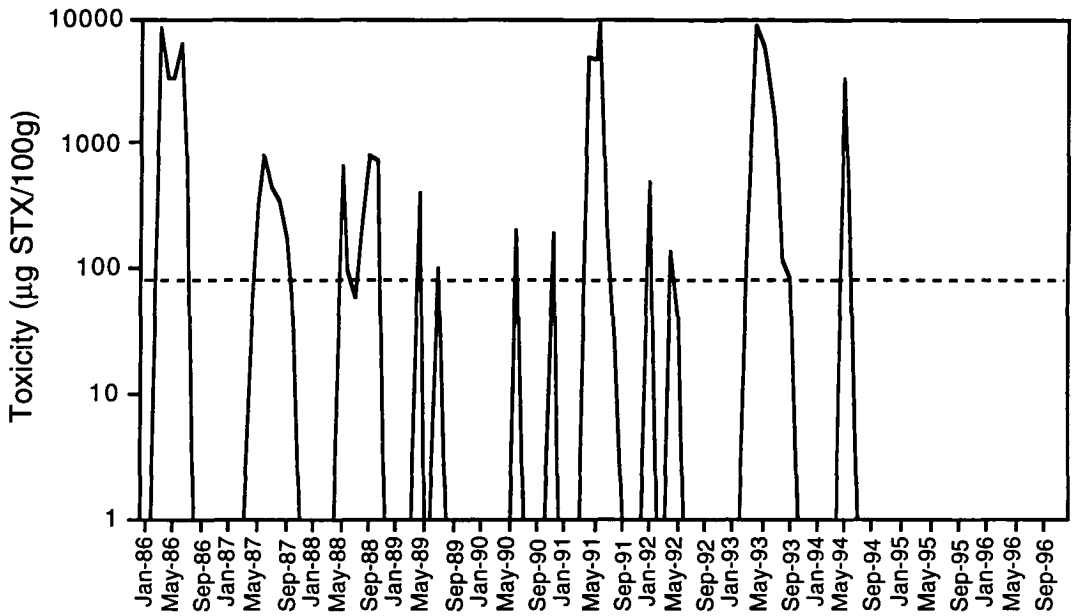


Fig. 1.5: Interannual variability in the concentration of *Gymnodinium catenatum* toxins (µg saxitoxin equivalent per 100 g shellfish meat) in Huon River longline mussels in the period 1986 - 1996. A logarithmic scale of shellfish toxicity has been used to emphasize seasonal bloom patterns. The dashed line indicates the USFDA quarantine level of 80 µg per 100 g, above which shellfish farm closures need to be instituted; from Hallegraeff *et al.* 1995.

### *1.5.5 Excretion of inhibitors or growth promoting substances and water preconditioning*

Stimulated growth and yields in culture with addition of soil extract (Blackburn *et al.* 1989) and the apparent inability to explain patterns of *G. catenatum* abundance from macronutrient distribution, indicates that water preconditioning by land drainage (input of terrestrially-derived humic substances) may provide appropriate chemical conditions for bloom development. In addition, *G. catenatum* has no marked allelochemical or allelopathic properties to deter competing species and grazers. Exudates of *G. catenatum* cultures have been tested for their toxicological and pathogenic effects on brine shrimp and juvenile flounder, with no apparent effect (G. Lush, unpublished data). There has however been some suggestion that the toxins which exist in different life history stages (cysts and vegetative cells) of *G. catenatum* (Bravo *et al.* 1997) are

grazing deterrants (Riegman 1998). Furthermore, phytoplankton monitoring in south-east Tasmania over the last 10-15 years has not identified any distinct patterns of plankton species abundance prior to *G. catenatum* blooms, suggesting that biotic interactions are not important for bloom formation (G. Hallegraeff, unpublished data).

## 1.6 Humic substances as a factor in *Gymnodinium catenatum* bloom initiation

The most important conditions for *G. catenatum* bloom development in south-east Tasmania appear to be favourable hydrological conditions (stable water column), a competitive nutrient uptake strategy through vertical migration and water preconditioning by input of humic substances (HS) in river runoff.

HS are a diverse group of compounds formed from the decomposition of organic matter, and comprise > 50% of the dissolved organic carbon (DOC) in aquatic systems (Fox 1983, Thurman 1985). They are considered refractory (unavailable for further breakdown), have widely varying composition and structure and are not known to carry out a specific biochemical function. HS vary in size from relative molecular masses of a few hundred (which are freely soluble), to insoluble macromolecular aggregates with molecular weights >100,000 (Kirk 1994, Thurman 1985). Humic substances are further distinguised by whether or not they are adsorbed, aggregated or co-precipitated with other particles (Beckett 1986). A proposed structure for humic acid (soluble at low pH) is shown in Fig. 1.6.

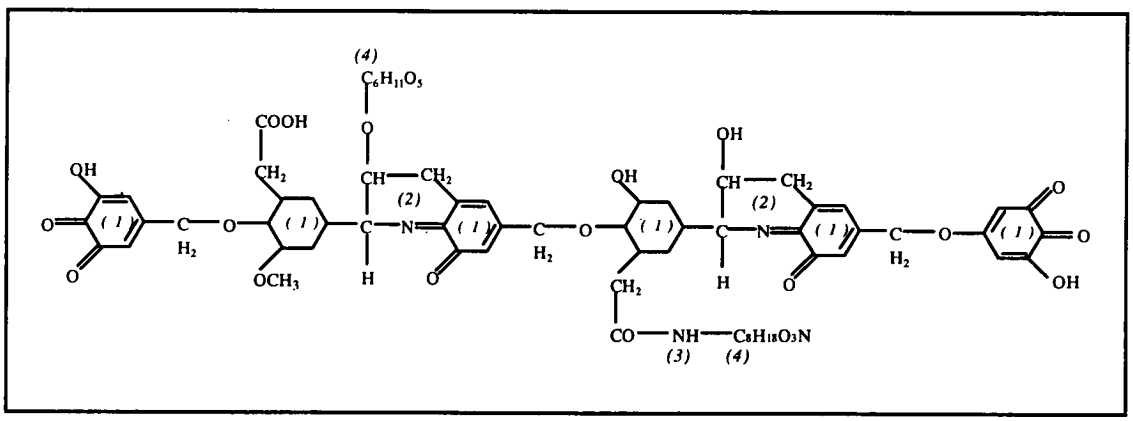


Fig. 1.6: Proposed structure for humic acid showing (1) aromatic ring of the di- and trihydroxyphenol type, part of which has the double linkage of a quinone grouping; (2) nitrogen in cyclic forms; (3) nitrogen in peripheral chains; (4) carbohydrate residues; from Rashid 1985.

## 1.7 Importance of micro-nutrients for phytoplankton

Some phytoplankton, and dinoflagellates in particular, are difficult to isolate and maintain in culture because of their fastidious growth requirements. Soil extract (a mixture of soil and water which has been autoclaved and filtered) has traditionally been added to cultures to enhance growth and biomass production of such "difficult" species (eg. Föyn 1933, Loeblich 1975). Greater understanding of phytoplankton nutrition and the subsequent development of culture media has resulted in the replacement of soil extract in some cases with more defined constituents such as vitamins (thiamine, biotin, vitamin B12), trace metals (eg. Ni, Co, Fe, Mn, Mo, Sr, Zn; added in conjunction with artificial chelators such as EDTA), and other elements such as selenium (Se). Despite this, some species, including *G. catenatum*, are still unable to grow in completely artificial media such as Aquil (Morel *et al.* 1979; Price *et al.* 1989) or ESAW (Harrison 1980), indicating that some growth factors remain to be elucidated. The incidence of *G. catenatum* blooms in humic-rich waters, and the demonstration that addition of small quantities of these micronutrients to *G. catenatum* cultures increases growth and biomass yields (Blackburn *et al.* 1989), prompted a study investigating the nutritive role of these organic growth factors on *G. catenatum*.

## 1.8 Study objectives

This study tests the hypothesis that river runoff and the concomitant input of HS is important in Tasmanian *G. catenatum* bloom development. Use of small scale batch cultures (<0.2 l) and large scale (approx. 9 l) column cultures allowed investigation of both chemical and physical factors associated with river runoff. The specific aims were to:

1. identify whether HS have a stimulatory effect on *G. catenatum* growth and biomass production;
2. assess whether HS affect nutrient availability and uptake by *G. catenatum*;
3. explore the mechanism behind humic stimulation by examining the requirement for selenium in *G. catenatum* and investigating whether humic substances affect selenium uptake by this dinoflagellate species;
4. examine the vertical migration behaviour of *G. catenatum* under nutrient-replete and nutrient-deficient conditions in vertically stratified water columns;
5. investigate the effect of HS on the nutrient status and vertical migration behaviour of *G. catenatum*.

A brief thesis outline is given below.

## 1.9 Thesis outline

The following chapter (2) discusses the potential role of HS in phytoplankton dynamics, and presents the variable effects of HS on *G. catenatum* growth and biomass yields. Chapter 3 introduces the micro-nutrient selenium and the importance it has in determining growth and biomass production of *G. catenatum*. This is followed by an assessment of the intra-specific variability in *G. catenatum* selenium requirement and the implications this has for population dynamics and bloom formation (Chapter 4). Chapter 5 reviews diel vertical migration as a successful ecological strategy in environments where light and nutrients are vertically separated, and tests the hypothesis that *G. catenatum* exhibits this behaviour to facilitate dark nutrient assimilation. This chapter also determines how *G. catenatum* vertical migration behaviour is modified in the presence of HS. Chapter 6 investigates the effect of HS and nutrients in *G. catenatum* sexual reproduction. The final chapters (7 and 8) contain a discussion of *G. catenatum* bloom formation in south-east Tasmanian waters, putting them into a global context, and includes a refinement of the current conceptual model, future research directions and bloom management strategies.

# Chapter 2: Interaction of dissolved organic substances with selenium and nutrients: Implications for growth and biomass production by *Gymnodinium catena tum*[1]

## 2.1 Introduction

Land runoff and the input of terrestrially-derived dissolved organic matter (DOM; otherwise known as "humic substances") have been implicated as important factors in the initiation of coastal dinoflagellate blooms (e.g. *Gymnodinium breve*, Florida, U.S.A.; *Alexandrium tamarense*, Maine, U.S.A. and St. Lawrence Estuary, Canada), and as a general stimulant for marine phytoplankton production since the early 1970s (Ingle and Martin 1971, Kim and Martin 1974, Prakash 1975, Glover 1978, Therriault *et al.* 1985, Carlsson *et al.* 1993) .

Riverine input of DOM into coastal waters can affect phytoplankton dynamics in a number of ways. The presence of DOM (1) alters the underwater light climate in reducing photosynthetically active radiation (PAR), particularly at the blue end of the spectrum (Kirk 1994) and (2) provides some protection from UV-B radiation (Nielsen and Ekelund 1993). Such compounds are also efficient natural chelators which could (3) decrease the toxic concentrations of metals e.g. Cu (Anderson and Morel 1978) or (4) increase the availability of other metal ions required as nutrients, e.g. Fe, Mn (Sunda 1988). DOM may also (5) provide cells with an additional nitrogen supply (Granéli *et al.* 1985; Berg *et al.* 1997). There is limited evidence that some dissolved organic substances are also (6) available for direct uptake (or indirect uptake through the microbial loop) by phytoplankton (Carlsson and Granéli 1998) and may (7) affect bloom dynamics by inducing production of sexual dinoflagellate resting cysts (Heil 1996).

In south-east Tasmanian waters, blooms of the toxic, PSP-producing dinoflagellate, *G. catenatum*, are recurrent events (with high inter-annual variability in spatial extent and toxicity). Extensive blooms appear to be preceded by a rainfall "trigger" (Hallegraeff *et al.* 1995) with an associated influx of DOM from land runoff. Furthermore, growth and production of *G. catenatum* in culture is stimulated by addition of soil extract (Blackburn *et al.* 1989).

[1]Doblin, M. A., Blackburn, S. I. and Hallegraeff, G. M. (1998a) Interaction of dissolved organic substances with selenium and other nutrients: implications for growth of the toxic dinoflagellate *Gymnodinium catenatum*. *J. Exp. Mar. Biol. Ecol.* in press.

This chapter describes the variable effect of DOM on *G. catenatum* growth and biomass production, and discusses the potential nutritive role of these organic compounds, particularly with regard to selenium, previously identified as an important factor for the growth of some phytoplankton taxa (e.g. Harrison *et al.* 1988).

## 2.2 Materials and Methods

### *2.2.1 Culture conditions*

*G. catenatum* (strain GCDE08 in Blackburn *et al.* 1989) was obtained from the CSIRO Collection of Living Microalgae, Tasmania, Australia (culture code CS-301) and was originally isolated in June 1987 from the Derwent Estuary, Tasmania as an 8-celled chain (Blackburn *et al.* 1989). This strain has been maintained in seawater medium with GPM nutrients (Loeblich, 1975) with the addition of $10^{-8}$ M selenium (IV). *G. catenatum* cultures were grown in filtered (1.0 µm, 0.45 µm and charcoal activated filters to remove dissolved organics), teflon-autoclaved oceanic seawater (collected off Maria Island, south-east Tasmania), with salinity adjusted to 28 ppt using sterile Milli-Q® (MQ) water. Filter sterilised GPM nutrients (Loeblich 1975) were added at a concentration of 1/10 of the original recipe, with the addition of selenium as selenite ($10^{-9}$ M $H_2SeO_3$) and no added soil extract (Table 2.1). Cultures were grown in acid-washed, 50ml Pyrex® or KIMAX® tubes (loosely capped teflon-lined lids) at a temperature of 18 °C with bottom illumination at 150 µmol photons PAR $m^{-2}s^{-1}$ from Philips Deluxe cool white fluorescent lights on a 12:12 h L:D cycle. Culture media were prepared at least 24 h before being inoculated with exponential-phase *G. catenatum* cells to allow time for equilibration. Initial cell densities for experiments were 1 x $10^5$ cells $l^{-1}$ and experimental cultures were grown for at least two transfers (≥ 10 generations) to ensure differences between treatments were not the result of cells being in different stages of acclimation. Cultures were transfered approximately every 18 days to maintain cells in the exponential phase of growth, with the inoculum (1 ml) being diluted 1:30 (the minimum carry over for sustained growth of *G. catenatum* in culture; M. Doblin, unpublished data). Each treatment (Table 2.2) had a minimum of three replicates plus at least one control (medium containing no algae) and exponential growth rates and yield were the indices of algal response. A summary of experimental conditions is provided in Table 2.2.

Table 2.1: GSe/10 medium used in experiments (based on GPM medium, Loeblich 1975)

| Additive | Nutrient | Amount (g $l^{-1}$) | Concentration (μM) |
|---|---|---|---|
| Nitrate | $KNO_3$ | 0.020 | 200 |
| Phosphate | $K_2HPO_4$ | $3.50 \times 10^{-3}$ | 20 |
| Metals (pH adjusted to 7.5): | $EDTA(Na,H_2O)_2$ | 0.127 | 10 |
| | $H_3BO_3$ | 0.114 | 55 |
| | $FeCl_3.6H2O$ | $4.84 \times 10^{-3}$ | 0.5 |
| | $MnCl_2.4H_2O$ | $1.44 \times 10^{-2}$ | 2.0 |
| | $CoCl_2.6H_2O$ | $4.00 \times 10^{-4}$ | 0.053 |
| | $ZnCl_2$ | $1.04 \times 10^{-3}$ | 0.23 |
| Selenium | $H_2SeO_3$ | $0.129 \times 10^{-6}$ | 0.001 |
| Vitamins: | Vitamin $B_{12}$ | $0.100 \times 10^{-6}$ | 0.74 |
| | Thiamin.HCl | $0.100 \times 10^{-3}$ | 3.0 |
| | Biotin | $0.200 \times 10^{-6}$ | 0.0004 |

### *2.2.2 Growth measurements*

Growth rates of cultures were calculated from changes in *in vivo* fluorescence (Brand *et al.* 1981, Levasseur *et al.* 1993), measured every 2-3 days at the same time each day on unopened culture tubes:

$k\ (day^{-1}) = \ln (F_1/F_0) / (t_1 - t_0)$ (Levasseur *et al.* 1993)

Fluorescence readings ($F_1$, $F_0$) at each time period ($t_1$, $t_0$) were corrected for fluorescence of control tubes (containing no algae) at the same time. The fluorometer set up included a 10-045 daylight white lamp, 10-050 excitation filter (340 - 500 nm; colour specification 5-60), 10-051 emission filter (>685 nm; colour specification 2-64) and 10-052 reference filter (neutral density 400 - 700 nm; colour specification 3-66).

### *2.2.3 Yield estimates*

Cultures were sampled at the end of exponential phase (as soon as fluorescence stopped increasing exponentially) for cell density and chlorophyll *a* (chl *a*). Duplicate

Table 2.2: Experimental conditions for humic investigations

| Experiment type | Figures | Treatments | Medium Abbreviation | $NO_3$ (µM) | $PO_4$ (µM) | Standard Humic Acid (mg $l^{-1}$) | Huon River DOM (0.0006 - 0.2 µm) (ml $l^{-1}$) | EDTA (µM) |
|---|---|---|---|---|---|---|---|---|
| *Effect of standard humic acid on growth of* G. catenatum *in the presence of EDTA* | 2.1a, b | GSe/10 (28 ppt) | GSe/10[a] | 200 | 20 | 0 | 0 | 10 |
| | | GSe/10 + standard humic acid | SHA[b] | 200 | 20 | 0.33, 1.64, 3.23 | 0 | 10 |
| *Effect of organic additives on growth of* G. catenatum *in the absence of EDTA* | 2.1c, d | GSe/10 (28 ppt) | GSe/10 | 200 | 20 | 0 | 0 | 0 |
| | | GSe/10 + standard humic acid | SHA | 200 | 20 | 3.23 | 0 | 0 |
| | | GSe/10 + DOM (DOC = 1.24 mg C $l^{-1}$) | DOM[c] | 200 | 20 | 0 | 22 | 0 |
| *Comparison of limiting nutrient for* G. catenatum *biomass production in the presence and absence of dissolved organic matter* | 2.2a, b, c | GSe/10 (28 ppt) | GSe/10 | 200 | 20 | 0 | 0 | 0 |
| | | GSe/10 + standard humic acid | SHA | 200 | 20 | 3.23 | 0 | 0 |
| | | GSe/10 + DOM (DOC = 1.24 mg C $l^{-1}$) | DOM | 200 | 20 | 0 | 22 | 0 |

[a] Seawater medium (28 ppt) with nutrients added to yield GPM medium (Loeblich 1975) at a concentration of 1/10 of the original recipe, with the addition of selenium as selenite ($10^{-9}$ M $H_2SeO_3$) and no added soil extract

[b] Seawater medium (28 ppt) as for a, but with addition of standard Suwannee River (U.S.A.) humic acid (purchased from the International Humic Substances Society)

[c] Seawater medium (28 ppt) as for a, but with addition of natural DOM isolated from the Huon River, Tasmania by tangential flow filtration (Douglas *et al.* 1993)

1 ml samples were taken for cell counts and immediately fixed with acid Lugol's solution (0.2%; Lovegrove 1960). Samples were counted with a Sedgwick-Rafter counting chamber under bright field and when possible, cell density estimates were based on counting a minimum of 400 cells. In cultures with very low yields, estimates are based on counting 50-100 cells. Yields are defined as the final number of cells in the medium, without subtraction of initial cell density. Subsamples (20-30 ml) were also filtered through 25mm GF/F filters under dim light and filters stored at -20 °C for later chl *a* analysis. Pigments on filters were extracted with 100% acetone, and the mixture sonicated for 45 sec while on ice. Extracts were left at 0 °C in the dark for a minimum of 30 min, centrifuged (2000g for 5 min) and chl *a* in the supernatant determined spectrophotometrically, using the appropriate equations of Jeffrey and Humphrey (1975).

### *2.2.4 Isolation of dissolved organic substances*

Two types of dissolved organic substances were added to cultures: standard aquatic humic acid (SHA) isolated from the Suwannee River, U.S.A. using the method of Thurman and Malcolm (1981) and natural dissolved organic matter (DOM) isolated from the Huon Estuary, Tasmania using tangential flow ultrafiltration (Douglas *et al.* 1993). Previous studies have either used commercial extracts or DOM isolated from local waters using a variety of methods (eg. vacuum distillation, freeze-drying, ultrafiltration, reverse osmosis, ion exchange, and sorption onto nonionic macroporous resins - see Aiken 1985). In general, commercial compounds are significantly different to soil and aquatic humic substances (MacCarthy 1985), despite their obvious advantage in supplying a relatively constant source of material. Aiken (1985, 1988) recommends the use of macroporous resins (such as XAD-8) for isolation of aquatic DOM and this is the method adopted by the International Humic Substances Society (IHSS) for isolation of their standard and reference materials (see Thurman and Malcolm 1981). Ultrafiltration has been used to successfully isolate humic substances of different size fractions from water (eg. Milanovich *et al.* 1975), with the advantage that large volumes of water can be efficiently processed with relatively low risk of modification (although there are membrane-solute interactions - see Douglas *et al.* 1993). Since previous studies had found variable effects of humic acid depending on molecular weight (eg. Prakash and Rashid 1968), isolation of size fractions was considered important. I therefore used two types of HS in experiments: (i) IHSS standard humic acid, to ensure comparability with other studies; and (ii) size fractionated dissolved organic matter (DOM), for a local source of HS which underwent minimal modification during isolation.

Several options were considered for sterilisation of DOM fractions: (i) filtration; (ii) autoclaving or microwave treatment; (iii) gamma irradiation; (iv) addition of antibiotics or other chemicals such as sodium azide $NaN_3$; and (v) UV irradiation. The first three options are common techniques used to sterilise media or cultureware, however filtration was not possible because it would interfere with the size characteristics of coarse colloidal DOM fractions (> 0.2 μm). In addition, concerns over irreversible changes to organic compounds at high temperature and pressure precluded autoclaving or microwave treatment. It was thought that addition of antibiotics or chemicals may prove effective in eliminating unwanted bacteria and algae, but residual levels would also be harmful to *G. catenatum* and other phytoplankton species in culture. UV-irradiation was not appropriate because of the potential for photodegradation of DOM (Kieber *et al.* 1989), so gamma-irradiation was therefore considered the best option. Gamma irradiation (dosage 25 kgray) is commonly used for sterilisation of plastic and culture ware. However dissolved organic carbon (DOC) analysis of DOM fractions before and after gamma irradition provided some evidence for modification during sterilisation, with 5 out of 8 samples showing a 10-20% reduction in DOC concentration. Absorbance spectra also changed after sterilisation (Fig. 2.1), so subseqent experiments (described in the following chapters) were carried out using whole river water which had been filter-sterilised (0.2 μm).

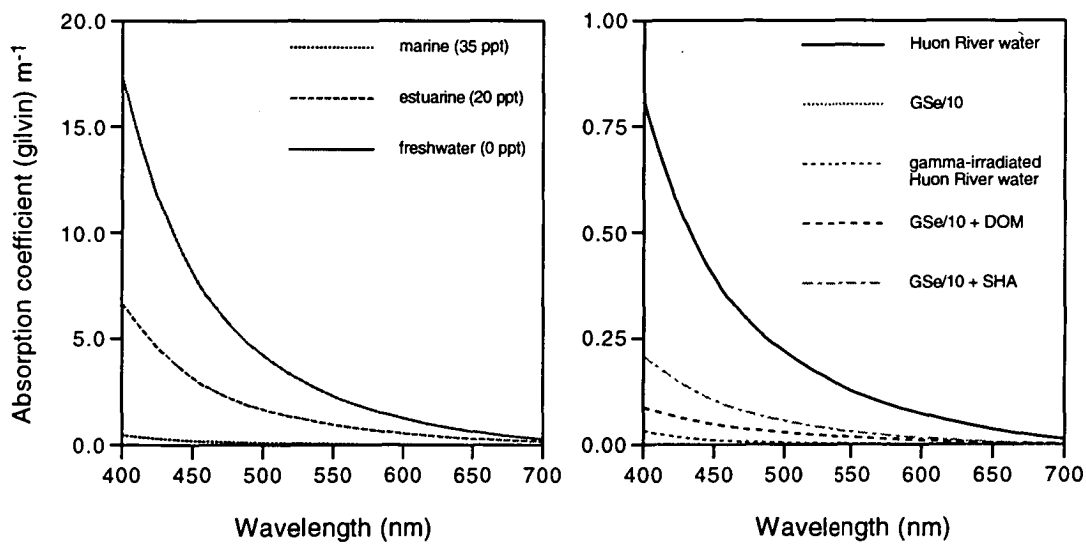


Fig. 2.1: (a) Absorption spectra of 0.2 μm filtered Huon Estuary water, showing a decrease in water colour (gilvin; Kirk 1994) from freshwater to marine endmembers; (b) Absorption spectra of culture media and Huon River water before and after gamma-irradiation.

### *2.2.5 Effect of standard humic acid on growth of* Gymnodinium catenatum *in the presence of EDTA*

Suwannee River Standard Humic Acid (SHA) prepared using standard methods (Thurman and Malcolm 1981) was obtained from the International Humic Substances Society and 0.22 µm filter sterilised before use. *G. catenatum* was grown in GSe/10 (including [EDTA+metals]; Table 2.1) with SHA at three different concentrations: 3.23, 1.64 and 0.33 mg $l^{-1}$ (equivalent to DOC concentrations of 1.24, 0.63 and 0.13 mg C $l^{-1}$; Table 2.3) for two transfers. To check whether bacterial densities increased with addition of organics, cultures were aseptically subsampled during exponential phase of the second transfer and plated out onto seawater agar (with added vitamins; Lewis *et al.* 1986). *G. catenatum* growth was monitored by *in vivo* fluorescence and at the end of the exponential phase during the second transfer, cultures were sampled for cell counts and chl *a*.

### *2.2.6 Effect of organic additives on growth of* Gymnodinium catenatum *in the absence of EDTA*

Two types of organic additives were tested for their effect on *G. catenatum* growth: SHA and natural dissolved organic matter (DOM; size range 0.006 - 0.2 µm), isolated from Huon River water by tangential flow ultrafiltration (Millipore-Pellicon Model ON141; Douglas *et al.*, 1993) in January 1996. DOM was sterilised by gamma-irradiation (25 kgray; Steritech Pty. Ltd., Victoria, Australia) before addition to cultures. SHA and DOM were analysed for total dissolved N and total dissolved P using a Technicon Autoanalyser and dissolved organic carbon was measured with a Shimadzu TOC-5000 carbon analyser. *G. catenatum* was grown in GSe/10 with SHA or DOM (DOC = 1.24 mg C $l^{-1}$) but without [EDTA+metals] for two transfers (approx. 40 d). The addition of SHA and DOM to cultures was equivalent to a final concentration of 237 and 246 µM $l^{-1}$ total dissolved nitrogen (TDN), respectively and 20.3 and 20.6 µM $l^{-1}$ total dissolved phosphorus (TDP), respectively (excluding the initial nitrogen and phosphorus content of the seawater; Table 2.3). *G. catenatum* growth was monitored by *in vivo* fluorescence and during the second exponential phase, estimates of bacterial abundance were made by aseptically subsampling cultures and plating out onto seawater agar (with added vitamins; Lewis *et al.* 1986). Cultures were sampled for cell counts and chl *a* at the end of the second exponential phase.

Table 2.3: Dissolved organic carbon (DOC), nitrogen (DON) and phosphorus (DOP) in standard humic acid (SHA) and natural dissolved organic matter (DOM) used in experiments. Total dissolved nitrogen (TDN) and phosphorus (TDP) = DON + DIN and DOP + DIP, respectively and % org. N and % org. P refer to the percentage of nitrogen and phosphorus SHA or DOM contribute to TDN and TDP, respectively.

| Organic additive | DOC (mg $l^{-1}$) | DON (μM $l^{-1}$) | DIN (μM $l^{-1}$) | TDN (μM $l^{-1}$) | % org. N | DOP (μM $l^{-1}$) | DIP (μM $l^{-1}$) | TDP (μM $l^{-1}$) | % org. P |
|---|---|---|---|---|---|---|---|---|---|
| SHA | 1.24 | 37.4 | 200 | 237.4 | 15.8 | 0.31 | 20 | 20.31 | 1.5 |
| | 0.63 | 18.7 | 200 | 218.7 | 8.6 | 0.15 | 20 | 20.15 | 0.7 |
| | 0.13 | 3.7 | 200 | 203.7 | 1.8 | 0.03 | 20 | 20.03 | 0.15 |
| | nominal | 0.0 | 200 | 200.0 | 0.0 | 0.0 | 20 | 20.00 | 0.0 |
| | ly 0.0 | | | | | | | | |
| DOM | 1.24 | ≤46.3 | 200 | 246.3 | ≤18.8 | ≤0.61 | 20 | 20.61 | ≤3.0 |

≤ is used for DOM to indicate that not all nitrogen or phosphorus species contained in this fraction (0.006 - 0.2 μm) are necessarily organic

### *2.2.7 Comparison of limiting nutrient for* Gymnodinium catenatum *biomass production in the presence and absence of dissolved organic matter*

*G. catenatum* was grown for two transfers in nutrient-enriched seawater (GSe/10; Table 2.1) with no SHA or DOM. Growth was monitored by fluorescence and after exponential phase during the second transfer, cultures were aseptically subsampled for cell counts and the remaining culture enriched with individual nutrients (nitrate, phosphate, vitamins, [EDTA+ metals] and selenium) at the same concentration as used in the beginning of the experiment (GSe/10), with Milli-Q water as the control. All treatments received the same volume (20 µl) of sterile-filtered nutrients, including Milli-Q. Cultures were monitored for an increase in biomass by fluorescence and once fluorescence started to decline, cultures were immediately sampled for cell numbers and chl *a*.

After growing in organically-enriched (SHA and DOM) GSe/10 with no [EDTA+metals] for two transfers, cells were transfered from cultures at the end of exponential phase during the second transfer to organically-enriched GSe/10 with all nutrients excluding [EDTA+metals] and nitrate (treatment -$NO_3^-$), or with all nutrients exluding [EDTA+metals] and phosphate (treatment - $PO_4^{3-}$), or all nutrients excluding [EDTA+metals] and selenium (treatment -Se), or all nutrients excluding [EDTA+metals] and vitamins (treatment -Vit), or all nutrients excluding [EDTA+metals] (treatment -Met). Medium replete in all nutrients (treatment ALL) served as the control. Growth was monitored by *in vivo* fluorescence and cultures were then sampled for cell counts and chl *a* at the end of exponential phase.

### *2.2.8 Statistical analysis*

Experiments were designed and analysed as random single-factor analyses-of-variance (ANOVA), ensuring sample independence. Before analysis, checks were made for normally distributed data and homogeneity of variances between treatments. Transformations (either logarithmic or square root) were performed where necessary and the analysis calculated using Systat 5.1 with a significance level of a = 0.05.

## 2.3 Results

### *2.3.1 Effect of standard humic acid on growth of* Gymnodinium catenatum *in the presence of EDTA*

In the presence of EDTA, growth of *G. catenatum* in the highest concentration of SHA (3.23 mg $l^{-1}$), decreased by approximately 25% (k = 0.19 $d^{-1}$) compared to growth in seawater with no SHA (k = 0.25 $d^{-1}$; Fig. 2.2a). In contrast, growth in lower concentrations of SHA (0.33 and 1.64 mg $l^{-1}$) was similar to that in seawater with no SHA (n = 3; F = 1.591; p = 0.243). Cultures containing no SHA achieved the highest number of cells (3.0 x $10^5$ cells $l^{-1}$), compared to SHA-enriched cultures where cell numbers were decreased by 30-90% (0.1 - 2.0 x $10^5$ cells $l^{-1}$), with the decline directly related to SHA concentration (Fig. 2.2b). Bacterial numbers in cultures were in the order of 2.0 - 4.0 x $10^7$ cells $l^{-1}$, with no differences in densities evident over all SHA concentrations (n = 2; F = 4.38; p = 0.06).

### *2.3.2 Effect of standard humic acid on growth of* Gymnodinium catenatum *in the absence of EDTA*

In the absence of [EDTA+metals], *G. catenatum* growth was greatest in seawater containing dissolved organic additives (k = 0.23 $day^{-1}$; Fig. 2.2c). In comparison, growth in the absence of chelators (either EDTA or SHA/DOM) decreased by approximately 15% (k = 0.19 $d^{-1}$). Cultures containing DOM had the highest biomass (120 µg chl *a* $l^{-1}$), 30% higher than seawater with SHA (75 mg chl *a* $l^{-1}$) and 60% higher than seawater with no organic additives (30 µg chl *a* $l^{-1}$; Fig. 2.2d). Bacterial numbers in cultures were in the order of 0.7 - 1.2 x $10^8$ cells $l^{-1}$ and there was no difference in densities with organic enrichment (n = 3; F = 0.79; p = 0.50).

### *2.3.3 Comparison of limiting nutrient for* Gymnodinium catenatum *biomass production in the presence and absence of dissolved organic matter*

Cultures enriched with selenium (treatment +Se) at the end of the second exponential phase in GSe/10 with no organic additives showed a slightly prolonged exponential phase, with dinoflagellate chains remaining intact for over 7 d; cultures also exhibited a slow decline in *in vivo* fluorescence. In contrast, cultures spiked with Milli-Q,

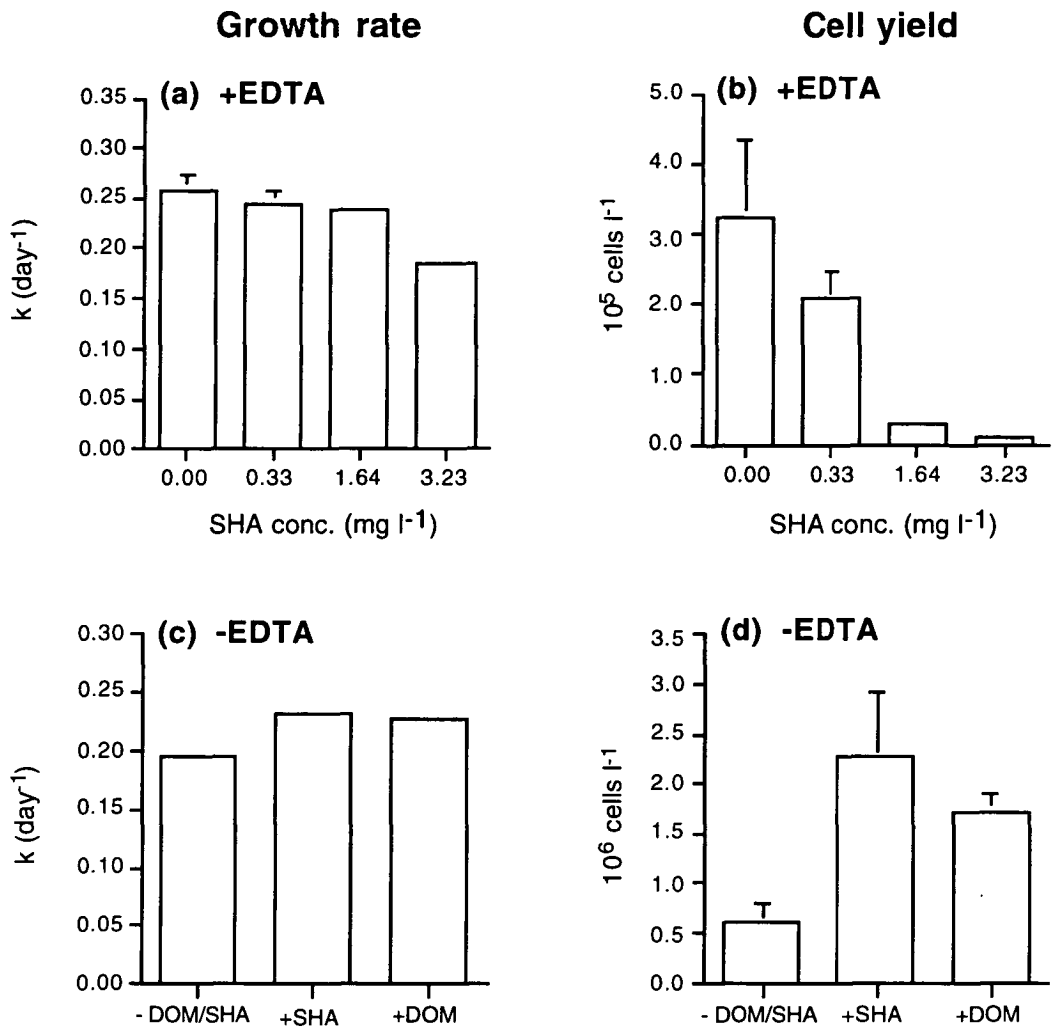


Fig. 2.2: (a) *Gymnodinium catenatum* growth rates and (b) cell yields after approx. 40 d. (≥ 10 generations) in enriched seawater medium (GSe/10; Table 2.1) with different concentrations of SHA (n = 4); (c) *G. catenatum* growth rates and (d) cell yields after approx. 40 d. (≥ 10 generations) in GSe/10 with 3.23 mg $l^{-1}$ SHA and 22 ml $l^{-1}$ DOM (DOC = 1.24 mg C $l^{-1}$) and no [EDTA+metals] (n = 3). All plots are drawn with error bars (SE), indicating low variability in exponential growth rates in (c).

nitrate, phosphate, vitamins or [EDTA+ metals] (treatments +MQ, +$NO_3^-$, +$PO_4^{3-}$, +Vit and +Met respectively) contained mostly single cells and had a faster decline in fluorescence. Selenium was the only nutrient which caused a substantial increase (approx. 1.0 x $10^6$ cells $l^{-1}$) in cell numbers after enrichment (Fig. 2.3a).

Following transfer of nutrient-deplete cells grown in GSe/10 + SHA or DOM at the end of the second exponential phase into GSe/10 + SHA or DOM deficient in individual

nutrients, biomass was greatest (75 - 120 µg chl *a* $l^{-1}$) when no [EDTA+metals] or all nutrients were supplied (treatments -Met and ALL; Figs. 2.3b and c). Lowest biomass (< 20 µg chl *a* $l^{-1}$) was noted in cultures deficient in nitrate and [EDTA+metals] (treatment -$NO_3^-$) and in cultures deficient in phosphate and [EDTA+metals] (treatment -$PO_4^{3-}$). In seawater containing SHA - [EDTA+metals], chl *a* biomass produced increased from cultures without nitrate, phosphate, selenium, vitamins, to a maximum when [EDTA+ metals] were absent or all nutrients were present: i.e. treatments - $NO_3^-$< - $PO_4^{3-}$ < -Se < -Vit < -Met = ALL. In seawater containing DOM - [EDTA+metals], biomass produced was also lowest in nitrate-deficient cultures, increasing from cultures without nitrate, phosphate, vitamins, selenium, to a maximum when [EDTA+metals] were absent or all nutrients were present: i.e. treatments - $NO_3^-$ < - $PO_4^{3-}$< -Vit < -Se < -Met = ALL.

## 2.4 Discussion

Many coastal dinoflagellate blooms develop after periods of rainfall and subsequent riverine input (Slobodkin 1953, Nümann 1957, Aldrich and Wilson 1960, Glover 1978, Therriault *et al.* 1985, Paerl *et al.* 1990, Hallegraeff *et al.* 1995, Paerl 1997). The inability to explain the incidence of some blooms purely in terms of altered hydrological conditions and the influx of inorganic macronutrients has led to formulation of the hypothesis that dissolved organic substances derived from land runoff contibute to bloom initiation (Prakash 1975, Granéli *et al.* 1989, Carlsson and Granéli 1993).

The results presented here are consistent with previous studies which have shown that organic additives can be stimulatory to marine dinoflagellates (Sweeney 1954, Prakash and Rashid 1968, Doig and Martin 1974, Granéli *et al.* 1985, Carlsson and Granéli 1993). Contrasting results in other studies have found negative or no effects of DOM on algal growth. For example, Devol *et al.* (1984) found that humic and fulvic acid (isolated from lake sediments) added to natural phytoplankton assemblages from Lago Jacaretinga, Central Amazon, Brazil did not change phytoplankton nutrient uptake kinetics or final chlorophyll yields. Addition of moss-peat material (leaching humic matter) to limnocorrals in Southern Indian Lake, northern Manitoba initially caused an increase in chlorophyll levels, but within 3-4 weeks of the addition, biomass was less than in the controls (Guildford *et al.* 1987).

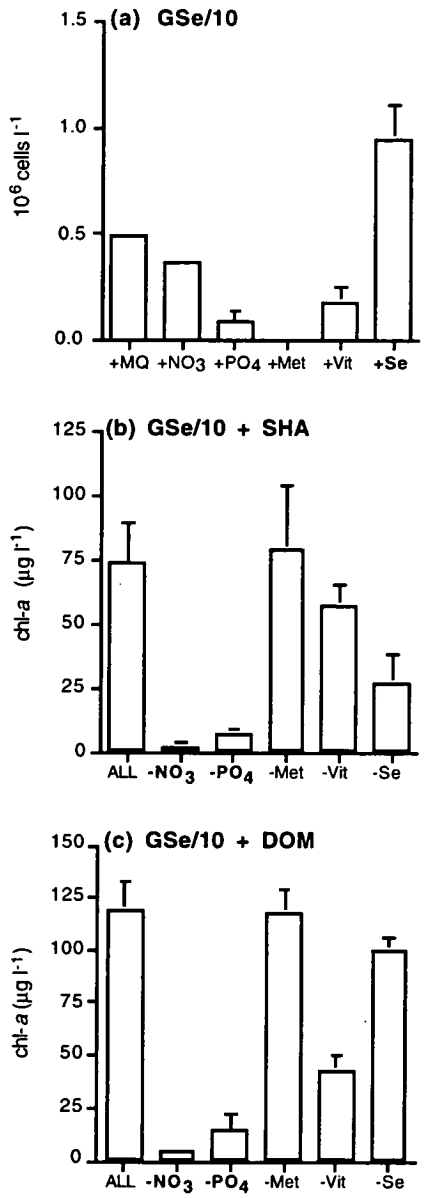


Fig. 2.3: (a) Increase in *Gymnodinium catenatum* cell abundance after enriching cultures (grown in GSe/10; Table 2.1), with no dissolved organic additives for approx. 40 d. (≥ 10 generations) with nutrients at the end of the exponential phase during the second transfer (n = 2); MQ = Milli-Q water added (control); $+NO_3$ = nitrate added, + $PO_4$ = phosphate added; +Vit = vitamins added; +Met = [EDTA+metals] added, +Se = selenium added; (b) *G. catenatum* chl *a* biomass yields after transfering cells at the end of exponential phase (grown for approx. 40 d in GSe/10 with 3.23 mg $l^{-1}$ SHA or (c) 22 ml $l^{-1}$ DOM (DOC = 1.24 mg C $l^{-1}$) and no [EDTA+metals]) into the same organically-enriched medium without certain nutrients: ALL = +DOM or SHA $+NO_3$ + $PO_4$ +Vit -[EDTA+metals] +Se; - $NO_3$ = +DOM or SHA $-NO_3$ + $PO_4$ +Vit -[EDTA+metals] +Se etc.; n = 3; error bars = SE.

The present study indicates that dissolved organic additives are only stimulatory to *G. catenatum* in the absence of other artificial chelators (compare Figs. 2.2a and b with Figs. 2.2c and d). When added to cultures with [EDTA+metals], standard humic acid causes a reduction in growth and biomass of *G. catenatum* which is concentration dependent. The most widely accepted mechanism for the stimulation of algal growth by DOM is its potential for altering the free ion concentration ("bioavailability") of trace metals. These compounds are natural ligands and have the ability to form metal-organic complexes of varying strength (see Mantoura *et al.* 1978), which may serve to enhance phytoplankton growth by increasing the availability of some metals (eg. Fe; Ingle and Martin 1971, Sunda, 1988) while concomitantly decreasing the availability (i.e. toxicity) of others (e.g. Cu; Anderson *et al.* 1978). Artificial ligands such as EDTA behave in the same way and at high concentrations have the potential to cause "over-chelation" due to the large reduction in free ion concentrations (Price *et al.* 1988). For example, Muggli and Harrison (1996) found that 100 µM EDTA inhibited cell division in two freshly isolated oceanic phytoplankton (the coccolithophorid *Emiliana huxleyi* and the diatom, *Actinocyclus* sp.) from the northeast Subarctic Pacific. High concentrations of inorganic or organic chelators or addition of both artificial and natural chelators (DOM), could therefore reduce free ion concentrations to limiting levels. Prakash *et al.* (1973) suggested that the decrease in growth of the diatom *Skeletonema costatum* when exposed to high concentrations (>28 mg $l^{-1}$) of organic extracts (mangrove leachates) was due to a reduction in metal concentrations. In addition, Guildford *et al.* (1987) suggested that the DOM leached from moss-peat material added to limnocorrals bound iron or some other metal which limited phytoplankton biomass production.

Other possible explanations for the observed decline in growth and production of *G. catenatum* in the presence of [EDTA+metals] and humic additives are light reduction, colloid formation (resulting in nutrient binding) or direct toxicity. The first and last alternatives are considered unlikely, given the relatively low concentration of DOM added to cultures (which despite causing an increase in colour (from 0.0 to 0.1 $m^{-1}$ gilvin at 440 nm; Fig. 2.3), would have a minimal effect on light absorption in culture tubes), and the absence of any obvious toxic effects (i.e. cessation of cell division, abnormal morphology) at the same concentrations in cultures without EDTA. There were no obvious signs of flocculation in the culture medium (this becomes visible when larger volumes of DOM are added to seawater), but colloids (<1 µm) may have formed at the DOM concentrations used, possibly affecting nutrient availability.

Transfer of nutrient-deplete cells grown in organically enriched seawater medium for two transfers at the end of exponential phase into the same medium deficient in

individual nutrients demonstrated that in the presence of DOM, selenium was no longer the limiting nutrient: cells were either nitrate or phosphate limited. The changed order of nutrient limitation in the presence of humic additives suggests that DOM changes overall nutrient availability and / or uptake by *G. catenatum*, either by addition of nutrients (e.g. N and P) to the system, or by interaction with other nutrients.

A significant proportion (30-50%) of DOM entering coastal systems is comprised of humic substances (ie. can be isolated by adsorption onto macroporous nonionic resins; Thurman and Malcolm 1981) and contains between 1-3% of nitrogen and approximately 0.2% of phosphorus (g:g; Thurman 1985). This pool of organic substances is relatively dynamic, with some high molecular weight compounds being rapidly cycled by bacteria (Amon and Benner 1994) and other compounds undergoing photochemical degradation into more labile low molecular weight compounds which are then directly utilised by plankton as a substrate (Kieber *et al.* 1989). Utilisation of DOM by bacteria and regeneration of inorganic nitrogen ($NH_4^+$) due to grazing activity on bacteria or on bacterial grazers (e.g. heterotrophic flagellates), may also lead to indirect uptake of DOM by phytoplankton (Carlsson *et al.* 1993, Carlsson and Granéli 1998). In the Huon Estuary, Tasmania, > 85% of the total dissolved nitrogen (TDN) is generally present as DON, and towards the end of summer when inorganic nitrogen levels are < 0.01 $\mu M\ l^{-1}$, this proportion increases to almost 100% (V. Latham, CSIRO Huon Estuary Study, unpublished data), suggesting that nitrogen bound to humic substances forms a significant pool for phytoplankton uptake. Berg *et al.* (1997) have recently demonstrated that DON uptake by the chrysophyte *Aureococcus anophagefferens* exceeds $NH_4^+$ uptake, and that growth rates were directly proportional to the organic:inorganic nutrient ratio in Long Island (U.S.A.) waters. The SHA and DOM fraction isolated from Huon River water used in experiments contained a considerable amount of dissolved nitrogen (Table 2.3), contributing 2 - 16% DON to the TDN pool (and up to 2% of the TDP pool) available for *G. catenatum* uptake in culture. Whether *G. catenatum* is mixotrophic and can utilise these compounds remains to be elucidated, but previous experiments have shown that low molecular weight nitrogenous compounds such as urea support *G. catenatum* growth (in the light) and that growth rates are similar when cells are supplied with nitrate or urea (M. Doblin, unpublished data). Uptake of such compounds in culture may have been facilitated by photo-degradation of DOM (as shown by the exponential decline in fluorescence in control tubes with no algae) or by the action of cell surface or extracellular peptidases associated with bacteria (Rosso and Azam 1987), which can hydrolyse peptides and provide algal cells with additional nitrogen (Carlsson and Granéli 1998). Stimulation of *G. catenatum* growth in the presence of organic matter

(DOM and SHA) may therefore have been partly due to increased N (and P) availability.

The present study found that bacterial densities (estimated during mid-exponential phase) were the same in all cultures, unlike other studies which have shown an increase in bacterial abundance with organic enrichment (e.g. Carlsson *et al.* 1993). This suggests that the DOM and SHA were relatively refractory (ie. had low nutritional value for bacteria), or that density estimates were made when the bacterial population had already utilised the available substrate. Observations of cultured *G. catenatum* cells and those in field samples show no evidence of intracellular bacteria after staining with the fluorochrome 4'6-diamidino-2-phenylindole (DAPI), and cell-surface bacteria are only abundant during cell senescence (M. Doblin, unpublished data). This suggests that the direct association of *G. catenatum* cells with bacteria is not important in this dinoflagellates nutrition. However axenic cultures of *G. catenatum* grow relatively slowly and are difficult to maintain because of their tendency to crash (C. Lovejoy, unpublished data), suggesting that an indirect association with bacteria is still important.

In seawater without HS, the element selenium limited growth rate and production of *G. catenatum*, at least for the Tasmanian strain used in this study. Selenium has been recognised as an important nutritional element for algal growth since Pinter and Provasali (1968) first demonstrated the stimulatory effects of selenium on the growth of three axenic marine *Chrysochromulina* spp. Numerous studies have shown that some phytoplankton species (eg. the dinoflagellate *Peridinium cinctum* fa. *westii*, Lindström and Rodhe 1978; the prasinophyte *Micromonas* sp., Keller *et al.* 1984; the prymnesiophyte *Chrysochromulina breviturrita*, Wehr and Brown 1985; and the green flagellate *Platymonas subcordiformis,* Wheeler *et al.* 1982; see Chapter 3 and Doblin *et al.* 1998b, submitted) have an absolute requirement for selenium or that its addition to culture medium considerably stimulates growth. Data to be presented in Chapter 4 show that the selenium requirement of *G. catenatum* differs between strains, with addition of selenium at concentrations from $10^{-9}$ - $10^{-7}$ M causing a variable increase in growth and biomass yields (Doblin *et al.* 1998c, submitted).

A substantial portion of the total selenium in aquatic systems exists in organic forms and suspended colloidal material is able to adsorb a considerable amount of both organic and inorganic selenium (Takayanagi and Wong 1985). Preliminary data show that most selenium present in the Huon Estuary, Tasmania is organic ("organo-selenide": Se II + Se 0 = total dissolved Se - Se IV - Se VI; Cutter 1989), with inorganic selenium (Se IV and Se VI) concentrations being < 0.01 nM $l^{-1}$ (G. Cutter,

personal communication). Organo-selenide concentrations are positively correlated with salinity, suggesting that the selenium is not associated with allochthonous DOM from land runoff. However, the interaction of DOM, colloids or particles with inorganic selenium at varying pH and salinity, and the competition of phosphate with selenium anions for adsorption onto particles (Barrow 1996) can have a major effect on the uptake of selenium by phytoplankton (e.g. Riedel and Sanders 1996). Riverine input of inorganic selenium and its interaction with DOM may therefore be important in the supply of this trace element for *G. catenatum* growth and biomass production, and may be a critical factor in bloom initiation during summer after rainfall events.

In conclusion, this study found that *G. catenatum* growth and biomass production was stimulated by addition of SHA and natural DOM in culture. Humic addition also caused a shift in nutrient availability and / or uptake so that the macronutrients nitrate and phosphate limited biomass production, in contrast to seawater without organic enrichment where the micronutrient selenium was limiting. Input of DOM into south-east Tasmanian coastal waters after rainfall may therefore play a critical role in *G. catenatum* bloom development.

# Chapter 3: Selenium requirement of *Gymnodinium catenatum* and two other bloom-forming phytoplankton: *Alexandrium minutum* (Dinophyta) and *Chaetoceros* cf. *tenuissimus* (Bacillariophyta)[1]

## 3.1 Introduction

Selenium (Se) is now widely recognised as an important nutritional element for microalgal growth. Pintner and Provasoli (1968) first demonstrated the stimulatory effects of Se on the growth of three axenic marine *Chrysochromulina* spp. (Prymnesiophytes), but since then, there have been numerous studies which have shown that some phytoplankton species (e.g. the dinoflagellate *Peridinium cinctum* fa. *westii*, Lindström and Rodhe 1978; the prymnesiophyte *Chrysochromulina breviturrita*, Wehr and Brown 1985; and the diatom *Thalassiosira pseudonana* , Price *et al.* 1987) have an absolute requirement for Se or that its addition to culture medium stimulates growth (Wheeler *et al.* 1982, Keller *et al.* 1984, Harrison *et al.* 1988).

Selenium is present in seawater in three different chemical forms: selenite ($SeO_3^{2-}$, Se IV), selenate ($SeO_4^{2-}$, Se VI) and organic selenides (eg. selenomethionine; Se -II). Particulate selenium can be found in any oxidation state (-II, 0, IV, VI), either as adsorbed/coprecipitated selenite and selenate, organic selenides or elemental selenium (Se 0) (Cutter 1985). Dissolved selenite appears to be the most bioavailable form of inorganic selenium (Wrench and Measures 1982), but selenate and organic selenides are generally the most abundant species in estuarine waters (e.g. Cooke and Bruland 1987, Cutter 1989a). Selenium sources in estuaries include riverine, atmospheric, sediment and point source (pollutant) inputs (e.g. oil refineries) (Cutter 1989b).

While Se is essential for some phytoplankton at low concentrations (0.1 - 100 nM), higher concentrations can be toxic (Price *et al.* 1987, Wheeler *et al.* 1982, Foe and Knight 1986, Boisson *et al.* 1995) and this depends on the chemical form of the element. Some studies have shown that selenate is more toxic than selenite (e.g. diatom *Thalassiosira pseudonana*, Price *et al.* 1987), while others have shown that for different species (e.g. prymnesiophyte *Cricosphaera elongata*, Boisson *et al.* 1995) the reverse is true.

[1]Doblin, M. A., Blackburn, S. I. and Hallegraeff, G. M. (1998b) Comparison of the selenium requirement of three species of phytoplankton: *Gymnodinium catenatum*, *Alexandrium minutum* and *Chaetoceros* cf. *tenuissimus*. *J. Plankton Res.*, submitted.

Studies on the uptake of Se into algal cells show that the element is incorporated into various biochemical components: amino acids and proteins, soluble carbohydrates, lipids, and polysaccharides (Bottino *et al.* 1984, Vandermeulen and Foda 1988), but the role of Se in cell function is not clear. Some evidence suggests that selenium is important in cell division processes and maintaining internal membrane integrity (Doucette *et al.* 1987), while it is also an essential part of the enzyme glutathione peroxidase, which protects cells against the destructive effects of hydrogen peroxide (Overbaugh and Fall 1982, Price and Harrison 1988).

In Australia, blooms of toxic dinoflagellates that produce paralytic shellfish toxins (PSTs), are frequent events in south-east Tasmanian waters (*Gymnodinium catenatum* Graham), and the Port Estuary, South Australia (*Alexandrium minutum* Halim). *G. catenatum* blooms occur within a seasonal temperature window (January to June) following a rainfall "trigger" and extended periods of calm weather (Hallegraeff *et al.* 1995). *A. minutum* blooms are also linked to heavy rainfall (and low salinity sewage effluent), causing resting cyst germination and bloom development in the upper Port Estuary (Cannon 1993). In addition, blooms of small diatoms such as *Chaetoceros* cf. *tenuissimus* Meunier are common in these estuarine and coastal waters, but are generally not coincident with dinoflagellate blooms (G. Hallegraeff, unpublished data). The incidence of toxic dinoflagellate blooms after rainfall and the potential riverine input of dissolved Se (e.g. Cutter 1989b) suggests that Se could play a role in phytoplankton species dominance and succession in southern Australian estuaries.

To gain a better understanding of the role of this trace element in phytoplankton bloom dynamics, the Se requirement of these bloom forming species was examined in culture. This study compares growth and biomass yields of *G. catenatum*, *A. minutum* and *C.* cf. *tenuissimus* in the absence of Se and with $10^{-9}$ M added selenite ($H_2SeO_3$). This is followed by a discussion of the importance of Se in coastal waters and the implications of species-specific Se requirements in bloom development.

## 3.2 Materials and Methods

### *3.2.1 Strain history*

Cultures were obtained from the CSIRO Collection of Living Microalgae, Hobart, Tasmania, where they are maintained in enriched seawater medium (salinity 28 ppt) with GPM nutrients (Loeblich, 1975), soil extract and $10^{-8}$ M Se (referred to as GSe medium in this laboratory). One strain of each species was used to determine the Se

requirement. Strain codes, isolation details and toxicity information are provided in Table 3.1. The diatom *C.* cf. *tenuissimus* Meunier sensu Rhines and Hargraves (1988) is synonymous with *C. galvestonensis*, *C. simplex* var. *calcitrans*, *C. calcitrans* and *C. calcitrans* forma *pumilus* (see Rhines and Hargraves 1988; Fig. 3.1).

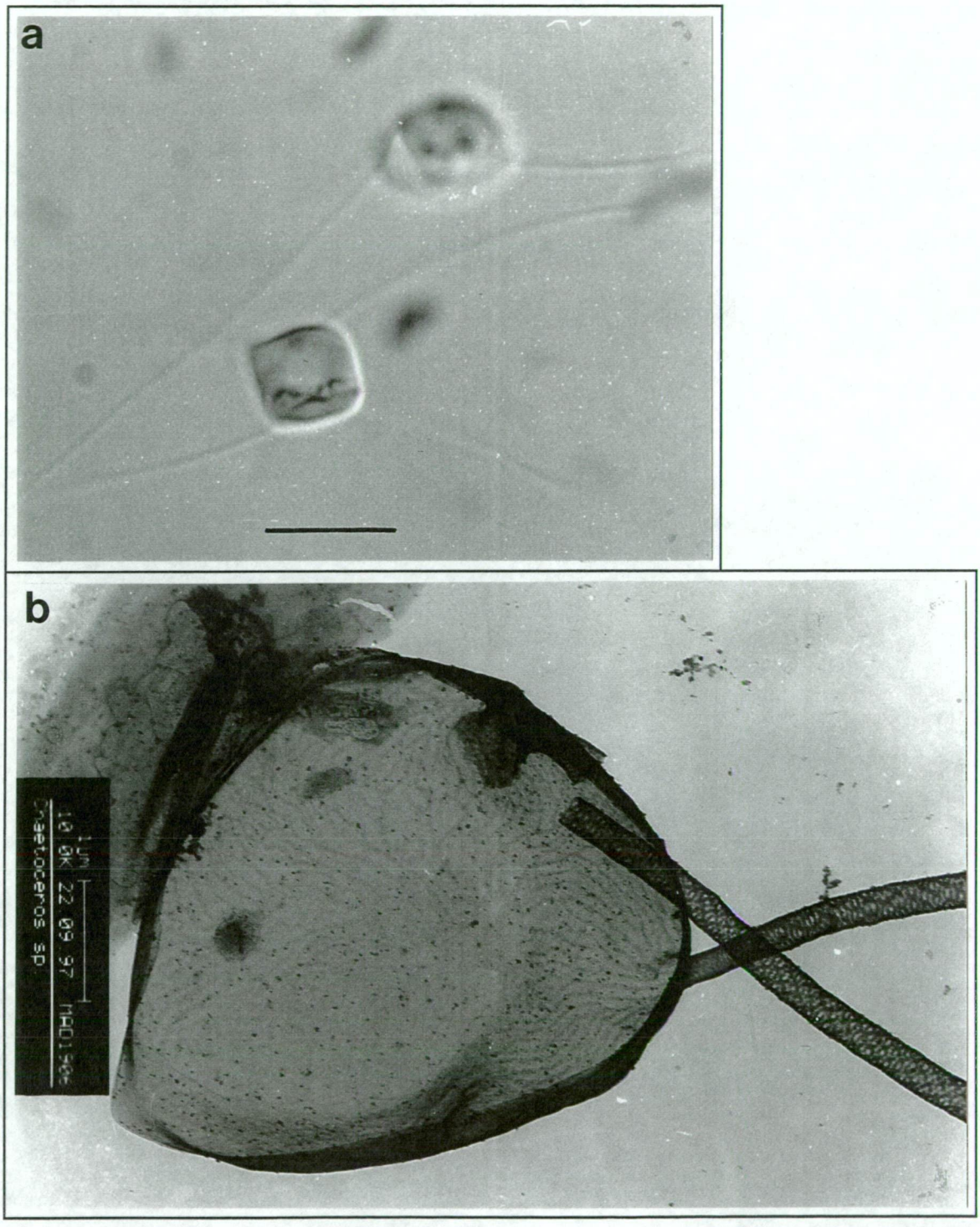


Fig. 3.1: (a) LM. The diatom *Chaetoceros* cf. *tenuissimus* in culture; Scale bar = 5 µm; (b) SEM. *C.* cf. *tenuissimus* showing structural detail of the setae; Scale bar = 1 µm.

Table 3.1: Characteristics of algal strains used in experiments

| Species | Strain code | Source | Date of isolation | Clonal | Toxic |
|---|---|---|---|---|---|
| *Gymnodinium catenatum* (GCDE08) | CS-301 | Derwent Estuary, Tasmania | 15 June 1987 | + | + (PSP) |
| *Alexandrium minutum* (AMAD06) | CS-323 | Port River, South Australia | 27 Oct. 1987 | + | + (PSP) |
| *Chaetoceros* cf. *tenuissimus* | CS-365/1 | St. Helens, Tasmania | 1989 | + | - |

### *3.2.2 Culture conditions*

Experimental cultures were grown in seawater (collected at an offshore station near Maria Island, south-east Tasmania), which was filtered through a series of glass-fibre, activated charcoal and 0.22 µm filters and autoclaved in teflon bottles. Salinity was adjusted to 28 ppt using sterile Milli-Q® (MQ) water. Nutrients were added to yield GPM medium (Loeblich 1975) at a concentration of 1/10 of the original recipe with the addition of Se as selenite ($10^{-8}$ M $H_2SeO_3$; Aldrich Chemical Company, Lot no. 1124KH) and no added soil extract (GSe/10; Table 2.1). Cultures were grown in acid-washed, 50ml Pyrex® or KIMAX® tubes (loosely capped teflon-lined lids) at a temperature of 18 °C with bottom illumination at 180 µmol photons PAR $m^{-2}s^{-1}$ (measured at the base of tubes using a Biospherical Instruments QSL-100 light sensor with integrating sphere) from Philips Deluxe cool white fluorescent lights on a 12:12 h L:D cycle. Cultures were not axenic but precautions were taken to minimise bacterial contamination by carefully timed transfers (late exponential phase) and minimal carry-over volumes. In addition, light microscope (Zeiss Axioplan) inspections were made on randomly selected exponential-phase cultures to check for bacterial colonisation.

Information about growth measurements, yield estimates and statistical analyses is provided in Chapter 2.2.2, Chapter 2.2.3 and Chapter 2.2.8, respectively.

### 3.2.3 *Growth of* G. catenatum *in the presence and absence of Se (IV)*

Experimental *G. catenatum* cultures were inoculated with GSe exponential-phase cells into GSe/10 (Table 2.1) containing no added Se (-Se) or GSe/10 with $10^{-9}$ Se (IV) added as $H_2SeO_3$ (+Se). The initial cell concentration in each culture was approximately 1 x $10^5$ cells $l^{-1}$. Cultures were transfered approximately every 18 days to maintain cells in the exponential phase of growth, with the inoculum (1 ml) being diluted 1:30 (the minimum carry over for sustained growth of *G. catenatum* in culture; M. Doblin, unpublished data). Growth was monitored by *in vivo* fluorescence and after exponential phase during the second transfer, cultures were sampled for cell density and chl *a*.

To test the recovery rate of cells after long-term selenium limitation, three replicates in each treatment (+Se, -Se) were transfered for a third time into Se-replete medium and another three replicates transfered to Se-deplete medium (yielding ++, +-, -+ and --Se treatments). Subsequent growth was monitored by fluorescence and cultures were sampled at the end of the third exponential phase for cell density and chl *a*.

### 3.2.4 *Recovery of* G. catenatum *after short-term Se limitation*

*G. catenatum* was grown for one transfer (approximately 3 weeks) in nutrient-enriched seawater (GSe/10; Table 2.1). At the end of the exponential phase, cultures were sampled for cell density and subsequently spiked with individual nutrients (nitrate, phosphate, vitamins, metals, selenium) at the same concentration used in the beginning of the experiment (GSe/10), with Milli-Q as the control. All treatments received the same volume of sterile-filtered nutrients. Cultures were monitored for an increase in biomass by fluorescence and once fluorescence started to decline, cultures were immediately sampled for cell numbers and chl *a*.

### 3.2.5 *Growth of* G. catenatum *in different Se (IV) concentrations*

To test the lower limit of requirement and level of tolerance of selenium in *G. catenatum*, cells were grown for two transfers (approximately 5 weeks) in nutrient-enriched seawater medium (GSe/10), with added $H_2SeO_3$ concentrations of $10^{-11}$, $10^{-9}$ and $10^{-7}$M. GSe/10 with no added Se formed the control. Growth was monitored by *in vivo* fluorescence and during the exponential phase of the second transfer, estimates of bacterial abundance were made by aseptically subsampling cultures and plating out onto seawater agar (with added vitamins; Lewis *et al.* 1986). Cultures were sampled for cell counts and chl *a* biomass at the end of the second exponential phase.

### *3.2.6 Se requirement of other phytoplankton species*

Two other phytoplankton species were tested for their Se requirement: another toxic (PSP-producing) dinoflagellate, *Alexandrium minutum* and a south-east Tasmanian, bloom-forming diatom, *Chaetoceros* cf. *tenuissimus*. Unialgal cultures of both species were maintained over 4-8 transfers (approximately 8 weeks) in GSe/10 containing no added Se (-Se) or GSe/10 with $10^{-9}$ M $H_2SeO_3$ (+Se). Dilution ratios between transfers were 1:100 for *A. minutum* and 1:300 for *C.* cf. *tenuissimus*. Differential growth rates between species resulted in an unequal number of transfers (*G. catenatum* = 3, *A. minutum* = 5, *C.* cf. *tenuissimus* = 8), but after a period of approximately 8 weeks, cultures were sampled for cell density and chl *a* at the end of exponential phase. Eight weeks was equivalent to approximately 12 generations for *G. catenatum*, >30 generations for *A. minutum* and >60 generations for *C.* cf. *tenuissimus*.

## 3.3 Results

### *3.3.1 Growth of* G. catenatum *in the presence and absence of Se (IV)*

Exponential growth rates of *G. catenatum* were identical in the absence of Se and with $10^{-9}$ M added Se during the first transfer (approximately 2.5 weeks). However, during the second transfer, cells in Se-deficient medium grew 35% slower than in Se-replete medium ($k = 0.17$ day$^{-1}$ compared to $k = 0.27$ day$^{-1}$; Fig. 3.2a and b; $n = 12$, $F = 168$, $p < 0.0001$). In addition, cell yields in Se-deficient cultures were 90% lower ($0.3 \times 10^5$ cells l$^{-1}$) compared to Se-replete cultures ($3.5 \times 10^5$ cells l$^{-1}$) (Fig. 3.2c; $n = 6$, $F = 44$, $p < 0.0001$).

Cells supplied with Se ($10^{-9}$ M) for two transfers and which were then, on the third transfer transfered into either Se-replete (++Se) or Se-deficient medium (+-Se) had the same growth rate ($k = 0.32$ day$^{-1}$; Fig. 3.3a and b; $n = 3$, $F = 0.13$, $p = 0.73$), but cell yields were considerably reduced in the +-Se treatments ($1.54 \times 10^5$ cells l$^{-1}$ compared to $1.42 \times 10^6$ cells l$^{-1}$; Fig. 3.3c; $n = 3$, $F = 14$ , $p = 0.01$). Cells which were not supplied with Se for two transfers and which were then transferred for a third time into Se-replete medium (-+Se) grew 25% more slowly than cells which were cultured in the presence of Se for all transfers ($k = 0.23$ day$^{-1}$; Fig. 3.3b; $n = 3$, $F = 73$, $p = 0.001$). Cells cultured in medium containing no Se for 3 transfers ceased to grow (Fig. 3.3a and b).

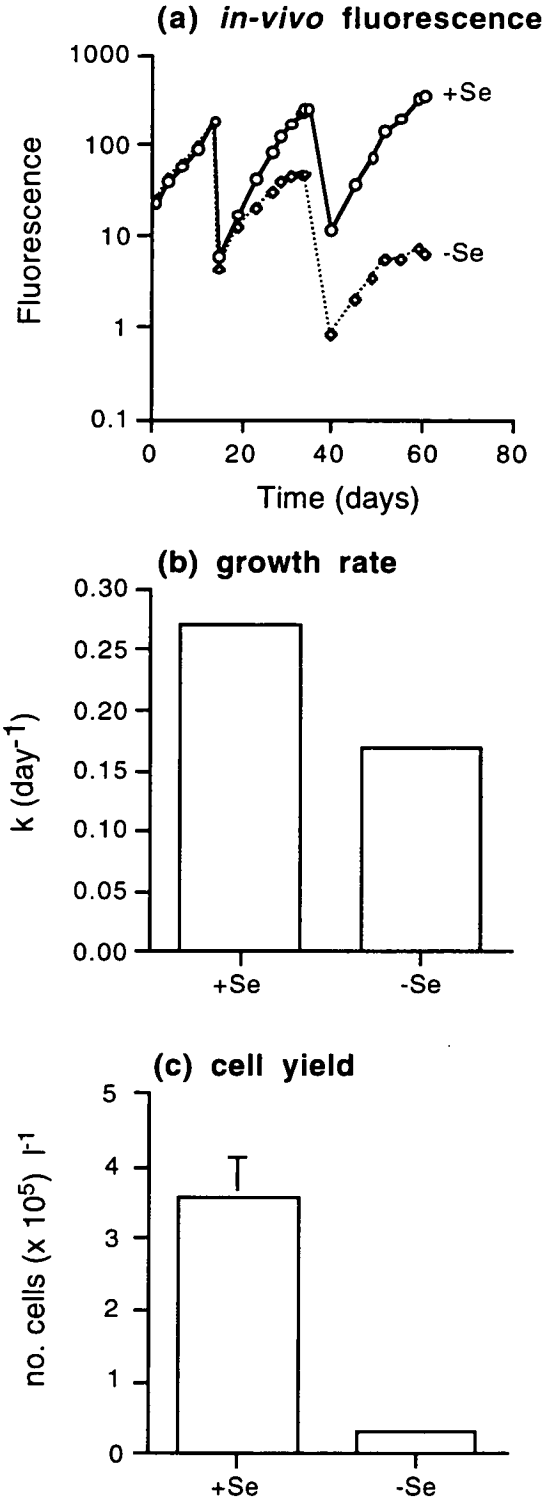


Fig. 3.2: (a) Growth of the dinoflagellate *Gymnodinium catenatum* determined by *in vivo* fluorescence over three transfers in enriched seawater medium (GSe/10; Table 2.1); (b) exponential growth rates and (c) cell yields of *G. catenatum* during the second transfer in enriched seawater medium (GSe/10); -Se = no added selenium; +Se = with $10^{-9}$ M $H_2SeO_3$; n = 12; error bars = SE.

There were no obvious morphological differences between *G. catenatum* cells grown in the presence or absence of Se observed at the light microcrope level, however there was a higher proportion of single cells and 2-4 celled chains in Se-deficient cultures compared with normal 8 celled chains in Se-replete medium (85% single cells compared to 57%; n = 3, F = 4.72, p = 0.10).

### *3.3.2 Recovery of* G. catenatum *after short-term selenium limitation*

Addition of $10^{-9}$ M $H_2SeO_3$ to Se-deplete stationary phase cultures caused a resumption in growth compared to other nutrients, shown by the substantial increase in cell density (approx. 1.0 x $10^6$ cells $l^{-1}$) in *G. catenatum* cells after spiking (Fig. 3.4a; n = 2, F = 3.05, p = 0.049). Addition of Milli-Q (control), nitrate, phosphate, metals and vitamins resulted in a much smaller increase in cell abundance, confirming that Se was the limiting nutrient (see Doblin *et al.*, in press). Cultures spiked with Se showed higher maximum fluorescence than the other treatments (Fig. 3.4b), and 2, 4 and 8-celled chains remained intact for ≥ 7 days. In contrast, cultures spiked with $NO_3^-$, $PO_4^{3-}$, vitamins or metals contained mostly single cells and had lower maximum fluorescence values after spiking. Cell counts and microscopic examination verified that the increase in *in vivo* fluorescence was due to an increase in cell abundance and not an increase in chlorophyll quota (although cultures spiked with metals showed no significant increase in cell density after spiking despite an increase in fluorescence; Fig. 3.4b).

### *3.3.3 Growth of* G. catenatum *in different Se (IV) concentrations*

During the second transfer into medium with no added Se and with $10^{-11}$ M Se *G. catenatum* cultures had the same exponential growth rate and biomass yields (Fig. 3.5), but at Se concentrations > $10^{-11}$ M, growth and biomass yields of *G. catenatum* were stimulated. Cells supplied with $10^{-9}$ M and $10^{-7}$ M Se grew 10-20% faster (k = 0.24 $day^{-1}$) than those supplied with 0 or $10^{-11}$ M added Se (k = 0.20 $day^{-1}$; Fig. 3.5a; n = 4; F = 28, p = 0.0003). Addition of $10^{-9}$ and $10^{-7}$ M Se caused a substantial increase in cell yields (0.9 x $10^6$ cells $l^{-1}$ and 2.0 x $10^6$ cells $l^{-1}$ respectively), compared to cultures yields (0.9 x $10^6$ cells $l^{-1}$ and 2.0 x $10^6$ cells $l^{-1}$ respectively), compared to cultures with no added Se (1.6 x $10^5$ cells $l^{-1}$ ; Fig. 3.5b; n = 4, F = 137, p < 0.0001). Chl *a* biomass in cultures with $10^{-9}$ and $10^{-7}$ M Se were also elevated (90 and 135 $\mu g \cdot l^{-1}$, respectively) compared to cultures with 0 and $10^{-11}$ M Se (approx. 15 $\mu g \cdot l^{-1}$; Fig. 3.5c; n = 4, F = 244 , p < 0.0001).

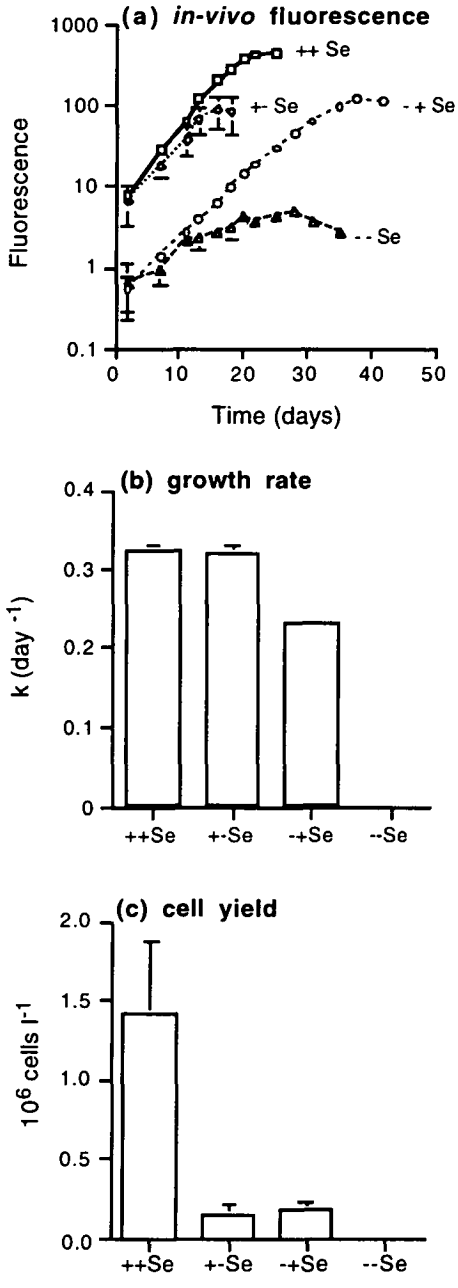


Fig. 3.3: (a) *Gymnodinium catenatum* growth curves determined by *in vivo* fluorescence, (b) exponential growth rates and (c) cell yields after two growth cycles in the presence or absence of Se and transfer of cells in late exponential phase into enriched seawater medium with no added selenium (-Se) or with $10^{-9}$ M $H_2SeO_3$ (+Se). ++Se/-- Se = cells grown for 3 consecutive transfers (approximately 8 weeks; equivalent to 12 generations) in +Se/-Se medium (respectively); +-Se = cells grown for two transfers in +Se medium and then transfered to -Se medium; -+Se = cells grown for two transfers in -Se medium and then transfered to +Se medium; n = 3; error bars = SE.

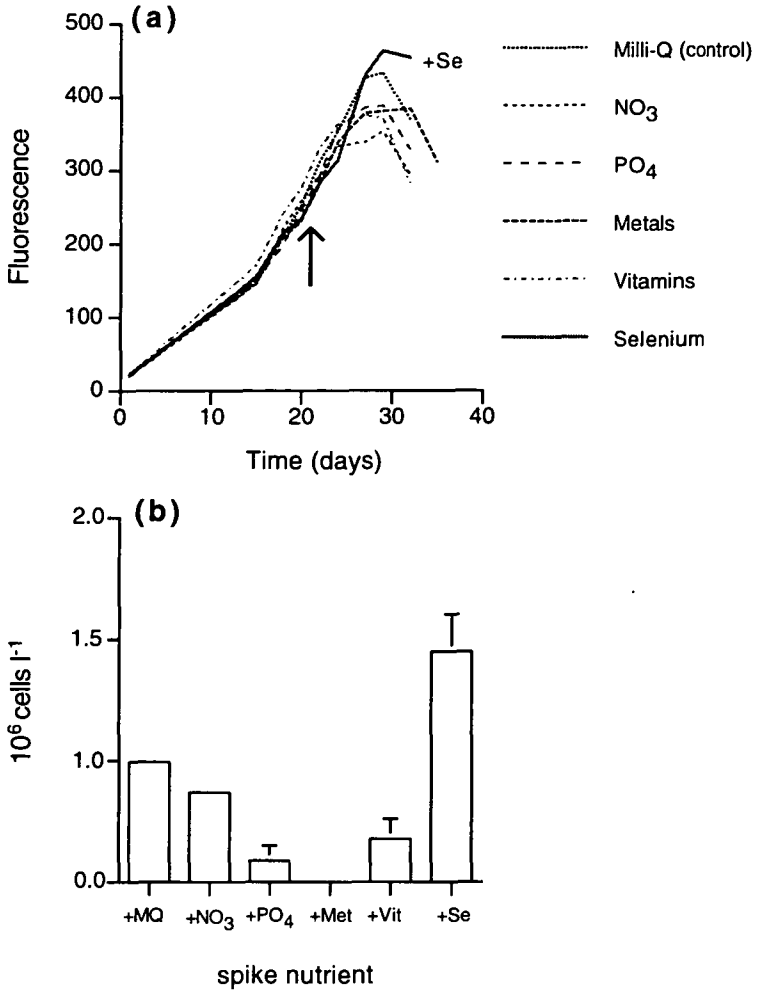


Fig. 3.4: (a) *In vivo* fluorescence and (b) increase in cell density of *G. catenatum* grown in enriched seawater medium (GSe/10) for approximately 3 weeks before enriching (arrow) with different individual nutrients at the end of exponential phase (day 20). +MQ = control, $+NO_3$ = nitrate, $+PO_4$ = phosphate, +Met = metals, +Vit = vitamins, +Se = $10^{-9}$ M selenium (other nutrients added at 1/10 GSe concentration, Table 2.1); n = 3; error bars = SE.

Bacterial abundance in cultures did not change as a result of Se addition (n = 3, F = 0.37, p = 0.71), and with the added precautions of inoculating and transfering cultures with exponential-phase cells using minimal culture volumes, we are confident that addition of Se caused a direct effect on algal growth and was not due to a bacterial interaction. Futhermore, microscopic examination of exponential-phase cultures verified that the low numbers of bacteria present were free-living and not associated with algal cells.

### *3.3.4 Se requirement of other phytoplankton species*

During the first, second and third transfers into media containing no or $10^{-9}$ M Se, exponential growth rates of *A. minutum* were the same (k = 0.65 $day^{-1}$; Fig 3.6a and b; n = 4, F = 0.20, p = 0.66). During the fourth and fifth transfers however, growth was 5-10% slower in cultures not supplied with Se (k = 0.61 $day^{-1}$ compared to 0.66 $day^{-1}$; n = 4, F = 8.0, p = 0.015 ). Cell yields were also lower in -Se cultures during the fourth and fifth transfer (Fig. 3.6c; 1.0 x $10^7$ cells $l^{-1}$ compared to 5.0 x $10^7$ cells $l^{-1}$; n = 4, F = 122, p <0.0001), as were chl *a* levels (Fig. 3.6d; 15-20 $\mu g \cdot l^{-1}$ compared to 110-130 $\mu g \cdot l^{-1}$; n = 4, F = 53, p <0.0001).

In contrast to both the dinoflagellates *A. minutum* and *G. catenatum*, growth rates and cell yields of the diatom *C.* cf. *tenuissimus* were the same over all 8 transfers (Fig. 3.7a, b, c and d). Furthermore, growth rates and biomass yields didn't decline when cells weren't supplied with Se (k = 1.4 - 1.6 $day^{-1}$; n = 4, F = 0.76, p = 0.40; cell density = 1.7 x $10^9$ cells $l^{-1}$, n = 4, F = 0.03, p = 0.87; chl *a* = 397 $\mu g \cdot l^{-1}$, n = 4, F = 0.01, p = 0.92).

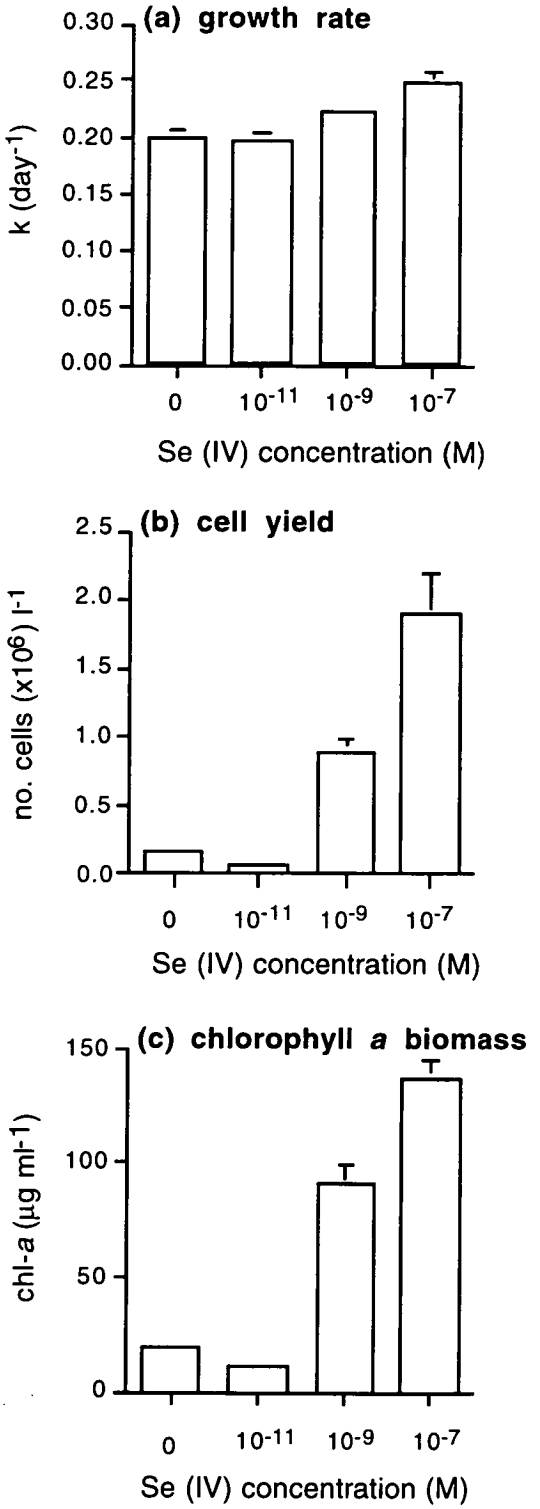


Fig. 3.5: (a) Exponential growth rates, (b) cell yields, and (c) chlorophyll *a* biomass of *Gymnodinium catenatum* after 2 transfers (approximately 5 weeks) in enriched seawater medium (GSe/10) with 0, $10^{-11}$, $10^{-9}$ and $10^{-7}$ M added Se (IV); n = 4, error bars = SE.

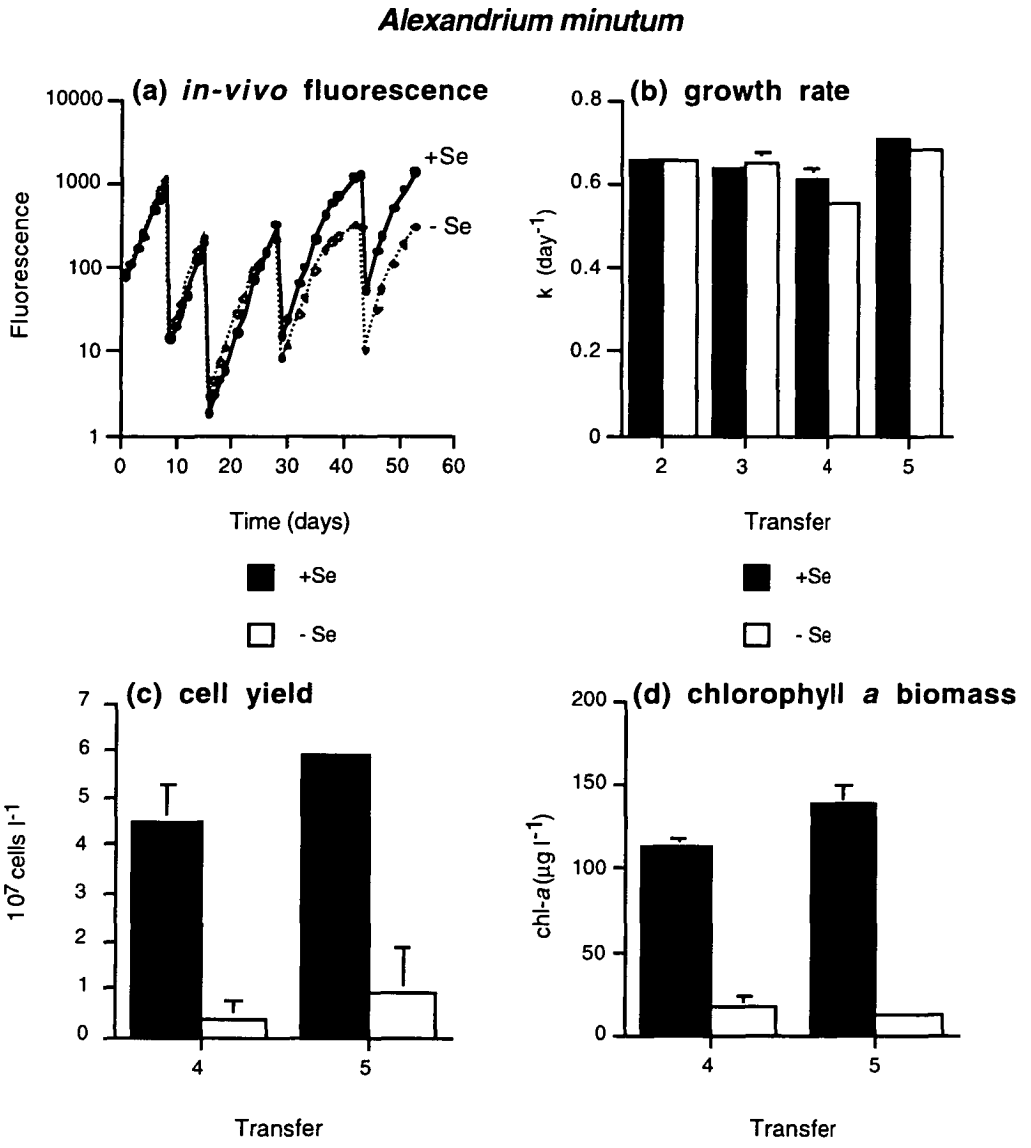


Fig. 3.6: (a) *In vivo* fluorescence, (b) exponential growth rates, (c) cell yields, and (d) chlorophyll *a* biomass of the dinoflagellate *Alexandrium minutum* during 5 transfers (approximately 8 weeks) in enriched seawater medium (Gse/10; Table 2.1) with no added selenium (-Se) and with $10^{-9}$ M $H_2SeO_3$ (+Se). Data for transfers 4 and 5 only are shown in c) and d); n = 4; error bars = SE.

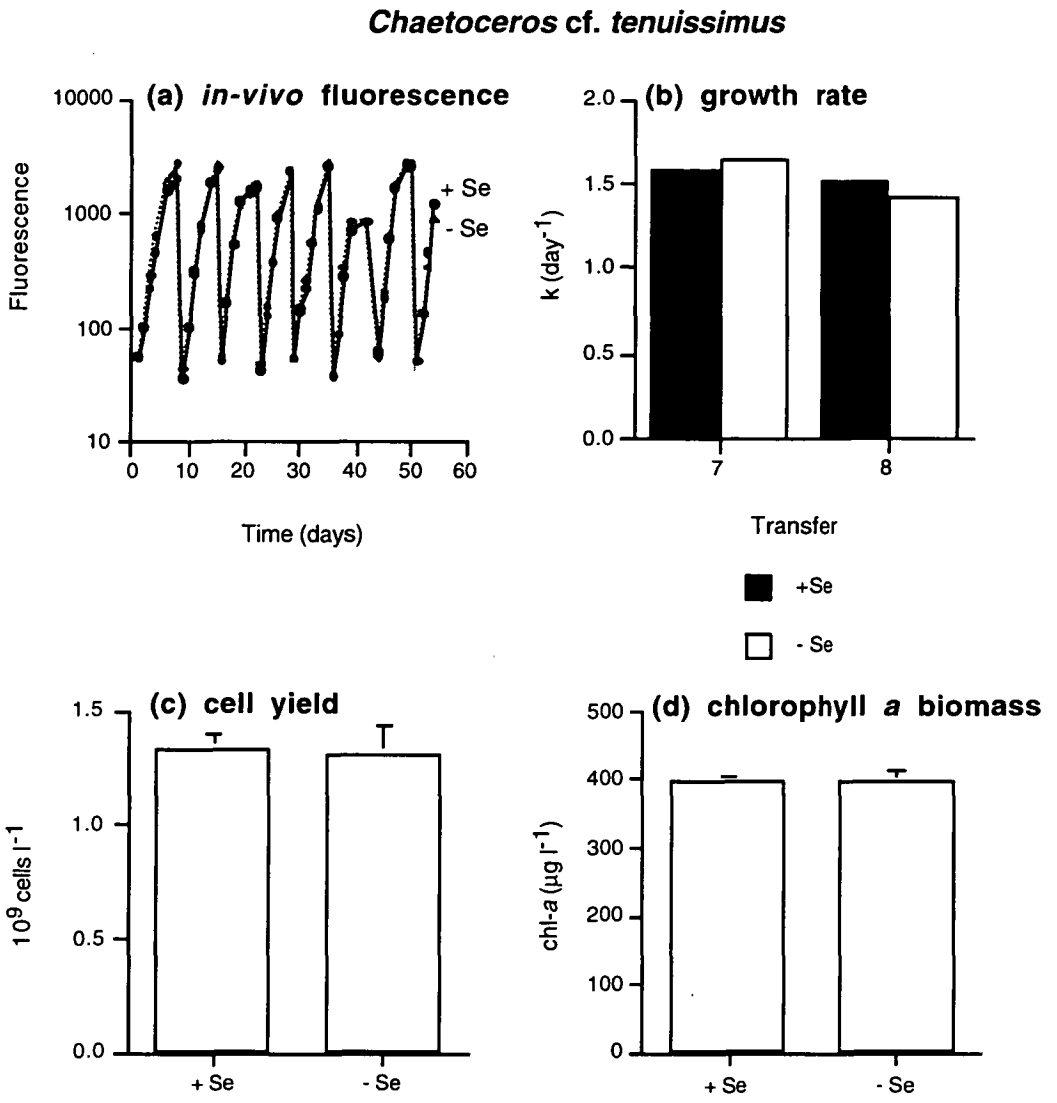


Fig. 3.7: (a) *In vivo* fluorescence, (b) exponential growth rates, (c) cell yields, and (d) chlorophyll *a* biomass of the diatom *Chaetoceros* cf. *tenuissimus* during 8 transfers (approximately 8 weeks) in enriched seawater medium (GSe/10) with no added selenium (-Se) and with $10^{-9}$ M $H_2SeO_3$ (+Se). Data for transfers 7 and 8 are shown in b), while data from transfer 8 only is shown in c) and d); n = 4; error bars = SE.

## 3.4 Discussion

### *3.4.1 Se requirement of* G. catenatum *and recovery from Se limitation*

The obligate requirement for selenium by *G. catenatum* was conclusively demonstrated by the reduction of exponential growth rates and biomass yields during the second

transfer into enriched seawater medium (GSe/10) with no added Se. Cessation of cell division in cultures which had not been supplied with Se for approximately 5 weeks (equivalent to 10 cell divisions) and the resumption of growth upon addition of $10^{-9}$ M $H_2SeO_3$ to Se-deplete stationary phase cultures, provide further evidence of a Se requirement.

Cells were able to recover from Se-starvation, dependent on the length of time exposed to Se-deficient conditions. Recovery from short-term Se limitation at the end of exponential phase was relatively rapid, in the order of a few days. When cells which had been cultured for two transfers in Se-deficient medium were resupplied with Se (-+Se; Fig. 3.2a), growth increased, but it was still slower than growth of cells which had received a continuous supply of selenium. Cells cultured without Se for $\geq 3$ transfers ($\geq 5$ weeks) ceased to grow, indicating that this is the length of time required to reach the minimum Se cell quota.

No obvious change in *G. catenatum* cell morphology could be observed at the light microscope level as a result of Se limitation, unlike other studies which have shown that Se deficiency causes morphological and ultrastructural changes to cells. For example, Price *et al.* (1987) found that Se-limited cells of *Thalassiosira pseudonana* had larger cell volumes, were elongated in the pervalvar axis and had a different mitochondrial, chloroplast and reticular membrane system configuration (Doucette *et al.* 1987). However, changes occurred in the growth habit of *G. catenatum* when grown under Se-deficient conditions, with an increasing (but not significantly different) proportion of single cells and shorter chains. The break up of chains is typical of *G. catenatum* at the end of exponential phase (Blackburn *et al.* 1989), however in this study, shorter chain lengths were observed even during early exponential phase in Se-deficient medium.

### *3.4.2 Growth of* G. catenatum *in different Se (IV) concentrations*

Growth and biomass yields of cultures with $10^{-11}$ M added Se were the same as those which had no added Se, indicating that background Se levels in the enriched seawater medium are of this order of magnitude. Price *et al.* (1987) estimate the background Se in ESAW medium (with nutrient solutions which have passed through a Chelex 100 column to eliminate cationic trace metal contaminants) to be $<10^{-12}$ M. In comparison, concentrations of Se from $10^{-9}$ - $10^{-7}$ M stimulated *G. catenatum* growth and biomass production (Fig. 3.4), with no indication of toxicity. Higher Se concentrations were not tested, mainly because they exceed natural levels in unpolluted estuarine and coastal waters (Takayanagi and Wong 1984, Cutter 1989b). While these experiments did not

determine the upper limit of Se tolerance, they clearly show that in seawater, low (nM) levels of Se are limiting to *G. catenatum* growth and biomass production.

### *3.4.3 Se requirement of other phytoplankton species*

Monitoring of cultures over consecutive transfers in Se-replete and Se-deficient medium showed that like *G. catenatum*, the dinoflagellate *A. minutum* also has a Se requirement. It took the same amount of time (approximately five weeks), but a greater number of generations (approximately 30) for *A. minutum* cells to show a reduction in exponential growth rates and biomass yields. In contrast, over the same time period (>60 generations), there was no change in growth or biomass yields in *C.* cf. *tenuissimus* cultures, indicating that this diatom species has no Se requirement, or that its Se requirement was met by the background Se levels in the culture medium. This adds to the findings of Harrison *et al.* (1988) who demonstrated the variable Se requirement within the *Chaetoceros* genus, with *C. debilis*, *C. pelagica* and *C. vixvisibilis* having a Se requirement and *Chaetoceros gracilis* and *C. simplex* not having one. The following Chapter (4) shows that intra-specific differences in Se requirements also exist, with some *G. catenatum* strains having a greater requirement for Se than others (Doblin *et al.* 1998c).

Addition of Se to cultures has been demonstrated to stimulate other toxic or harmful bloom forming species of phytoplankton. For example, Usup and Azanza (1998) have shown that Se (added in the form of selenite and organic selenide) increases cell yields of the dinoflagellate *Pyrodinium bahamense*. In addition, growth of the red tide raphidophyte flagellate, *Chattonella verruculosa*, is increased by addition of selenite at concentrations of 1.0 - 10 nM (Imai *et al.* 1996). There appears to be no consistency in the Se requirements of toxic versus non-toxic phytoplankton or those belonging to certain algal classes, but a review of the literature indicates that the number of species with demonstrated Se requirements is increasing (Table 3.2).

### *3.4.4 Role of Se in structuring phytoplankton populations*

Several field investigations have demonstrated that the distribution of Se is directly related to phytoplankton abundance and productivity. Wrench and Measures (1982) found that temporal changes in Se levels (relative proportion of Se VI and Se IV) were correlated with pulses of primary productivity in Bedford Basin, Nova Scotia, Canada, a fjord ecosystem. A similar pattern was observed by Lindström (1983) in Lake Erken, Sweden, where the vertical distribution of Se IV was correlated with the

Table 3.2: Phytoplankton species with demonstrated selenium requirement

| Species | No. transfers | Reference |
|---|---|---|
| **Diatoms** | | |
| *Amphipora hyalina* | 2 | Harrison *et al.*, 1988 |
| *Chaetoceros debilis* | 2 | Harrison *et al.*, 1988 |
| *Chaetoceros pelagica* | 1 | Harrison *et al.*, 1988 |
| *Chaetoceros vexvisibilis* | 1 | Harrison *et al.*, 1988 |
| *Coscinodiscus asteromphalus* | 2 | Harrison *et al.*, 1988 |
| *Corethron criophilum* | 2 | Harrison *et al.*, 1988 |
| *Ditylum brightwellii* | 1 | Harrison *et al.*, 1988 |
| *Skeletonema costatum* (strain 18c NEPCC) | 2 | Harrison *et al.*, 1988 |
| *Skeletonema costatum* (strain 611 NEPCC) | 2 | Harrison *et al.*, 1988 |
| *Skeletonema costatum* (strain 616 NEPCC) | 1 | Harrison *et al.*, 1988 |
| *Stephanodiscus hantzschii* var. *pusillus* ? | 1 | Lindström, 1983 |
| *Stephanopyxis palmeriana* | 3 | Harrison *et al.*, 1988 |
| *Thalassiosira pseudonana* | 2 | Price *et al.*, 1987; Harrison *et al.*, 1988 |
| *Thalassiosira oceanic* | 2 | Harrison *et al.*, 1988 |
| *Thalassiosira rotula* | 1 | Harrison *et al.*, 1988 |
| *Thalassiosira aestivalis* | 5 | Harrison *et al.*, 1988 |
| **Dinoflagellates** | | |
| *Alexandrium minutum*[h] | 4 | present work |
| *Gymnodinium catenatum*[h] | 2 | present work |
| *Gymnodinium nagasakiense*[h] | 1 | Ishimaru *et al.*, 1989 |
| *Katodinium rotundatum* ? | 3 | Harrison *et al.*, 1988 |
| *Peridinium cinctum* fa. *westii* | 1 | Lindström and Rodhe, 1978 |
| *Pyrodinium bahamense*[h] | 1 | Usup and Azanza, 1998 |
| **Prymnesiophytes** | | |
| *Chrysochromulina breviturrita* | 2 | Wehr and Brown, 1985 |
| *Chrysochromulina kappa* | ? | Pintner and Provasoli, 1968 |
| *Chrysochromulina brevefilum* | ? | Pintner and Provasoli, 1968 |
| *Chrysochromulina strobilis* | ? | Pintner and Provasoli, 1968 |
| *Chrysochromulina polylepsis*[h] | 1 | Dahl *et al.*, 1989; Edvardsen *et al.*, 1990 |
| **Raphidophytes** | | |
| *Chattonella verruculosa*[h] | 2 | Imai *et al.*, 1996 |
| **Chlorophytes** | | |
| *Platymonas subcordiformis* | 1 | Wheeler *et al.*, 1982 |
| **Chrysophytes** | | |
| *Aureococcus anophagefferens*[h] | 4 | Cosper *et al.*, 1993 |

[h] Harmful Algae

? uncertainty about requirement or number of transfers

abundance of the dinoflagellate *Woloszynskia ordinata*, showing a decline between 1-5 m coincident with the sub-surface bloom maximum. Ishimaru *et al.* (1989), in their investigations of the toxic dinoflagellate *Gymnodinium nagasakiense*, found that Se IV levels during the month preceding the 1988 bloom in Wakayama waters, Japan were low (< 0.1 nM) and greatest concentrations (2.8 nM) coincided with maximum cell density.

Inorganic selenium (Se IV and VI) concentrations in the Huon Estuary, south-east Tasmania are < 0.01 nM (below detection; G. Cutter, pers. commn.) in spring during low flow periods and are thus potentially limiting for the toxic dinoflagellates *G. catenatum* and *A. minutum*, (but not for the diatom *C.* cf. *tenuissimus*). Riverine input of Se during early summer runoff events, when water temperatures are elevated (>15 °C), may therefore be a critical trigger for dinoflagellate blooms in southern Australian estuaries. Preliminary data indicate that the major Se fraction in the Huon Estuary is "organic selenide" (Se II + 0) (operationally defined as the difference between total Se and Se VI + VI ; Cutter 1989b) and that organic selenide concentrations are positively correlated with phytoplankton biomass and salinity (G. Cutter, personal communication). The low concentration of inorganic Se and relatively high levels of organic selenide is typical of unpolluted estuaries and indicates rapid biological utilisation of inorganic Se, resulting in minimal accumulation of Se IV and VI (Lindström 1983). Uptake of organo-selenium compounds may occur if inorganic Se concentrations are limiting. Direct uptake of organic Se (-II, 0) has been demonstrated in the diatom *Stephanodiscus hantzschii* var. *pusillus* (seleno-methionine and seleno-cystine; Lindström 1983) and in the prymnesiophyte *Chrysochromulina breviturrita* (DL-seleno-methionine only; Wehr and Brown 1985). The interaction of humics, colloids or particles with Se at varying pH and salinity, and the competition of phosphate with Se anions for adsorption onto particles (Barrow 1996) can also have a major affect on the uptake of Se by phytoplankton (e.g. Riedel and Sanders 1996; Chapter 2).

This Chapter demonstrates that the toxic dinoflagellates *G. catenatum* and *A. minutum* have a requirement for Se, while the small diatom *C.* cf. *tenuissimus* does not. Very low (< 0.01 nM) concentrations of Se found in the Huon Estuary suggest that this trace element could be limiting for growth of some phytoplankton (e.g. *G. catenatum* and *A. minutum* ) and that riverine input of Se after rainfall may be a critical trigger for *G. catenatum* blooms in this region. In comparison, blooms of the diatom *C.* cf. *tenuissimus*, with no Se requirement, have no such pre-requisite for river runoff (Se input) but their incidence is limited by other factors such as turbulence and silicate (Margalef 1978; see further discussion in Chapter 7). Humic substances, while stimulating growth and biomass production of *G. catenatum* (see Chapter 2), may also play an important role in *G. catenatum* bloom initiation by regulating the supply of bioavailable Se.

# Chapter 4: Intraspecific variation in the selenium requirement of different geographic strains of the toxic dinoflagellate *Gymnodinium catenatum*[1]

## 4.1 Introduction

Blooms of the toxic, paralytic shellfish poisoning (PSP) chainforming dinoflagellate *Gymnodinium catenatum* Graham pose a serious risk to human health, aquaculture developments and the coastal environment. During the last twenty years, *G. catenatum* has been found in an increasing number of locations worldwide, with vegetative cells being identified in both temperate and tropical waters: e.g. north-west Spain, Mexico, Japan, Portugal, Venezuela, Thailand, Phillipines, Palau, Uruguay, Morocco, Malasia and south-east Tasmania, Australia (Table 1.1; see Hallegraeff and Fraga 1998 for review).

Several hypotheses have been put forward to explain *G. catenatum* bloom development. Bloom formation in the Huon Estuary, Tasmania appears to be autochthonous and linked to the incidence of freshwater input after rainfall (contributing organic and inorganic growth factors) and is associated with extended periods of low wind-stress (Hallegraeff *et al.* 1995). In contrast to blooms in Tasmanian waters, *G. catenatum* outbreaks in the Spanish Rias may be the result of either: (i) shoreward transport of allochthonous oceanic seed populations into the Rias (Fraga *et al.* 1988); or (ii) germination of resting cysts from Rias sediments (Figueiras and Pazos 1991, Blanco 1995). In addition, Spanish blooms are associated with upwelling relaxation which results in a deepening of the nutricline and selection for strongly migratory species such as *G. catenatum* (Fraga and Bakun 1993). These bloom formation mechanisms involve changes in environmental conditions (e.g. before and after rainfall, transition from sediment to water column or offshore to inshore waters), so that cysts or motile vegetative cells are subject to different salinity, temperature, nutrient and turbulence regimes. Phenotypic and genetic diversity in nutritional and physiological requirements within and between populations may therefore comprise a successful ecological strategy for *G. catenatum*.

Micro-nutrients such as iron (Fe), copper (Cu) and cobalt (Co) have been implicated in the incidence of harmful algal blooms of other species (e.g. Fe: dinoflagellate

[1]Doblin, M. A., Blackburn, S. I. and Hallegraeff, G. M. (1998c) Intraspecific variation in the selenium requirement of different geographic strains of the toxic dinoflagellate *Gymnodinium catenatum*. *J. Phycol.*, submitted.

*Gymnodinium breve*, southern Florida (Ingle and Martin 1971); Cu: dinoflagellate *Alexandrium tamarense*, Maine, U.S.A. (Anderson and Morel 1978); Co: prymnesiophyte *Chrysochromulina polylepis*, Kattegat, south-east Sweden (Granéli and Risinger 1994)). The trace element selenium has also been identified as potentially important in *Gymnodinium nagasakiense* dinoflagellate blooms in Japanese waters off Wakayama, where in 1988 a good correlation between selenium concentrations and cell density was reported (see Boyer and Brand 1998). Furthermore, addition of selenium to Temma Bay water stimulated growth of *G. nagasakiense* test cultures (Ishimaru *et al.* 1989). Other harmful algae with a selenium requirement, or those which have shown an increase in growth or biomass yields upon addition of nanomolar concentrations of selenium include the raphidophyte *Chattonella verruculosa* (Imai 1996), dinoflagellate *Pyrodinium bahamense* (Usup and Azanza 1998) and several *Chrysochromulina* prymnesiophyte species (Pintner and Provasoli 1968, Wehr and Brown 1985). Data presented in Chapter 3 demonstrate that *G. catenatum* (strain GCDE08) also has a selenium requirement, with addition of selenite (Se IV) at concentrations from $10^{-9}$ - $10^{-7}$ M causing an increase in both growth and biomass yields (Doblin *et al.* submitted).

This chapter reports on the variability in selenium (IV) requirement in five strains of *G. catenatum*, representing different regional and globally distributed populations. Comparisons were made between strains collected from the same location (Derwent Estuary, Tasmania) in different years (1988 and 1993) and between strains from adjacent (Derwent and Huon Estuaries, Tasmania) and widely separated areas (Tasmania, Japan and Spain).

## 4.2 Materials and Methods

### *4.2.1 Strain history*

The strains used in this study were all provided by the CSIRO Collection of Living Microalgae (Australia). *G. catenatum* strains were isolated from two locations in Tasmania, Australia: the Derwent Estuary in 1987 and 1993 (GCDE08 and GCDE9305 respectively), and the Huon Estuary in 1986 (GCHU02). Other strains were obtained from Ria de Vigo, Spain in 1985 (GCSP01) and Senzaki Bay, Japan in 1986 (GCJP10). Further information about these strains is provided in Table 4.1.

Information about culture conditions, growth measurements and yield estimates is provided in Chapter 3.2.2, Chapter 2.2.2. and Chapter 2.2.3, respectively.

Table 4.1: Isolation history of *Gymnodinium catenatum* strains used in this study; ND = not determined

| Strain | CS-code | Source | Date of isolation | Isolator | Clonal | Toxic |
|---|---|---|---|---|---|---|
| GCDE08 | CS-301 | Derwent Estuary, Tasmania | 15 June 1987 | S. Blackburn | + (8 celled chain) | + |
| GCDE9305 | no CS # | Derwent Estuary, Tasmania | 13 May 1993 | H. Ling | + | ND |
| GCHU02 | CS-302 | Huon Estuary, Tasmania | 6 June 1986 | S. Blackburn | + (8 celled chain) | + |
| GCJP10 | CS-305 | Senzaki, Japan | 1986 | T. Ikeda | unknown | + |
| GCSP01 | CS-306 | Ria de Vigo, Spain | 5 Nov. 1985 | I. Bravo | - 4 chains of 16-30 cells | + |

### *4.2.2* G. catenatum *growth and biomass production without and with $10^{-9}$ M added selenium*

*G. catenatum* strains were inoculated from GSe medium into GSe/10 medium containing no added selenium (-Se) or GSe/10 with $10^{-9}$ M selenium (IV) as $H_2SeO_3$ (+Se). To ensure that differences in growth rates and biomass yields were not the result of different stages of acclimation (see Brand *et al.* 1981), experimental cultures were transfered three times (>8 generations), with the inoculum (1 ml) being diluted 1:30 (the minimum carry over for sustained growth of *G. catenatum* in culture tubes). I am therefore confident that the observed phenotypic differences in selenium requirement between strains are due to genotypic variation. The initial cell concentration in each culture was $10^5$ cells $l^{-1}$. Cultures were not axenic but precautions were taken to minimise bacterial levels by carefully timed transfers (late exponential phase). Growth of cultures was monitored by *in vivo* fluorescence, and at the end of exponential phase during the third transfer, cultures were subsampled for cell counts and chl *a*.

### *4.2.3 Statistical analyses*

The experiment was designed and analysed as a random single-factor analyses-of-variance (ANOVA). Before analysis, checks were made for normally distributed data and homogeneity of variances between treatments. Transformations (logarithmic) were performed where necessary and the analysis calculated using Systat 5.1 at a significance level of a = 0.05. Comparisons were performed to test for differences

between strains isolated from (i) the same location in different years (Tasmania Derwent Estuary 1987 and 1993); (ii) locations in close proximity (Tasmania Huon and Derwent Estuaries); and (iii) widely separate locations (Tasmania, Japan and Spain).

## 4.3 Results

### *4.3.1* G. catenatum *growth and biomass production without and with $10^{-9}$ M added selenium*

Despite similar cell morphology, strains differed from one another in their growth habit (Fig. 4.1) and rate of exponential growth (Fig. 4.2a). The Japanese strain (GCJP10) typically formed long chains of ≥ 16 cells, while the Huon strain (GCHU02) formed mostly single cells. The remaining strains (GCDE08, GCDE9305 and GCSP01), formed chains of medium length (4-8 cells). Long term observations on these clones in culture confirm that the observed differences in chain length are consistent between transfers and are not related to nutrient deficiency, even though chains generally shorten during stationary phase (Blackburn *et al.* 1989).

Growth rates of different *G. catenatum* strains were variable, ranging from 0.19 - 0.34 $day^{-1}$ (Fig. 4.2a). In Se-replete medium, the Spanish (GCSP01) and Japanese (GCJP10) strains exhibited the lowest growth rates (k = 0.24 $day^{-1}$). Of the Tasmanian strains, the Huon strain (GCHU02) grew more slowly (k = 0.28 $day^{-1}$) than the two Derwent strains, which had comparable growth rates (k = 0.34 $day^{-1}$). In Se-deficient medium, growth of strains GCDE08 and GCJP10 decreased by 10% (from 0.34 to 0.31 $day^{-1}$) and 20% (from 0.24 - 0.19 $day^{-1}$), respectively, while there was no difference in growth of other strains (GCDE9305, GCHU02 and GCSP01).

Strain GCDE08 and GCSP01 showed the highest cell yields when grown in Se-replete medium (approx. 6.0 x $10^5$ cells $l^{-1}$; Fig. 4.2b). Cell yields of GCJP10 and GCHU02 were slightly lower (4.09 x $10^5$ cells $l^{-1}$ and 4.65 x $10^5$ cells $l^{-1}$, respectively), while those of strain GCDE9305 were lowest (1.16 x $10^5$ cells $l^{-1}$). Chl *a* biomass showed a similar pattern (Fig. 4.2c), with highest levels found in GCDE08 (74 μg $ml^{-1}$), intermediate levels in GCSP01, GCJP10 and GCHU02 (22- 25 μg·$ml^{-1}$) and lowest levels in GCDE9305 (11 μg $ml^{-1}$).

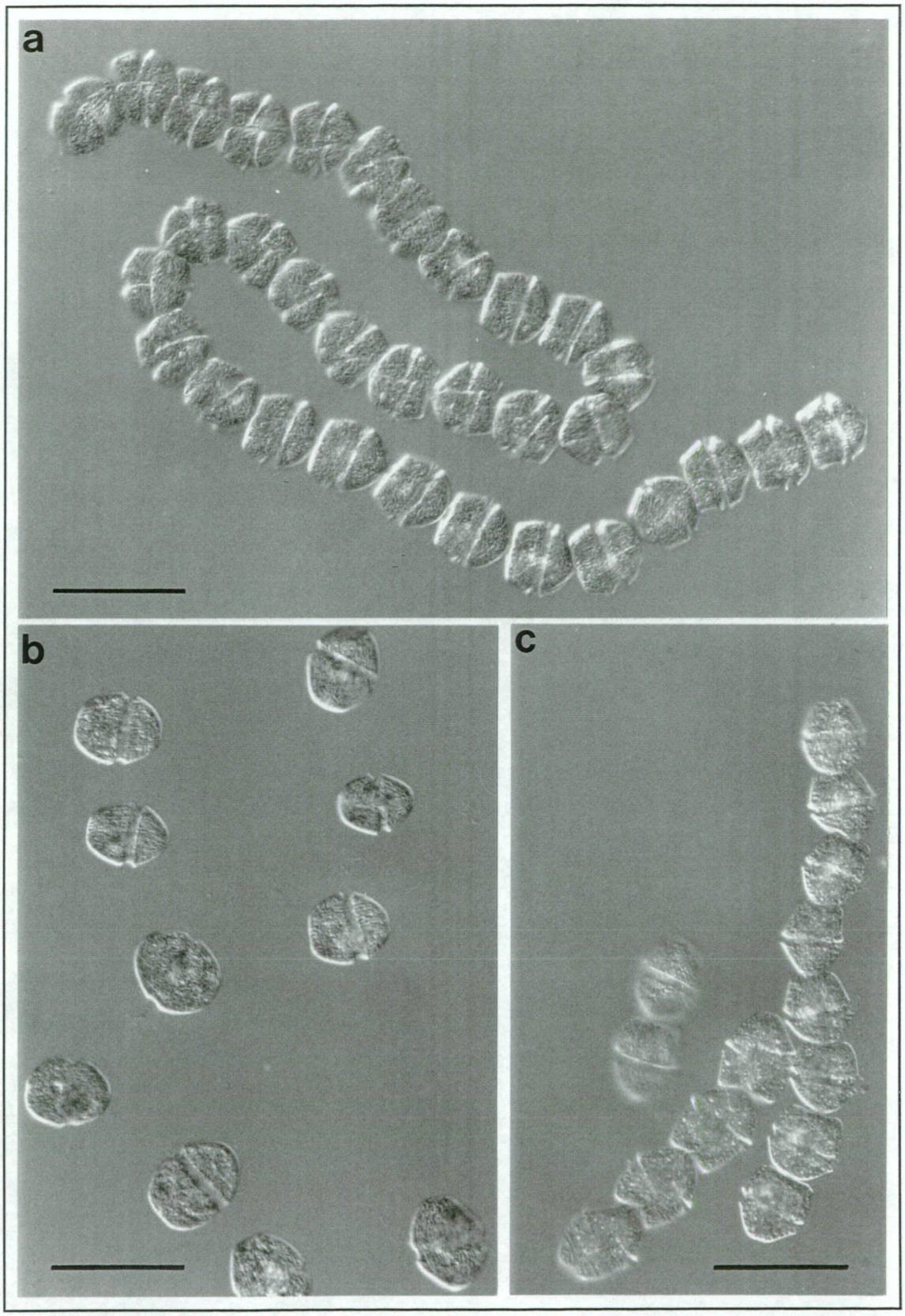


Fig. 4.1: LM. Vegetative chains and solitary cells of the toxic dinoflagellate *Gymnodinium catenatum* in culture: (a) strain GCJP10; (b) strain GCHU11; (c) strain GCDE08; scale bars = 50 μm.

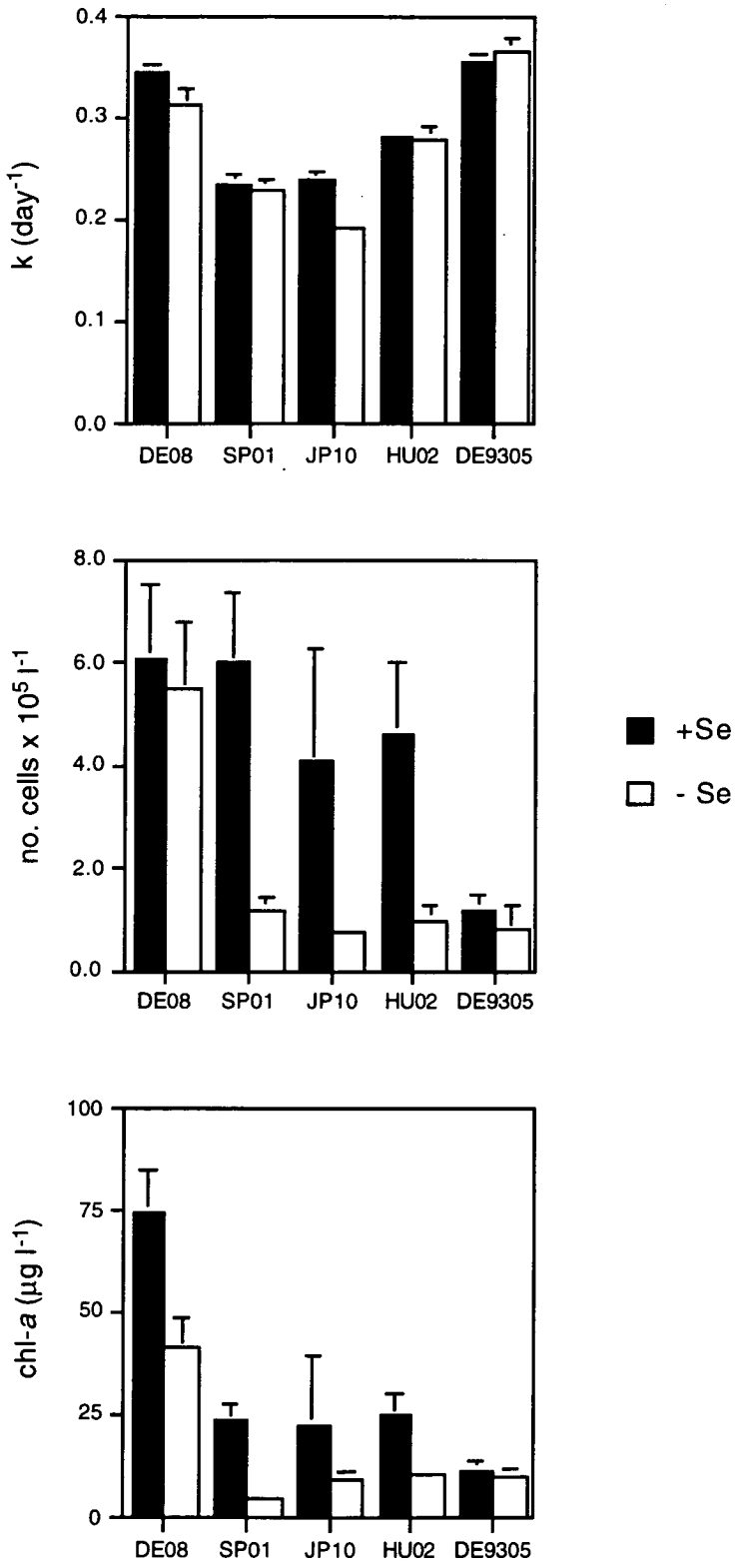


Fig. 4.2: Exponential growth rates (k) (a), cell yields (b), and chl *a* biomass (c) of *Gymnodinium catenatum* strains after approximately 40 days (>8 generations) in nutrient enriched seawater medium with no added selenium (-Se) or with $10^{-9}$ M selenium added as selenite ($H_2SeO_3$); n = 4, error bars = SE.

All strains except GCDE9305 exhibited reduced biomass yields when cultured in the absence of selenium (Fig. 4.2b and c). The Spanish, Japanese and Tasmanian Huon strain (GCSP01, GCJP10 and GCHU02 respectively) showed the greatest decrease (approximately 80% ) in cell abundance and chl *a* biomass after three transfers in Se-deficient medium. Cell yields decreased from approximately 5.99 x $10^5$ to 1.18 x $10^5$ cells $l^{-1}$, 4.09 x $10^5$ to 7.67 x $10^4$ cells $l^{-1}$ and 4.65 x $10^5$ to 9.7 x $10^4$ cells $l^{-1}$ (respectively) with no addition of selenium. Similarly, chl *a* biomass decreased from 23.1 to 4.6 µg $ml^{-1}$ in GCSP01, from 22.3 to 9.3 µg $ml^{-1}$ in GCJP10 and 24.5 to 10.2 µg $ml^{-1}$ in GCHU02. In contrast, there was only a 10% reduction in cell yield in the Tasmanian Derwent strain (GCDE08: from 6.09 x $10^5$ to 5.47 x $10^5$ cells $l^{-1}$) and a 45% decrease in chl *a* (from 74.2 - 41.3 µg $ml^{-1}$) in Se-deficient medium. The other Tasmanian Derwent strain (GCDE9305) showed no difference in cell yields or chl *a* biomass in Se-replete and Se-deficient medium (Fig. 4.2b and c).

### *4.3.2 Selenium requirement of* G. catenatum

The five strains investigated exhibited three different types of responses to Se-deficiency: (i) reduction in both exponential growth rates and biomass yields (GCDE08, GCJP10); (ii) no reduction in growth, but a reduction in biomass yields (GCSP01, GCHU02); and (iii) no reduction in growth or biomass yields (GCDE9305).

High intraspecific variability in exponential growth rates of *G. catenatum* strains in Se-replete and Se-deficient medium resulted in no significant difference in growth between all strains as a result of Se-deficiency ($n = 4$, $F = 3.23$, $p = 0.084$). However, comparison of growth rates between the (i) Derwent Estuary strains isolated in different years (GCDE08: 1987 and GCDE9305: 1993; $n = 4$, $F = 6.54$, $p = 0.02$); (ii) Tasmanian strains, originating from adjacent Estuaries (Derwent: GCDE08, GCDE9305 and Huon: GCHU02; $n = 4$, $F = 39.54$, $p < 0.001$); and (iii) Tasmanian strains and those from outside Australia, isolated from widely separated populations (Tasmanian: GCDE08, GCDE9305, GCHU02 and Spanish: GCSP01, Japanese: GCJP10; $n = 4$, $F = 186.76$, $p < 0.001$) were significant but showed no obvious pattern of variability related to the temporal or spatial differences in source populations (Table 4.2).

Cell yields of *G. catenatum* were much reduced under Se-deficient conditions ($n = 4$, $F = 13.23$, $p = 0.001$), despite strain variability in the reduction of cell yields with no

Table 4.2: ANOVA comparing growth rates between different *Gymnodinium catenatum* strains grown for three transfers in enriched seawater medium with no added selenium (-Se) and with $10^{-9}$ M $H_2SeO_3$ (+Se); n = 4; data untransformed

| Source | SS | DF | MS | F | P |
|---|---|---|---|---|---|
| Strain | 0.10 | 4 | 0.03 | 53.67 | <0.001 |
| **Selenium** | **0.00** | **1** | **0.00** | **3.23** | **0.084** |
| Strain*Selenium | 0.00 | 4 | 0.00 | 2.51 | 0.066 |
| i. DE08 vs DE9305 | 0.00 | 1 | 0.00 | 6.54 | 0.017 |
| ii. (DE08, DE9305) vs HU02 | 0.02 | 1 | 0.02 | 39.54 | <0.001 |
| iii. (DE08, DE9305, HU02) vs (JP10, SP01) | 0.09 | 1 | 0.09 | 186.76 | <0.001 |
| Error | 0.01 | 27 | 0.00 | | |

added selenium (with Derwent strains showing a smaller decrease in biomass in -Se medium compared to all other strains; Fig. 4.2b and c). The greatest variability was between strains isolated from the Derwent Estuary at different times (GCDE08 and GCDE9305; n = 4, F = 22.76, p < 0.001), and not between Tasmanian strains from adjacent areas (n = 4, F = 0.03, p = 0.959) or between Tasmanian and Japanese or Spanish populations (n = 4, F = 0.21, p = 0.652; Table 4.3).

Table 4.3: ANOVA comparing cell yields between different *Gymnodinium catenatum* strains grown for three transfers in enriched seawater medium with no added selenium (-Se) and with $10^{-9}$ M $H_2SeO_3$ (+Se); n = 4; data $\log_{10}$ transformed

| Source | SS | DF | MS | F | P |
|---|---|---|---|---|---|
| Strain | 3.53 | 4 | 0.88 | 6.57 | <0.001 |
| **Selenium** | **1.78** | **1** | **1.78** | **13.23** | **0.001** |
| Strain*Selenium | 0.62 | 4 | 0.15 | 1.15 | 0.354 |
| **i. DE08 vs DE9305** | **3.06** | **1** | **3.06** | **22.76** | **<0.001** |
| ii. (DE08, DE9305) vs HU02 | 0.00 | 1 | 0.00 | 0.03 | 0.959 |
| iii. (DE08, DE9305, HU02) vs (JP10, SP01) | 0.03 | 1 | 0.03 | 0.21 | 0.652 |
| Error | 4.03 | 30 | 0.13 | | |

*G. catenatum* chl *a* biomass was also significantly reduced as a result of Se-deficiency ($n = 4$, $F = 15.33$, $p < 0.001$), even though strain GCDE9305 showed no difference in biomass yields under Se-replete or Se-deplete conditions (Fig. 4.2c). The greatest variability between strains was between those isolated from the Derwent Estuary in different years (GCDE08 and GCDE9305; $n = 4$, $F = 22.76$, $p < 0.001$), but also between Tasmanian strains and those isolated from Japanese or Spanish populations ($n = 4$, $F = 14.76$, $p = 0.001$). Chl *a* quotas were different between strains ($n = 4$, $F = 5.64$, $p < 0.01$), with the Spanish strain having a lower quota (approximately 35 pg) compared to Tasmanian and Japanese strains (approximately 100 pg), however they did not change as a result of selenium deficiency ($n = 4$, $F = 0.20$, $p = 0.66$; Table 4.4).

Table 4.4: ANOVA comparing chl *a* biomass between different *Gymnodinium catenatum* strains grown for three transfers in enriched seawater medium with no added selenium (-Se) and with $10^{-9}$ M $H_2SeO_3$ (+Se); n = 4; data ln transformed.

| Source | SS | DF | MS | F | P |
|---|---|---|---|---|---|
| Strain | 17.20 | 4 | 4.30 | 14.27 | 0.000 |
| **Selenium** | **4.62** | **1** | **4.62** | **15.33** | **<0.001** |
| Strain*Selenium | 2.80 | 4 | 0.70 | 2.32 | 0.080 |
| **DE08 vs DE9305** | **11.84** | **1** | **11.84** | **39.29** | **<0.001** |
| (DE08, DE9305) vs HU02 | 0.88 | 1 | 0.88 | 2.91 | 0.099 |
| **(DE08, DE9305, HU02) vs (JP10, SP01)** | **4.45** | **1** | **4.45** | **14.76** | **0.001** |
| Error | 8.74 | 29 | 0.30 | | |

## 4.4 Discussion

### *4.4.1 Selenium requirement of* G. catenatum

This is the first study which has attempted to characterise the response of different strains of *G. catenatum* to the micronutrient selenium. Previous physiological investigations on other phytoplankton species (eg. the dinoflagellate *Peridinium cinctum* fa. *westii*, Lindström and Rodhe 1978; diatom *Thalassiosira pseudonana*, Price *et al.* 1987; dinoflagellate *Gymnodinium sanguineum*, prymnesiophyte

*Chrysochromulina polylepis*, diatom *Chaetoceros gracilis* and others, Harrison *et al.* 1988) have all examined selenium requirements using only a single clonal isolate and then made comparisons with other species. However, proper evaluation of interspecific differences is only possible when the magnitude of within-species variation is defined (Wood and Leatham 1992).

The present investigation demonstrated a variable requirement for selenium between *G. catenatum* strains, as shown by the three different responses to Se-deficiency: (i) a reduction of exponential growth rate and biomass yields; (ii) a reduction in biomass yields only; and (iii) no reduction in growth or biomass yields. The absence of an effect on exponential growth rates in some strains indicates that they either have a very low requirement for selenium which is met by background selenium levels in the culture medium (estimated to be about $10^{-11}$ M - see Chapter 3.4.2) or that they have no absolute requirement for selenium. Due to the variability between strains, the ANOVA detected no effect on *G. catenatum* growth as a result of Se-deficiency. There was, however, a clear reduction in *G. catenatum* biomass yields (cell density and chl *a*) in Se-deficient medium, with the Japanese (GCJP10) and one Tasmanian Derwent strain (GCDE08) showing a more marked response to Se-deficiency.

Exponential growth rates of the Tasmanian Derwent strain (GCDE08) were higher than in the previous experiment (Fig. 3.2, p. 36) and the effect of Se on growth and biomass yields much less. This may be due to differences in cell physiology (e.g. internal nutrient concentrations) when experimental cultures were started, despite being maintained under similar conditions. Nevertheless, the effect of Se deficiency was consistent in both experiments, with GCDE08 showing both a decrease in growth and biomass yields when grown in the absence of Se.

While the metabolic function of selenium in algae has not yet been clearly defined, an increasing body of evidence indicates that the addition of ≤ nanomolar quantities of selenium can stimulate or influence growth of a wide variety of marine and freshwater phytoplankton (eg. Lindström and Rhode 1978, Wehr and Brown 1985, Wheeler *et al.* 1982, Keller *et al.* 1984, Price *et al.* 1987, Harrison *et al.* 1988). There does not appear to be a greater requirement for selenium amongst neritic compared to oceanic species, although further testing with a larger number of species from different algal classes is necessary to confirm this (Harrison *et al.* 1988).

The concentration of selenium added to cultures ($10^{-9}$ M) was approximately equal to levels commonly found in coastal waters, including Tasmania (0.4 - 1.8 x $10^{-9}$ M $l^{-1}$:

Wrench and Measures 1982, Cutter and Bruland 1984, Takayanagi and Wong 1984, Chapter 3.4.4). In previous *G. catenatum* experiments with 0 - $10^{-7}$ M added selenium (Chapter 3.3.3) or with natural dissolved organic matter and selenium (Chapter 2.3.2), bacterial abundance did not change, indicating that the decrease in *G. catenatum* growth in the absence of selenium is unlikely to be related to a selenium-bacteria interaction.

The phenotypic variation in selenium requirement of *G. catenatum* found in this study adds to previous reports on strain variation for other characters within and between *G. catenatum* populations. For example, Oshima *et al.* (1993) compared the relative abundances of saxitoxin derivatives in a number of *G. catenatum* strains, including three used in this study (GCDE08, GCHU02 and GCSP01), and found that there were small but consistent differences between Tasmanian, Japanese and Spanish populations. Furthermore, Tasmanian strains were unique in their production of deoxy-decarbamoyl-STX toxins, suggesting this could be used as a biochemical marker to distinguish different geographic populations. Bolch *et al.* (1998) using RAPD-PCR (Randomly Amplified Polymorphic DNA-Polymerase Chain Reaction) analyses, found that the Japanese (GCJP10) and Spanish (GCSP01) strains have approximately equal genetic similarity compared to the Tasmanian Derwent (GCDE08, GCDE9305) strains. These authors also identified a greater genetic distance between Tasmanian Huon and Derwent strains compared to Tasmanian Huon and Japanese/Spanish strains, indicating relatively high genetic diversity within a small area.

Comparisons of the Se-requirement between strains in this study revealed that there was an equivalent or greater amount of phenotypic variation between strains isolated from the Derwent Estuary, Tasmania on different dates compared to the variation between strains isolated from adjacent areas in Tasmania and between Tasmanian and Spanish/Japanese strains. Other investigations have shown both an unexpectedly small amount of genetic variation between microalgal clones from widely separated locations (Brand *et al.* 1981, Hayhome *et al.* 1989, Chinain 1997) and a high degree of genetic variation between strains from the same or adjacent areas (Gallagher 1980, Bolch 1998). Some phytoplankton species also exhibit intra-specific variability in their nutrient requirements. For example, selected strains (Narragansett Bay and Osaka Bay) of the ichthyotoxic raphidophyte, *Heterosigma akashiwo*, cannot synthesize alkaline phosphatase, required for organic P assimilation (Nakamura 1985). This suggests that considerable phenotypic and genotypic variability exists within and between populations (on different temporal and spatial scales), highlighting the need for testing multiple strains in ecophysiological investigations.

The reasons behind the observed intraspecific differences in *G. catenatum* selenium requirement remain to be determined. Although is likely that populations from widely separated locations have evolved different physiological and nutritional requirements as a result of natural selection under different "selenium regimes", the variability in selenium requirement between *G. catenatum* strains demonstrated in this study suggests that these selection pressures may operate over much smaller spatial and temporal scales. Furthermore, it is not clear how rapidly the selenium requirement of a population can shift as a result of changing selenium bioavailability, but populations with higher genetic (and hence phenotypic diversity) may have an advantage over less diverse populations. Alternatively, selenium availability may play a minor role in structuring *G. catenatum* populations - the demonstrated diversity may be due to chance (such that all variations in selenium requirement are possible). Information about the variability in selenium concentrations in coastal waters and assessment of the selenium requirement of a larger number of strains will provide a better understanding of the role of such micronutrients in determining *G. catenatum* population structure and bloom formation.

This Chapter has demonstrated that the toxic dinoflagellate, *G. catenatum* has a variable selenium requirement and that populations of this species exhibit high phenotypic diversity in their response to this trace element. Subtle strain variability in micro-nutrient requirements of *G. catenatum*, as documented in this work, may therefore provide a partial explanation for the perplexing complexity of bloom patterns exhibited by the same taxon in different geographic regions (e.g. Tasmania and Spain) or in different seasons / years.

# Chapter 5: Vertical migration of *Gymnodinium catenatum* under different humic and nutrient conditions in culture[1]

## 5.1 Introduction

Blooms of the toxic dinoflagellate *Gymnodinium catenatum* Graham are regular events in south-east Tasmanian waters (Australia) during summer when surface nutrients are depleted and the water column is vertically stratified. Input of river water with high concentrations of humic substances (allochthonous dissolved organic matter) after rainfall is critical in bloom initiation, while bloom biomass can only accumulate during prolonged periods of calm weather (Hallegraeff *et al.* 1995). Blooms in Spanish Rías occur when warm nutrient depleted surface water moves towards the coast during upwelling relaxation (Fraga *et al.* 1990, Fermín *et al.* 1996). Field observations of *G. catenatum* in Ria de Vigo, northwest Spain, and in the Huon Estuary, Tasmania, have confirmed that this chainforming species undergoes vertical migration behaviour (swimming speed = 1.1 - 1.4 m $h^{-1}$, Fraga *et al.* 1989; 0.45 m $h^{-1}$, Hallegraeff *et al.* 1995), sinking in the late afternoon and rising in the early hours of the morning to form subsurface maxima during the day (Figueras and Fraga 1990, Hallegraeff and Fraga 1998).

Diel vertical migration (DVM) has been identified as a competitive strategy for phytoplankton under conditions where light and nutrients are spatially separated (Ganf and Oliver 1982). Coordination of this behaviour with the necessary physiological adaptations for dark nutrient assimilation provides access to nutrients below the photic zone and may confer an adaptive advantage over co-existing non-migratory or non-motile phytoplankton such as diatoms (Cullen 1985).

Field nutrient data suggest that nitrogen is the principal nutrient which induces DVM of *G. catenatum*, as a significant nitrate gradient develops in both Tasmanian and Spanish waters during blooms (CSIRO Huon Estuary Study, unpublished data, Fermín *et al.* 1996; see Chapter 7.4.3). Nutrient limitation bioassays using Huon River water collected during a *G. catenatum* bloom have demonstrated that nitrate addition causes an increase in biomass production (M. Doblin, unpublished data). Furthermore, addition of humic substances (natural dissolved organic matter and standard aquatic

[1]Doblin, M. A., Thompson, P., Blackburn, S. I. and Hallegraeff, G. M. (1998d). Vertical migration of the toxic dinoflagellate *Gymnodinium catenatum* under different nutrient and humic conditions in culture. *Mar. Ecol. Prog. Ser.*, submitted.

humic acid) to *G. catenatum* cultures changes nutrient availability so that biomass production is limited by nitrate instead of the trace element selenium (Chapter 2).

Humic substances (HS) contained in Huon River water may also affect DVM of phytoplankton because of their potential to change the underwater light climate. These chromophoric organic compounds reduce the total amount of photosynthetically active irradiation and alter the underwater PAR spectrum by the preferential absorption of UV-blue light (Kirk 1994). HS may also affect the nitrogenous nutrition of phytoplankton by forming an alternative organic nitrogen supply (Granéli *et al.* 1985).

This is the first study of *G. catenatum* DVM behaviour in vertically stratified laboratory water columns under different nutrient and humic conditions. The specific objectives were to test whether: (i) nutrient depletion in surface layers induced vertical migration of *G. catenatum*; (ii) nitrate was the principal nutrient determining DVM behaviour; and (iii) humic substances (with their potential for changing nutrient and light availability) altered the pattern of *G. catenatum* migration.

## 5.2 Materials and Methods

### *5.2.1 Strain history*

The *G. catenatum* strain GCDE08 (code CS-301) used in this study was obtained from the CSIRO Collection of Living Microalgae, Hobart, Tasmania, where it has been maintained in enriched seawater medium (salinity 28 ppt) with GPM nutrients (Loeblich 1975), soil extract and $10^{-8}$ M selenium (referred to as GSe medium in our laboratory). This strain was isolated from the Derwent Estuary, Tasmania on 15 June 1987 from an eight celled chain and produces Paralytic Shellfish toxins (PSTs) (Blackburn *et al.* 1989; Table 3.1).

### *5.2.2 Culture conditions*

A salinity and temperature stratified water column (fitted with 10 equally spaced side-sampling ports) was established in four transparent perspex cylinders (1.0 m in height with an internal diameter of 0.10 m; volume = 8.7 l; Fig. 5.1). The microcosms were kept in an environmentally controlled room at a temperature of 18 ± 1 °C. The bottom layer (35 ppt) extended 0.2 m up the column, and above this, salinity was 20 ppt. The pycnocline (10 - 12 ppt) was maintained by a temperature-controlled circulating water bath used to cool the bottom 0.2 m of the columns, generating a 2 °C temperature

gradient. Irradiance was provided by six 50W halogen lights on a 12 h light:12 h dark cycle with the light period from 06:00 to 18:00 h. *In situ* irradiance, measured with a Li-Cor 4π sensor and PAR photometer, ranged from 165 (surface) - 15 (bottom) µmol PAR $m^{-2}$ $s^{-1}$.

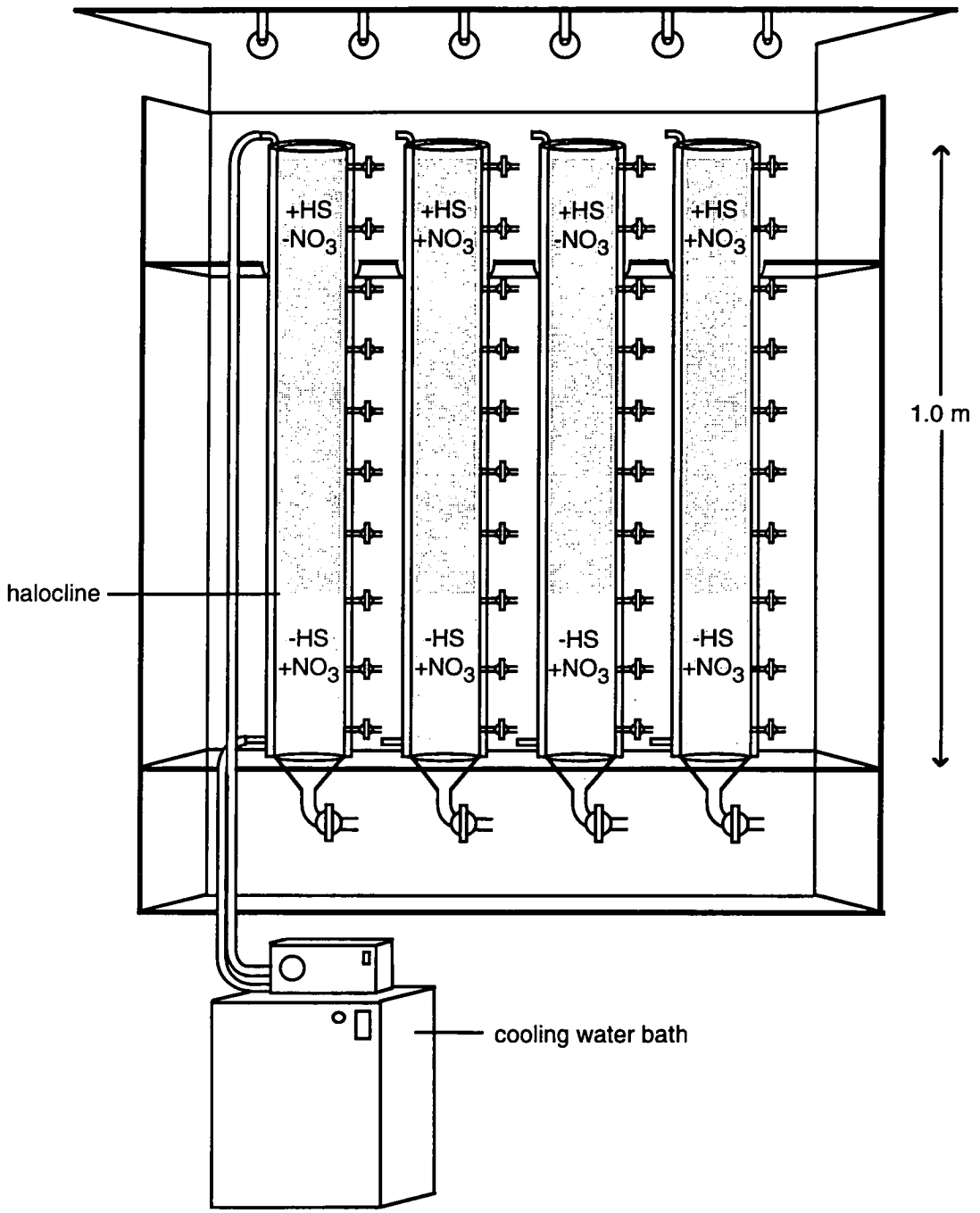


Fig. 5.1: Diagram of laboratory columns used in the present experiments (design based on the settling columns of Bienfang 1981).

Columns were scrubbed with hot water and thoroughly rinsed with Milli-Q before use. Seawater was collected from Crayfish Point, Taroona (Tasmania) on 8 September 1997, 0.2 µm filtered and autoclaved in 10 l polycarbonate Nalgene® carboys before addition to columns. Huon River water was collected from Judbury, Tasmania on the 14th of November 1997 and filter sterilised (0.22 µM) before use. Surface salinity was adjusted using autoclaved Milli-Q water or filtered Huon River water for non-humic or humic surface layers, respectively.

Nutrients (filter sterilised) were added to top and bottom layers of nutrient-replete and nitrate-replete columns and only the bottom layer of nutrient-deplete columns (Table 5.1). Nutrients were also added to top and bottom layers of nitrate-deplete columns, but surface layers were not enriched with nitrate (Table 5.1). Columns were fitted with transparent perspex lids and refilled with sterile seawater using sterilised Nalgene® tubing to minimise bacterial contamination.

### *5.2.3 Inoculum preparation and experiment set up*

Inoculum cultures were grown in enriched seawater medium (GSe/40 with $PO_4$ concentrations adjusted to 10 µM; Table 5.1) with salinity adjusted to 28 ppt using Milli-Q® in 1 l Erlenmeyer flasks at 180 µM $m^{-2}s^{-1}$ PAR (12:12 L:D cycle) at 17.5 °C. Cells were preconditioned for 5 days before addition to columns: cells added to nutrient-deplete columns were transferred (1:1 dilution) into seawater with no added nutrients, while cells used to inoculate nutrient-replete columns remained in GSe/40. Cells added to humic-enriched columns were grown in GSe/40 with salinity adjusted to 28 ppt using sterile Huon River water rather than Milli-Q®. Cells added to nitrate-deplete, humic enriched columns were preconditioned for 5 days in GSe/40 with no added $NO_3^-$, while cells added to nitrate-replete columns remained in GSe/40.

Columns were inoculated by gently adding 1-2 l of *G. catenatum* cells while columns were held as close to horizontal as possible. Once vertical, the columns were immediately topped up with medium using a variable speed Masterflex peristaltic pump, fitted with sterilised Nalgene® tubing. Filling involved pumping a further 4.5 - 5.5 l of surface water (salinity 25 ppt) at room temperature into the columns from the bottom (flow rate = 0.06 l $min^{-1}$). To generate the halocline, 2.5 l of cold (<10 °C) seawater (salinity 34 ppt) was then slowly pumped, also from the bottom (flow rate = 0.04 l $min^{-1}$) to fill the column. The cooling water baths (14.5 °C; one for two columns) were switched on during the second stage of filling and were operational for

the remainder of the experiment. To ensure ease of sampling, the halocline was set up to coincide with the eighth (from the top) side-sampling port (Fig. 5.1). Columns were refilled with appropriate water (25 or 35 ppt) through side-sampling ports to replace the volume removed by sampling.

Table 5.1: Concentration of nutrients in GSe/40 culture medium

| Additive | Nutrient | Concentration (μM) |
|---|---|---|
| Nitrate | $KNO_3$ | 50 |
| Phosphate | $K_2HPO_4$ | 5.0 |
| Metals (pH adjusted to 7.5): | $EDTA(Na,H_2O)_2$ | 2.5 |
| | $H_3BO_3$ | 13.75 |
| | $FeCl_3.6H2O$ | 0.125 |
| | $MnCl_2.4H_2O$ | 0.5 |
| | $CoCl_2.6H_2O$ | 0.013 |
| | $ZnCl_2$ | 0.058 |
| Selenium | $H_2SeO_3$ | $2.5 \times 10^{10}$ |
| Vitamins: | Vitamin $B_{12}$ | 0.185 |
| | Thiamin.HCl | 0.75 |
| | Biotin | 0.0001 |

### *5.2.4 Sampling and Analyses*

Columns were periodically subsampled through side-sampling ports to monitor the spatial distibution of cells at different stages of vertical migration. Subsamples (20-30ml) were withdrawn from 6 ports (always including the surface and halocline), filtered onto 25mm Whatman GF/C filters under dim light, before being stored at -20 °C for later chlorophyll *a* (chl *a*) analysis. Pigments on filters were extracted with 100% acetone overnight before measuring chl *a* and phaeopigments fluorometrically (USEPA 1992).

The nitrogen and carbon content of cells (particulate organic nitrogen, particulate organic carbon; PON and POC, respectively) and intracellular pools of nitrate were measured by sampling the biomass maximum (which coincided with either the surface or the halocline). For PON and POC analyses, 20-40 ml of culture was filtered onto

precombusted 25mm GF/C filters, rinsed twice with 5 ml of Milli-Q® water to remove any salt and stored at -20 °C before analysis. Samples were then oven dried (50 °C) overnight, pelletised and weighed into tin cups (Elemental Microanalysis Ltd., U.K.) and analysed for nitrogen, carbon, $d^{15}N$ and $d^{13}C$ using a Carlo Erba NA1500 CNS analyser (interfaced via a Conflo II to a Finnigan Mat Delta S isotope ratio mass spectrometer operating in the continuous flow mode). Combustion and oxidation were achieved at 1090 °C and reduction at 650 °C. Where necessary the carbon signal was diluted using helium. Subsamples for intracellular $NO_3^-$ pools were prepared using the method (C-2) of Thoresen *et al.* (1982) and analysed using a Technicon AutoAnalyser with the procedures of Airey and Sandars (1987).

Salinity and dissolved oxygen profiles were measured with a WTW Microprocessor Conductivity Meter LF 196 and Oximeter OXI 196 (respectively) on day 5 to check the integrity of vertical salinity stratification and degree of oxygenation (particularly in bottom layers). Dissolved nutrient samples were withdrawn from top and bottom layers and stored frozen (-20 °C) until analysis. Samples were analysed for dissolved $NO_3^-$+ $NO_2^-$, $PO_4^{3-}$, total dissolved N and total dissolved P using a Technicon Autoanalyser with the procedures of Airey and Sandars (1987). Nutrient concentrations in water used for refilling columns were also determined so that nutrient uptake could be corrected for any nutrient input as a result of column replenishment.

### *5.2.5 Vertical migration in nutrient-deplete and nutrient-replete columns without humic substances*

Columns were set up with a salinity gradient of approximately 10 ppt (top: 24 ppt; bottom: 34 ppt), with sterile Milli-Q® being used to adjust the salinity of water in the surface layers. Two columns contained nutrients (Table 5.1) in both surface (SL) and bottom (BL) layers (replete: $SL_rBL_r$), while another two columns contained nutrients only in the bottom layer (deplete: $SL_dBL_r$). Vertical migration was monitored quantitatively starting 2 days after column filling, with columns being sampled for cell distribution and nutrient concentration every 3 hours through an entire L:D cycle. Dissolved nutrients were sampled from one side-sampling port in the top and bottom layers of the columns. Visual observation of *G. catenatum* cells in the columns (seen as a dark band at the surface and halocline or as individual swimming chains) was used to select up to 5 sampling ports with maximum spatial resolution from the 10 available for monitoring the vertical distribution of *G. catenatum*. A summary of experimental conditions is provided in Table 5.2.

Table 5.2: Experimental conditions for *Gymnodinium catenatum* vertical migration investigations

| Experiment | Control | Treatment | Abbreviations | Top | | Bottom | |
|---|---|---|---|---|---|---|---|
| | | | | Nutrient conc. | Salinity (ppt) | Nutrient conc. | Salinity (ppt) |
| *Vertical migration in nutrient-deplete and nutrient-replete columns without humic substances* | surface layer nutrient-replete; bottom layer nutrient-replete | surface layer nutrient-deplete; bottom layer nutrient-replete | $SL_r$ $BL_r$ or $SL_d$ $BL_r$, repectively | $NO_3$ = 50 µM $PO_4$ = 10 µM other nutrients = GSe/40* | 25 | $NO_3$ = 100 µM $PO_4$ = 20 µM other nutrients = GSe/40 | 35 |
| *Vertical migration in nitrate-deplete and nitrate-replete columns with humic substances* | surface layer nitrate-replete and enriched with humic substances (Huon River water); bottom layers nutrient-replete | surface layer nitrate-deplete and enriched with humic substances (Huon River water); bottom layers nutrient-replete | $HSL_{-N}$ $BL_r$ or $HSL_r$ $BL_r$, respectively; | $NO_3$ = 50 µM $PO_4$ = 10 µM other nutrients = GSe/40 | 25 | $NO_3$ = 50 µM $PO_4$ = 10 µM other nutrients = GSe/40 | 35 |

* GSe medium is based on Loeblich's (1975) medium but is modified to include selenium ($10^{-8}$ M $H_2SeO_3$) and does not include soil extract

### *5.2.6 Vertical migration in nitrate-deplete and nitrate-replete columns with humic substances*

Huon River water, containing humic substances was added to surface layers to allow investigation of their effect on DVM. Columns were set up with a salinity gradient of approximately 12 ppt (top: 24 ppt; bottom: 36 ppt), with sterile 0.2 µm filtered Huon River water (rather than Milli-Q®) being used to adjust the salinity in the surface layers of all columns. Two columns contained nutrients (Table 5.1) in both top and bottom layers (replete: $HSL_rBL_r$), while another two contained nutrients but no nitrate in the top layer and all nutrients in the bottom layer (deplete: $HSL_{-N}BL_r$). Vertical migration was monitored from days 2-6, with columns being sampled for the vertical distribution of cells, PON, POC and intracellular $NO_3^-$ pools every 12 hours, 3 hours before the start and end of the light period (03:00 and 15:00 respectively). These times were chosen to allow sampling just before the onset of upwards or downwards migration, when cells were expected to have respectively, the lowest or the highest internal nutrient concentrations. Estimates of bacterial abundance in top and bottom layers were made after the experiment (day 6) by serially diluting 0.1ml aliquots from the top and bottom of the columns and plating out onto seawater agar (with added vitamins; Lewis *et al.* 1986). Dissolved nutrient samples were withdrawn through side-sampling ports at depths of 0.1, 0.3, 0.5, 0.7, 0.8 and 0.9m on day 1 and 5 to allow calculation of nutrient uptake by *G. catenatum*.

## 5.3 Results

### *5.3.1 Vertical migration in nutrient-deplete and nutrient-replete columns without humic substances*

Nitrate concentrations in surface layers of nitrate-deplete ($SL_dBL_r$) and nitrate-replete ($SL_rBL_r$) columns did not change significantly during the experiment (ANOVA, $p = 0.53$), but surface phosphate levels declined (approximately 1.5 µM) over the 24-hour period (ANOVA, $p = 0.02$). In comparison, nitrate and phosphate concentrations declined by 10-30% in bottom layers of all columns (ANOVA, $p = 0.02$, $p = 0.04$, respectively).

The vertical density stratification was stable during the three day incubation and *in situ* PAR levels in nutrient-replete and nutrient-deplete columns ranged from approximately 165 (surface) - 10 (bottom) µmol PAR $m^{-2}s^{-1}$ (Fig. 5.2).

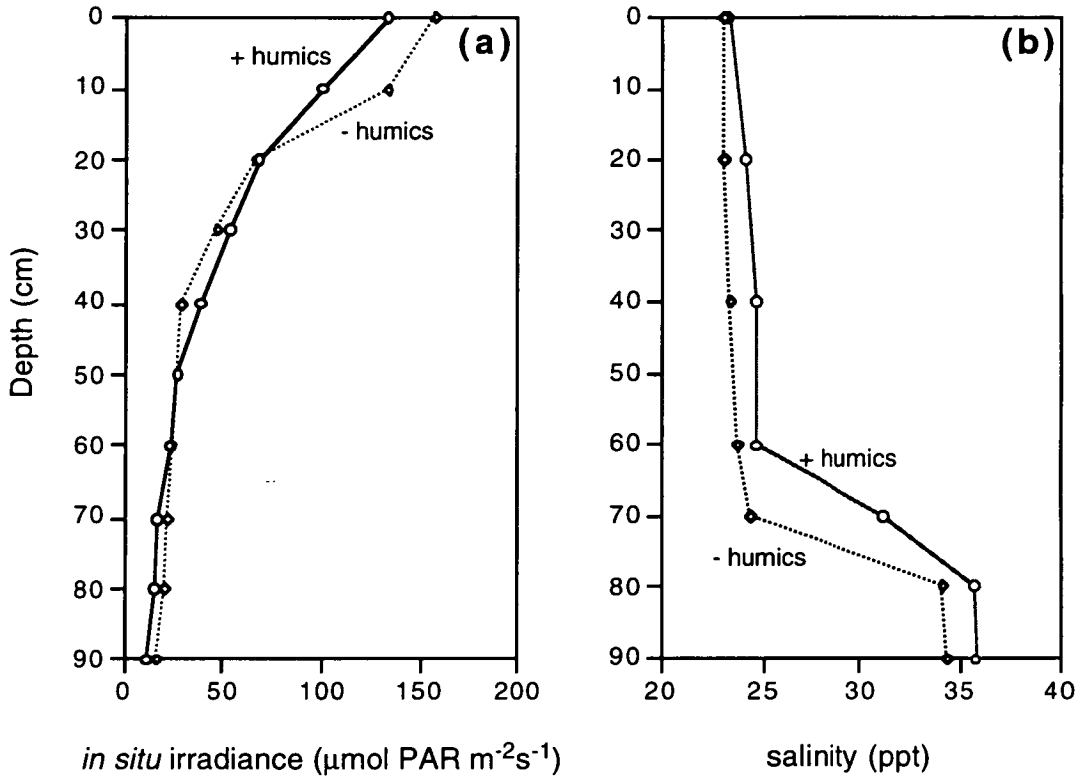


Fig. 5.2: *In situ* irradiance (a) and salinity (b) profiles in columns with and without humic substances in surface layers (0 - 70 cm).

Cells in nutrient-deplete ($SL_dBL_r$) columns exhibited DVM, starting their downwards migration approximately three hours before the end of the light period (15:00), accumulating at the pycnocline (0.7m deep) from 21:00 - 03:00 and beginning their upwards migration approximately three hours before the start of the light period (03:00) (Fig. 5.3). In contrast, cells in nutrient-replete ($SL_rBL_r$) columns didn't undergo DVM, although they were slightly more dispersed in the upper layer during the dark period. Cells in all columns accumulated at the surface (forming a thick band) during the light period. The sampling resolution (5 out of 10 side-sampling ports), while capturing the biomass peak at the surface and halocline, resulted in an apparent "disappearance" of biomass during DVM, which reappeared either at the surface or the halocline during the next sampling period (Fig. 5.3).

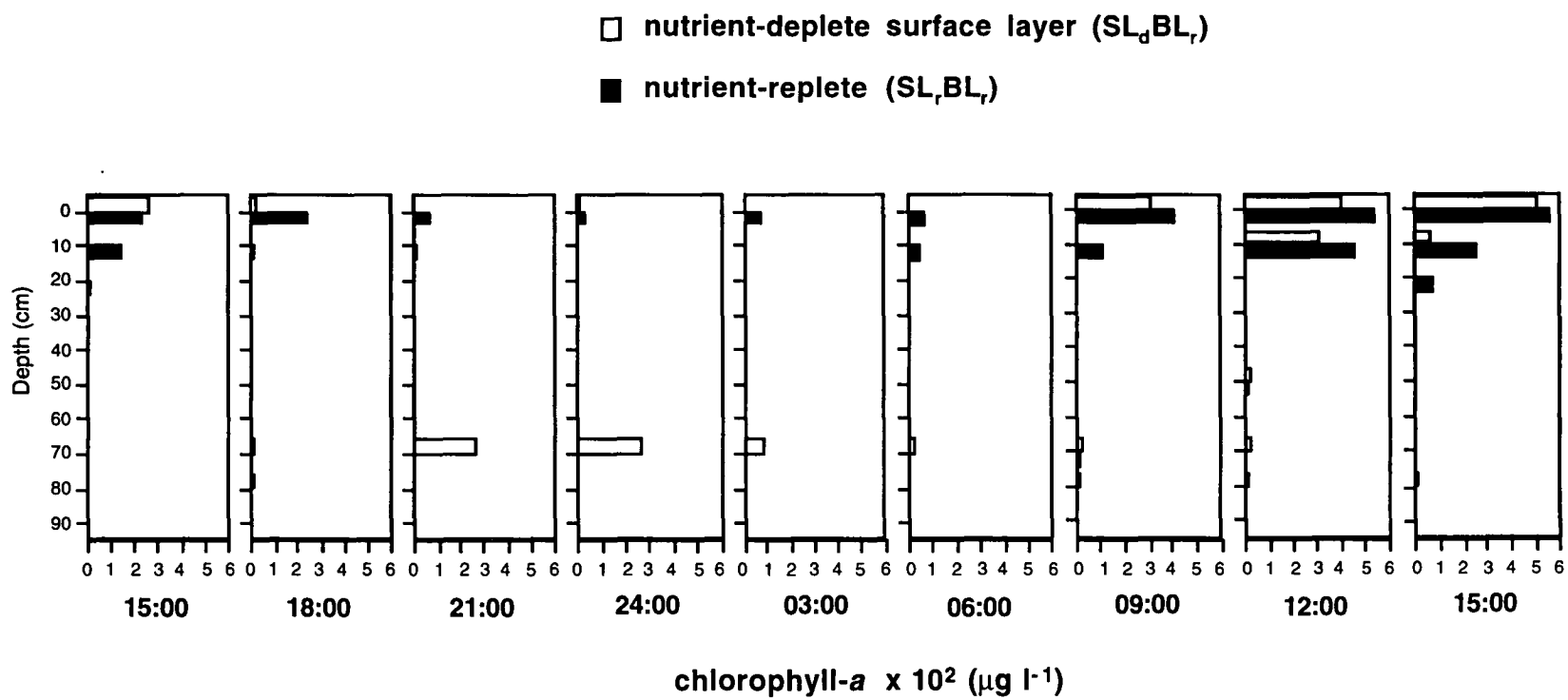


Fig. 5.3: Spatial distribution of *Gymnodinium catenatum* cells during a 24 h L:D cycle after 2 days in columns with nutrient-deplete surface layers + nutrient-replete bottom layers ($SL_dBL_r$) or columns with nutrient-replete surface and bottom layers ($SL_rBL_r$) conditions. Empty bars = nutrient-deplete columns; filled bars = nutrient-replete columns; n = 2.

### *5.3.2 Vertical migration in nitrate-deplete and nitrate-replete columns with humic substances*

*G. catenatum* cells in nitrate-deplete columns underwent DVM in the presence of humic substances, but unlike the pattern shown in columns without humic substances when *G. catenatum* cells in nutrient-deplete columns returned to the surface during the light period, there was a proportion (approximately 50%) of cells which remained at the pycnocline (70 cm deep) throughout the incubation (Fig. 5.4). In addition, unlike the situation in columns without humic substances, *G. catenatum* cells in nitrate-replete humic-enriched columns also underwent DVM, however there was a proportion (30%) of cells which remained at the surface during the dark period.

Phosphate concentrations in nitrate-deplete ($HSL_{-N}BL_r$) and nitrate-replete ($HSL_rBL_r$) columns were relatively constant during the experiment (Fig. 5.5), indicating minimal phosphate uptake by *G. catenatum* ($\leq 0.05$ µg P µg chl $a^{-1}$ $h^{-1}$), with no significant difference in phosphate uptake between treatments (ANOVA, $p = 0.17$) or between layers (ANOVA, $p = 0.43$; Table 5.3). However nitrate concentrations decreased considerably, with nitrate uptake occurring in both surface and bottom layers in nitrate-replete columns (Fig. 5.5). Nitrate uptake in nitrate-deplete ($HSL_{-N}BL_r$) columns was 0.40 µg N µg chl $a^{-1}h^{-1}$ in bottom layers compared to 0.0 µg N µg chl $a^{-1}$ $h^{-1}$ in surface layers, but in nitrate-replete columns, there was a much smaller difference between nitrate uptake in bottom compared to surface layers (bottom: 0.13 µg N µg chl $a^{-1}$ $h^{-1}$; top: 0.10 µg N µg chl $a^{-1}$ $h^{-1}$; Table 5.3). Overall, nitrate uptake rates were not significantly different in nitrate-deplete and nitrate-replete columns (ANOVA, $p = 0.10$), but they were higher in bottom compared to surface layers (ANOVA, $p = 0.005$). In addition, there was a greater rate of nitrate uptake in bottom layers of nitrate-deplete columns compared to nitrate-replete ones (significant treatment x layer interaction term; $p = 0.008$; Table 5.3). Analyses for total N showed that 50 - 100% of the dissolved nitrogen present in humic enriched surface layers was organic, with the greater proportions of organic N occuring in nitrate-deplete compared to nitrate-replete columns (ANOVA, $p = 0.02$). The proportion of organic P in humic enriched surface layers was considerably smaller (4-19%), with the greater proportions of organic P in nitrate-deplete columns compared to nitrate-replete columns (ANOVA, $p = 0.007$).

Physiological acclimations of cells to the different conditions were largely not statistically significant, but included cells in nitrate-deplete columns with lower chl *a* quotas (44.5 compared to 52.9 pg $cell^{-1}$), higher C:N (7.49 compared to 5.15 g:g) and higher C:chl *a* ratios (approximately 79 compared to 61) than cells in nitrate-replete

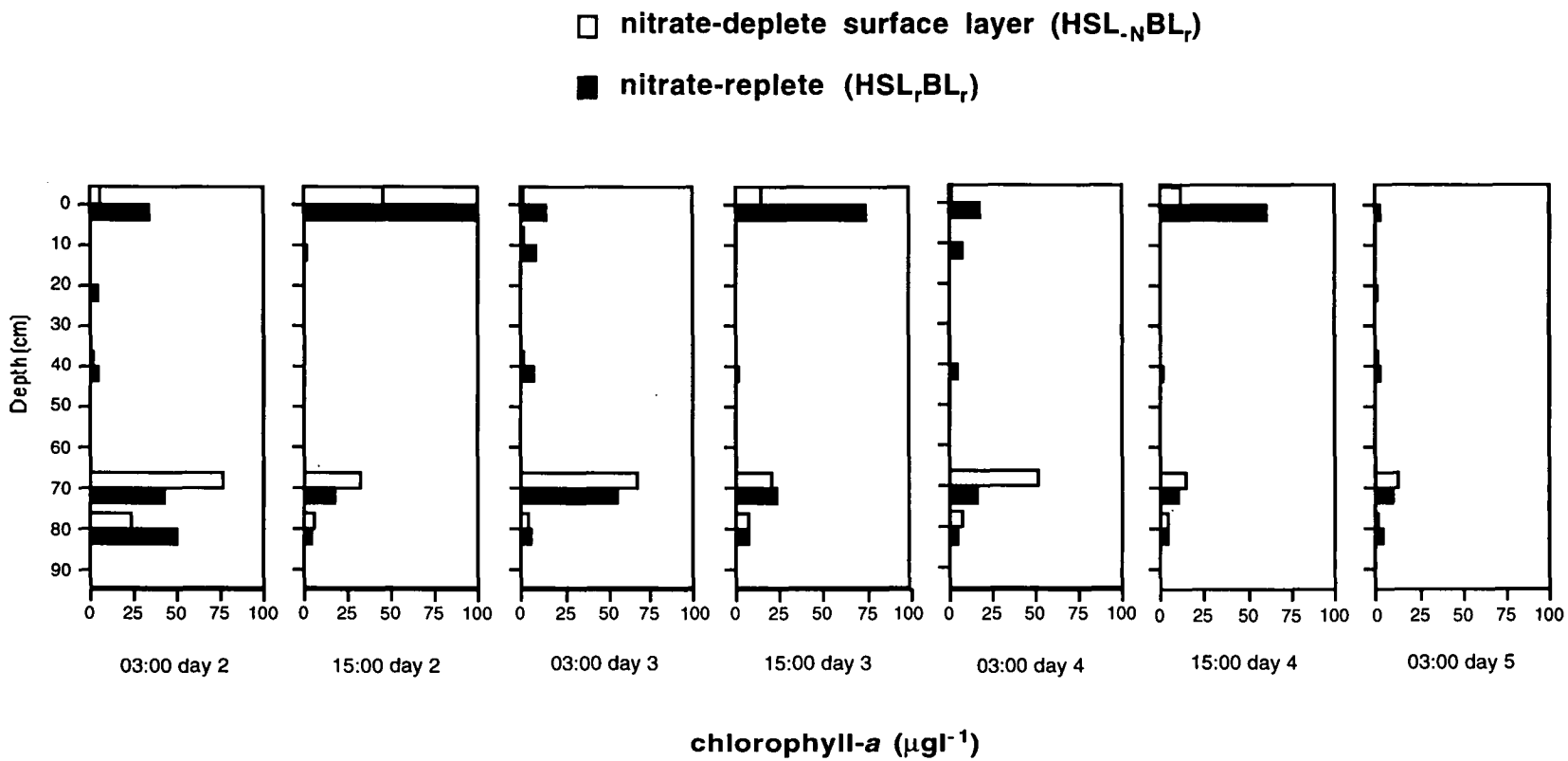


Fig. 5.4: Spatial distribution of *Gymnodinium catenatum* cells from days 2-6 under nitrate-deplete ($SL_{-N}BL_r$) or nitrate-replete ($SL_rBL_r$) conditions. Empty bars = nitrate-deplete columns; filled bars = nitrate-replete columns; n = 2.

columns at the start of the experiment (Table 5.3). Despite access to nitrate in bottom layers, vertically migrating cells in nitrate-deplete ($HSL_{-N}BL_r$) columns showed no consistent pattern of increasing N quota, decreasing C:N, C:chl-*a* ratios and / or increasing chl-*a* quotas during the DVM cycle. Cells in both types of columns showed no pattern of increasing C quota during the day (to indicate internal carbohydrate accumulation during photosynthesis) or increasing N quota during the night (to indicate nitrate uptake and assimilation). Cellular nitrogen quotas increased in both

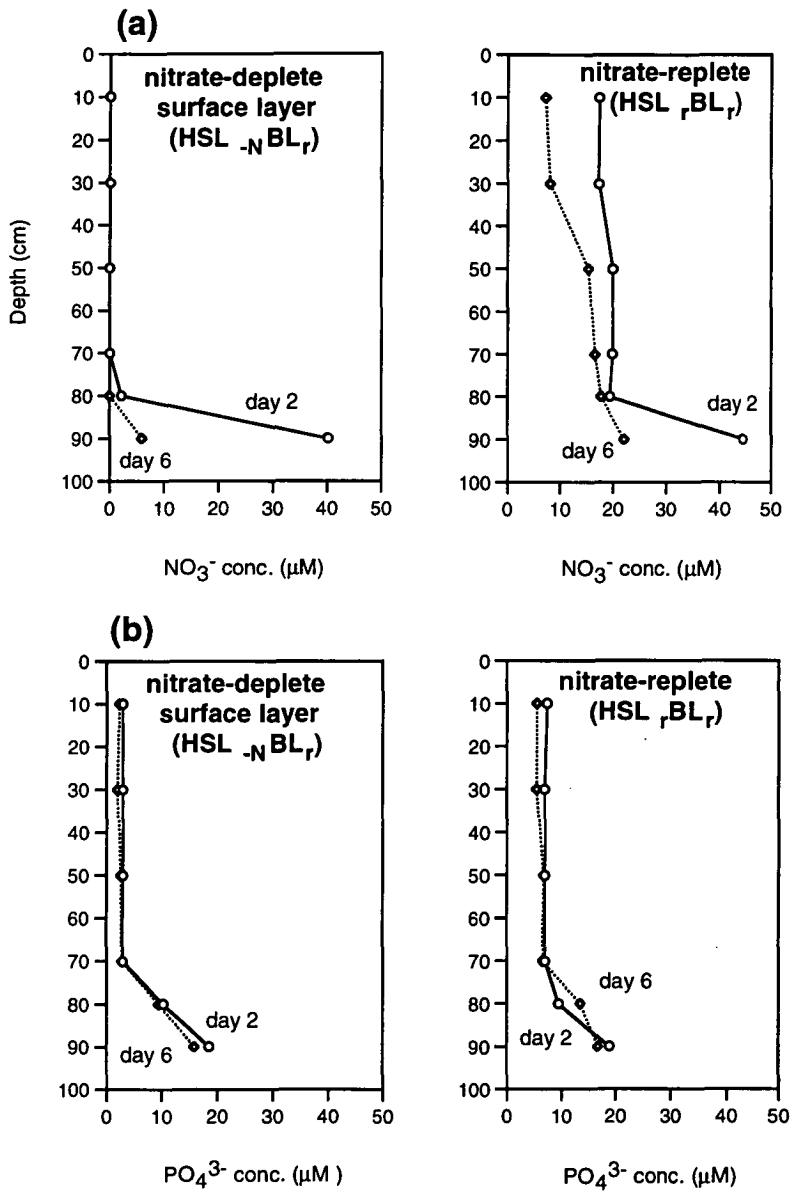


Fig. 5.5: (a) $NO_3^-$ and (b) $PO_4^{3-}$ concentrations in nitrate-deplete ($SL_{-N}BL_r$) and nitrate-replete ($SL_rBL_r$) columns from days 2-6; n = 2.

Table 5.3: Comparison of physiological parameters of cells from laboratory columns with humic enriched surface layers that were either nitrate-deplete ($HSL_{-N}BL_r$) or nitrate-replete ($HSL_rBL_r$) after 36 and 96 h; n = 2; F and P values are derived from two-way ANOVA, using time (36, 96 h) and treatment ($HSL_{-N}BL_r$, $SL_rBL_r$) as the analysis factors

| Parameter | nitrate-deplete ($HSL_{-N}BL_r$) | | nitrate-replete ($HSL_rBL_r$) | | Comparison 36 vs 96 h | | Comparison N-deplete vs N-replete | |
|---|---|---|---|---|---|---|---|---|
| | 36 h | 96 h | 36 h | 96 h | F | P | F | P |
| C quota (pg cell$^{-1}$) | 3520 | 3200 | 2562 | 7661 | 4.31[l] | 0.29 | 12.56 | 0.18 |
| N quota (pg cell$^{-1}$) | 470 | 497 | 497 | 1344 | 2.06[l] | 0.29 | 1.41 | 0.36 |
| chl-a quota (pg cell$^{-1}$) | 44.5 | 52.9 | 50.0 | 67.2 | 7.88[u] | 0.22 | 4.69 | 0.28 |
| C:N (g:g) | 7.49 | 6.44 | 5.15 | 5.70 | 87.6[u] | 0.07 | 2.42 | 0.36 |
| C:chl-a (g:g) | 79.1 | 60.5 | 51.2 | 114 | 0.55[u] | 0.59 | 1.65 | 0.42 |
| internal $NO_3$ pool (mM l cell volume$^{-1}$) | 28.2 | 93.64 | 2.74 | 29.63 | 5.60[l] | 0.08 | 1.27 | 0.32 |
| $\delta^{15}N$ | 8.64 | 7.99 | 8.62 | 7.94 | 10.67[u] | 0.03 | 0.24 | 0.65 |
| | | | | | Comparison surface vs bottom | | Comparison N-deplete vs N-replete | |
| | surface | bottom | surface | bottom | F | P | F | P |
| N uptake (μg N μg chl-$a^{-1}$ h$^{-1}$) | 0.00 | 0.40 | 0.10 | 0.13 | 32.6i | 0.10 | 4.43 | 0.10 |
| P uptake (μg P μg chl-$a^{-1}$ h$^{-1}$) | 0.04 | 0.07 | 0.04 | -0.02 | 0.82[l] | 0.43 | 3.19 | 0.17 |

[l] = ln transformed data
[u] = untransformed data
i = significant interaction between analysis factors (time x treatment); F = 24.5, p = 0.008.

nitrate-deplete (from 470 - 497 pg cell$^{-1}$) and nitrate-replete (from 497 - 1344 pg cell$^{-1}$) columns during the 4 day experiment, but there was no significant difference in N quotas between 36 and 96 h or between treatments (Table 5.3). Similarly, C:N and C:chl *a* ratios decreased (15-20%) in nitrate-deplete columns and increased (10-100%) in nitrate-replete columns, however the variability in these cell parameters meant that differences were not significant (Table 5.3). The $\delta^{15}N$ of *G. catenatum* cells in nitrate-deplete columns showed a decreasing trend from 8.5 - 8.0 ‰ during the 4 day experiment (Fig. 5.6a; $p = 0.09$) but the decline in $\delta^{15}N$ of *G. catenatum* cells in nitrate-replete columns was greater, decreasing from 8.9 to 7.9 ‰ (Fig. 5.6b; $p = 0.003$). In comparison, the $\delta^{13}C$ of *G. catenatum* cells in both nitrate-deplete and nitrate-replete columns showed an decreasing (more negative) trend from -20.0 to -21.5 ‰ during the 4 day experiment (Fig. 5.7; $p > 0.05$).

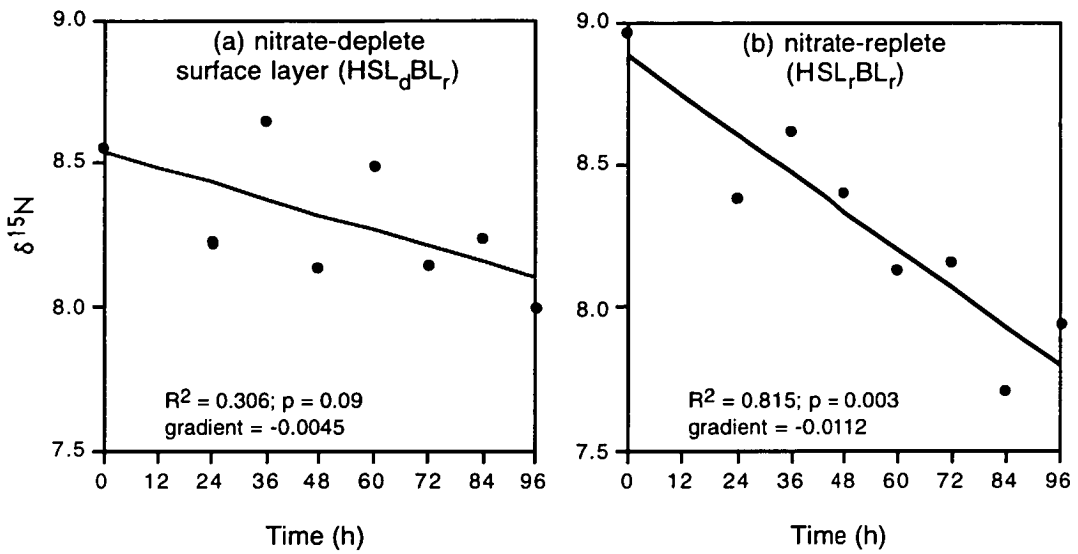


Fig. 5.6: (a) $\delta^{15}N$ values (‰) of *G. catenatum* cells in laboratory columns with nitrate-deplete surface layers and in (b) nitrate-replete columns during the 4 day experiment. Symbols represent means of two columns (SE < 0.4 ‰).

Nitrate-replete cells showed a 10-fold increase in internal $NO_3^-$ pools compared to a three-fold increase in nitrate-deplete cells during the four day experiment (Table 5.3). Intracellular $NO_3^-$ pools were low (<3 mM l$^{-1}$ cell volume) in nitrate-replete cells at the start of the incubation, and increased after 4 days to approximately 30 mM l$^{-1}$. In

comparison, intracellular $NO_3^-$ pools were initially 30 mM $l^{-1}$ in nitrate-deplete cells and increased after 4 days to approxiately 90 mM $l^{-1}$.

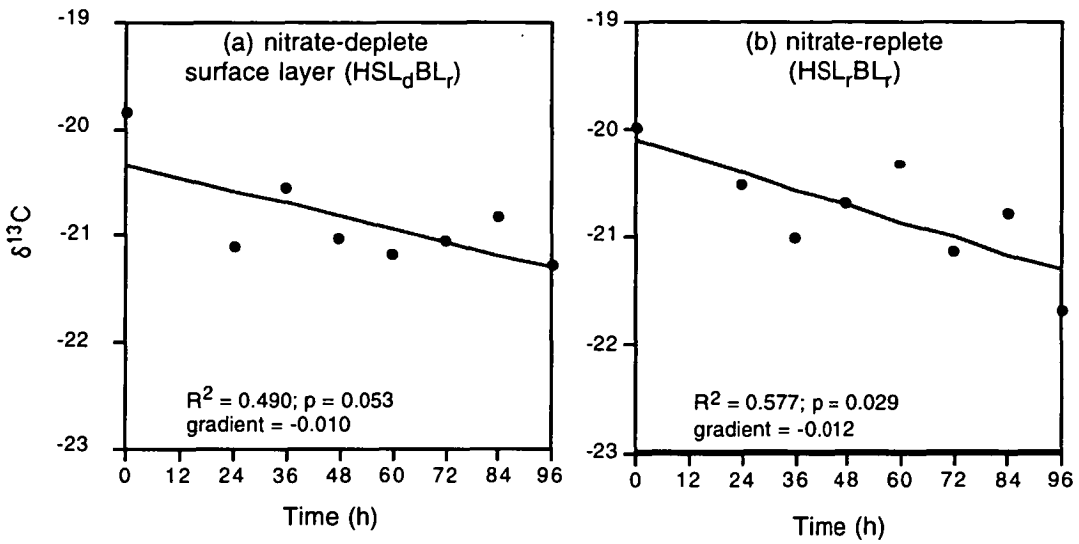


Fig. 5.7: (a) $\delta^{13}C$ values (‰) of *G. catenatum* cells in laboratory columns with nitrate-deplete surface layers and in (b) nitrate-replete columns during the 4 day experiment. Symbols represent means of two columns (SE < 0.5 ‰).

Bacterial abundance in columns ranged from 0.6 - 1.0 x $10^8$ cells $l^{-1}$, with no increase in density in nutrient enriched layers (n = 2, F = 0.53, p = 0.51) or with addition of humic substances (n = 2, F = 0.87, p = 0.40). Dissolved oxygen concentrations were greater in surface layers (4.7 - 5.0 mg $l^{-1}$) compared to bottom layers (1.2 - 1.4 mg $l^{-1}$; n = 2, F = 1048, p < 0.001), but there was no difference between nitrate-deplete ($HSL_{-N}BL_r$) and nitrate-replete ($SL_{-N}$ $BL_{-N}$) columns (n = 2, F = 7.66, p = 0.05). *In situ* surface irradiance in humic enriched columns was reduced by approximately 20% relative to non-humic enriched columns (from approximately 165 to 130 µmol PAR $m^{-2}s^{-1}$; p = 0.02) but irradiance did not differ between columns with non-humic or humic surface layers at depths greater than 20 cm (p = 0.12).

## 5.4 Discussion

This is the first study which has demonstrated vertical migration of *G. catenatum* in vertically-stratified laboratory columns. Diel vertical migration (DVM) of motile

phytoplankton has been widely observed in both natural populations (e.g. *Ceratium furca*: Eppley *et al.* 1968, Edler and Olsson 1985; *Heterosigma akashiwo*: Yamochi and Abe 1984; *Gonyaulax catenata*: Passow 1991; *Gymnodinium bogoriense*: Lieberman *et al.* 1994; *Gymnodinium mikimotoi*: Koizumi *et al.* 1996) and in laboratory cultures (e.g. *Ceratium hirundinella*: Heaney and Eppley 1981; *Heterocapsa niei*: Cullen 1985; *Chattonella antiqua*: Watanabe *et al.* 1991; *Alexandrium tamarense* MacIntyre *et al.* 1997). The potential adaptive significance of DVM has been linked to: (i) nutrient retrieval at depth (Eppley *et al.* 1968, Eppley and Harrison 1975, Harrison 1976, Cullen 1985, Lieberman *et al.* 1994); (ii) selective retention or flushing avoidance in dynamic estuarine environments (Anderson and Stolzenbach 1985, Crawford and Purdie 1992); and (iii) grazing avoidance (e.g. Stich and Lampert 1981); all of which are mechanisms that either enhance the specific growth rate of the organism or reduce losses due to predation, sinking or physical transport to unfavourable growth regions (Cullen 1985).

Field nutrient data (from the Huon Estuary, Tasmania and Ria de Vigo, Spain) suggest that nitrate is the principal nutrient which induces DVM of *G. catenatum*, since a significant vertical nitrate gradient develops over summer when DVM is observed (CSIRO Huon Estuary Study unpublished data, Fermín *et al.* 1996). The present study confirms that the DVM behaviour of *G. catenatum* is influenced by cellular nitrogen status and that vertically migrating *G. catenatum* cells take up nitrate in bottom layers, thereby increasing intracellular $NO_3$ pools and nitrogen quotas. However there was a considerable proportion (70%) of cells in nitrate-replete (compared to nutrient-replete) columns which migrated despite adequate supply of nitrate in both top and bottom layers. Similarly, not all cells in nitrate-deplete rather than nutrient-deplete columns underwent DVM, indicating that under these experimental conditions, nitrate wasn't the only factor controlling DVM behaviour.

External factors which affect DVM include nutrients (Eppley *et al.* 1968, Cullen 1985), irradiance (Harris *et al.* 1979, Heaney and Furnass 1980), anoxia (George and Heaney 1978), and temperature or density gradients (Kamykowski and Zentrar 1977, Tyler and Seliger 1978). Experiments with exponential and stationary phase cells indicate that growth stage may also be important (Heaney and Furnass 1980). Downwards migration of *G. catenatum* cells before the end of the light cycle and upwards migration before the end of the dark cycle demonstrate that the vertical migrations of *G. catenatum* (similar to *Ceratium furca*, *Heterosigma akashiwo*, *G. mikimotoi* and other species; Weiler and Karl 1979, Yamochi and Abe 1984, Koizumi *et al.* 1996 respectively) are also controlled by endogenous factors such as circadian rhythms. In addition, observations of *G. catenatum* cells undergoing DVM regardless of nutrient

status, suggests that there are other factors modulating this behaviour which remain to be elucidated.

The addition of humic substances (HS) (contained in river water) to surface layers may have affected DVM by altering the rate of photosynthesis through a reduction or change in spectral irradiance (Kirk 1994). *In situ* irradiance measurements confirm that there was a significant reduction (20%) of surface PAR in humic-enriched columns (but not in the degree of light attenuation at depths >20 cm), so that mean irradiance levels may have been sub-saturating for growth (Blackburn *et al.* 1989). HS may also increase the supply of nitrogen to *G. catenatum* cells (Granéli *et al.* 1985) thereby reducing the nitrogen gradient in the columns, with possible effects on DVM behaviour. Total dissolved N concentrations in columns with HS show that between 50-100% of the nitrogen present in surface layers was in organic form. Previous experiments have demonstrated that growth rates of *G. catenatum* cells are similar when supplied with nitrate or urea (M. Doblin, unpublished data), but whether cells are able to take up high molecular weight organic N compounds such as humic substances remains to be determined. Uptake of amino acids (low molecular weight compounds which could be associated with humic substances) may be possible through the action of cell surface or extracellular peptidases associated with bacteria (Rosso and Azam 1987), which can hydrolyse peptides and provide phytoplankton with an additional nitrogen source (Carlsson and Granéli 1998). Although bacterial abundance didn't increase with addition of HS, it is possible that aminopeptidases increased the pool of organic nitrogen available for phytoplankton uptake. In any case, addition of HS did not ameliorate cellular nitrogen depletion sufficiently to preclude the need for nutrient uptake at depth. This is somewhat at odds with the results of experiments described in Chapter 2 which demonstrate that humic substances stimulate *G. catenatum* biomass yields and shift nutrient availability and / or utilisation so that *G. catenatum* yields are limited by nitrogen rather than selenium. If HS provide DON then it seems counter-intuitive for nitrogen to be the limiting nutrient under such conditions. One explanation may be related to the energy cost of assimilating such compounds (see Thompson *et al.* 1989). In addition, the character of aquatic humic substances is spatially and temporally variable, so it is likely that the proportion of DOC or DON which can be utilised by phytoplankton or bacteria is also variable. The interaction of humic substances with other nutrients under varying environmental conditions (e.g. salinity, pH) may result in different bioavailable nutrient concentrations, with possible effects on DVM behaviour. The apparent inconsistency highlights the complexity of nutritional and behavioural relationships and their net effect on species abundance in natural phytoplankton communities. Further investigation is necessary to fully characterise this aspect of *G. catenatum* ecology.

DO concentrations in vertically-stratified columns were significantly lower in bottom layers compared to surface layers. Relatively low DO concentrations at depth could arise from (i) photosynthesis in surface layers during the light period; (ii) respiration at low ($< I_c$) irradiance or during the dark in bottom layers; and / or (iii) nitrate uptake (which has a high energy requirement - see Thompson *et al.* 1989) at the halocline. If the light compensation point of *G. catenatum* was similar to the toxic dinoflagellate *Alexandrium tamarense* (*Gonyaulax tamarensis*) (35 µmol PAR $m^{-2}s^{-1}$; Langdon 1986), then our laboratory columns provide conditions of net respiration at depths > 50 cm (Fig. 5.2a). In addition the energy cost of $NO_3$ reduction is 22% of the total energy costs by any cell (Thompson *et al.* 1989), increasing the respiratory burden of *G. catenatum* cells assimilating nitrate in bottom layers. Low DO levels in bottom layers are thus likely to be the result of high phytoplankton respiration rates. Bacterial metabolism of organic matter (derived from sedimenting *G. catenatum* biomass) may have also contributed to the relatively low DO levels found in bottom layers.

The $\delta^{15}N$ of *G. catenatum* cells in both nitrate-deplete and nitrate-replete columns declined by 6-12 ‰ during the 4 day experiment indicating preferential uptake of $^{14}N$ over $^{15}N$ (fractionation; Pennock *et al.* 1996). The continued decline of *G. catenatum* $\delta^{15}N$ during the experiment towards the $\delta^{15}N$ of the N source (1.2 for $NaNO_3$; A. Revill, personal communication) suggests that cells did not become N limited, undergoing DVM to access $NO_3^-$ at depth. Pennock *et al.* (1996) demonstrated that under nitrogen ($NO_3^-$ or $NH_4^+$) -replete culture conditions, the $\delta^{15}N$ of the diatom *Skeletonema costatum* initially decreased, but when nitrogen became exhausted, $\delta^{15}N$ increased to its original value. Controlled field experiments have also shown an initial decline and subsequent increase in $\delta^{15}N$ during phytoplankton growth, concomittant with a decline in external N concentrations. For example, Nakatsuka *et al.* (1992) found that the $\delta^{15}N$ in suspended particulate organic matter during a phytoplankton bloom in an ecosystem enclosure (70 $m^3$) in Saanich Inlet, British Columbia, Canada decreased by approximately 80% during the first 4 days of the bloom (from approximately 7.2 - 1.2 ‰), after which it returned to about 7 ‰ and remained constant for the remainder of the 23 day experiment. The much slower decline in $\delta^{15}N$ values in our experiment (particularly in nitrate-deplete columns), may be due to the slow rate of nitrogen assimilation by *G. catenatum* cells, and the very low ($< 0.1$ µM $l^{-1}$ $NO_3^-$) external nitrate concentrations in surface layers of nitrate-deplete columns. Preliminary data show that the $\delta^{15}N$ signals of inorganic nitrogen salts (e.g. $KNO_3$,

$NaNO_3$) and dissolved aquatic humic substances are different (A. Revill, personal communication), indicating a potential means to trace uptake of different nitrogen sources by phytoplankton, particularly those that are separated under vertically stratified conditions. A similar approach was adopted by McClelland and Valiela (1998), who used the isotopic ratios of N in groundwater and primary producers in Waquoit Bay, Cape Cod, Massachusetts to determine the link between nitrogen input and estuarine production.

The $\delta^{13}C$ values reported here (-19.5 to -21.5‰) fall within the range (-34 to -5‰) reported for photosynthetic marine organisms (Johnston 1996). The declining trend in $\delta^{13}C$ is unexpected, since under closed culture conditions, the $\delta^{13}C$ should increase (Johnston and Kennedy 1998). This suggests that the inoculum was relatively enriched in $^{13}C$ (due to high cell density) and was returning to a more normal value once cells were transfered into columns (with normal $^{12}C$: $^{13}C$ ratios). Environmental and physiological factors affecting phytoplankton carbon isotopic composition include temperature and pH (e.g. Johnston 1996), photon flux density (e.g. Thompson and Calvert 1994, 1995) and phytoplankton growth rate (Laws *et al.* 1995). Johnston and Kennedy (1998) also discuss the potential for nutrients to affect carbon isotopic composition, since nutrient limitation has a profound impact on growth rates and is also likely to determine which mechanism of inorganic carbon assimilation cells will adopt. The similarity of $\delta^{13}C$ values in nitrate-replete and nitrate-deplete columns (which differed only in their nitrate distribution) suggests that *G. catenatum* isotopic composition was unaffected by external nutrient conditions during the four day experiment, possibly because of DVM behaviour. Taken together, the $\delta^{15}N$ and $\delta^{13}C$ data indicate a similar carbon metabolism for the cells in both columns, whereas they suggest a modest difference in N metabolism, with cells in N-replete columns assimilating N faster, probably as a result of constant exposure to $NO_3^-$.

Measurement of nitrogen, carbon and chl *a* quotas and internal $NO_3^-$ pools showed that *G. catenatum* cells in columns altered their physiology in response to external nitrate supply. Cells used to inoculate nitrate-deplete columns had higher C:N and C:chl *a* ratios compared to cells in nitrate-replete columns, indicating they became N-deficient during preconditioning. Although DVM facilitated nitrate uptake in bottom layers (as shown by the reduction in external $NO_3^-$ concentration), cells in columns with nitrate-deplete surface layers did not recover sufficiently during the experiment to have similar C:N or N quotas compared to nitrate-replete cells. This may be because of a reduced ability to take up nitrate after a prolonged period of nitrogen stress (e.g. Dortch *et al.*

1982) and/ or a relatively slow rate of $NO_3^-$ uptake (as compared with $NH_4^+$) following N-starvation (Flynn *et al.* 1996). The fact that approximately 50% of cells in nitrate-deplete ($HSL_{-N}BL_r$) columns remained at the halocline during the experiment and did not migrate upwards during the light period suggests that cells were sufficiently stressed during preconditioning to have lost their vertical migration ability.

*G. catenatum*, like other phytoplankton species (e.g. the diatoms *Chaetoceros debilis*, *Skeletonema costatum*, *Rhizosolenia* and *Ethmodiscus*, the dinoflagellates *Amphidinium carterae* and *Pyrocystis*, and the prasinophyte *Halosphaera* (Dortch 1982, Dortch *et al.* 1984, Villareal and Lipschultz 1995), accumulates intracellular $NO_3^-$ pools (Table 5.4). Internal nitrate pools of *G. catenatum* (cell volume = 1.7 x $10^4$ $\mu m^3$) ranged from 2.7 - 94 mM $l^{-1}$, similar to natural populations of the prasinophyte *Halosphaera* (cell volume = 5.4 x $10^7$ $\mu m^3$) isolated from the Sargasso Sea (Villareal and Lipschultz 1995; Table 5.4). In the present study, pools were larger in nitrate-deplete cells compared to nitrate-replete cells, suggesting that N-stress causes a decrease in the rate of nitrate assimilation (resulting in a build-up of internal $NO_3^-$). Decreased nitrate assimilation may be related to a decrease in nitrate reductase activity, which has previously been demonstrated to be affected by light quality (particularly blue vs red light; e.g. Carmona *et al.* 1996) and quantity (e.g. Fritz *et al.* 1996), as well as nitrogen supply (Sanz *et al.* 1995).

Previous studies have demonstrated that internal nitrate concentrations are significantly lower in sinking compared to floating cells, suggesting that buoyancy is regulated by internal nutrient status (Villareal and Lipschultz 1995). The high variability in $NO_3^-$ pools in the present study obscures any cyclic pattern in the accumulation of $NO_3^-$ during the dark period (when nitrate-deplete cells were periodically exposed to high levels of nitrate), and the subsequent disappearance of $NO_3^-$ during the light period. However, internal pool sizes increased in both nitrate-deplete and nitrate-replete cells during the 4 day incubation, indicating exposure to $NO_3^-$ (Dortch 1982, Dortch *et al.* 1984) and recovery from internal nitrate depletion during preconditioning. The increase in $NO_3^-$ pools in nitrate-replete cells is difficult to explain, but shows that despite adequate supply of external $NO_3^-$, cells were slow to assimilate $NO_3^-$.

Vertically migrating *G. catenatum* cells took up more $NO_3^-$ than $PO_4^{3-}$ in bottom layers, with greater N uptake rates in nitrate-deplete ($HSL_{-N}BL_r$) columns. N uptake rates were low in comparison to other studies, (1 - 5 $\mu g$ N $\mu g$ chl $a^{-1}$ $h^{-1}$; Table 5.4) and may be related to the slow growth rate or the accumulation of $NO_3^-$ pools as a result of reduced N assimilation rates. It is unlikely that cellular nitrogen uptake is the major

Table 5.4: Comparison of nitrate uptake rates and cell physiology parameters in this study with other investigations

| Parameter | Values | Reference | Comments |
|---|---|---|---|
| N quota (pg $cell^{-1}$) | 470 - 1340 | this study | *Gymnodinium catenatum*; supplied with $NO_3$, 18 °C, 160 µmol PAR $m^{-2}s^{-1}$ |
| | 50 - 70 | Flynn *et al.* 1996 | *Gymnodinium catenatum*; supplied with $NO_3$, 18 °C, 180 µmol PAR $m^{-2}s^{-1}$ |
| | 688 | Montagnes *et al.* 1994 | *Gymnodinium sanguineum*; supplied with $NO_3$, 16 °C, 20-60 µmol PAR $m^{-2}s^{-1}$ |
| | 94 | Montagnes *et al.* 1994 | *Gyrodinium aureolum*; supplied with $NO_3$, 16 °C, 20-60 µmol PAR $m^{-2}s^{-1}$ |
| C:N (mass) | 5.2 - 5.7 (N-replete) | this study | *Gymnodinium catenatum*; supplied with $NO_3$, 18 °C, 160 µmol PAR $m^{-2}s^{-1}$ |
| | 7.5 (N-deplete) | this study | |
| | 6.0 (N-replete) | Flynn *et al.* 1996 | *Gymnodinium catenatum*; supplied with $NO_3$, 18 °C, 180 µmol PAR $m^{-2}s^{-1}$ |
| | 7.5 (N-deplete) | Flynn *et al.* 1996 | |
| internal $NO_3$ pool | 2.7 - 93.6 | this study | *Gymnodinium catenatum* |
| (mM l cell $volume^{-1}$) | 26.9 | Dortch *et al.* 1984 | *Skeletonema costatum*; max. pool after $NO_3$addition to $NO_3$-starved cultures |
| | 6.9 | Dortch *et al.* 1984 | *Amphidinium carterae* |
| | no measurable pool | Dortch *et al.* 1984 | *Thalassiosira pseudonana, Dunaliella tertiolecta* |
| | 0 - 20 | Villareal & Lipschultz 1995 | *Rhizosolenia* spp. chains collected in the Sargasso Sea |
| | 0 - 100 | Villareal & Lipschultz 1995 | *Halosphaera* spp. nautral population collected in the Sargasso Sea |
| $NO_3$ uptake | 0.1 - 0.4 | this study | *Gymnodinium catenatum* uptake in laboratory columns |
| (µg N µg chl-$a^{-1}$ $h^{-1}$) | 0 - 0.9 | Hamilton *et al.* 1997 | Uptake measured during dinoflagellate bloom in Swan River Estuary, Australia |
| | 2.0 | Matsuoka *et al.* 1986 | Lake Kasumigaura, Japan |
| | 1.0 - 5.0 | Jørgensen *et al.* 1986 | Review of nutrient uptake rates in eutrophication modelling |

factor involved in the decrease in nutrient concentrations in bottom layers during the 24 h experiment, given the absence of DVM in nutrient-replete ($SL_rBL_r$) columns. Nutrient concentrations were corrected for nutrient addition during column refilling, and adsorption of nutrients onto column surfaces or nutrient uptake by bacteria were considered minimal. The apparent decrease in nutrient concentrations may be related to nutrient diffusion across the halocline, but is more likely to be the result of poor spatial resolution during sampling (one port in the surface and bottom layers). Profiles of dissolved nutrients were therefore carried out in the second experiment to reduce sampling artefacts.

Other investigations of phytoplankton nutrition and its effect on DVM have produced results which are at variance to this study. For example, Eppley *et al.* (1968) showed that nitrogen-replete cells of the dinoflagellate *Gonyaulax polyedra* (Dinophyceae) underwent DVM, but nitrogen-deplete cells could not. In addition, the raphidophyte *Heterosigma akashiwo* was found to undergo DVM in response to phosphate (rather than nitrate) gradients (Watanabe *et al.* 1988). Identification of strain-specific differences in nutrient requirements of *G. catenatum* (Doblin *et al.* submitted) suggests that future studies on DVM should focus on both intra-specific and inter-specific differences in the factors controlling DVM in phytoplankton populations.

This Chapter has shown that DVM of nutrient-deficient *G. catenatum* cells provides access to light for photosynthesis in surface layers and high concentrations of nutrients in bottom layers, resulting in an effective strategy to increase growth and/or biomass accumulation. Nitrate-deplete *G. catenatum* cells undergoing vertical migration showed increased N quotas and internal $NO_3^-$ pools after exposure to nutrients at depth. In addition, nitrate-deplete cells had greater N-uptake rates compared to nitrate-replete cells. Nitrate-deplete (in comparison to nutrient-deplete) cells showed an altered pattern of DVM, with some cells migrating upwards to surface layers during the light period and other cells remaining at the halocline. The vertical migration of nitrate-deplete cells demonstrated that addition of humic substances did not ameliorate cellular nitrogen deficiency to preclude the need for nitrate uptake at depth. Furthermore, the varied behaviour within a *G. catenatum* population suggests that nitrate gradients are not the only factor controlling DVM. Overall, DVM may play an important role in this organism's ecological strategy, enabling it to exploit environments where light and nutrients are vertically separated. Investigations using vertically-stratified laboratory columns provide valuable opportunity to test hypotheses regarding DVM of *G. catenatum* in response to environmental gradients.

# Chapter 6: The importance of humic substances and other nutrients in *Gymnodinium catenatum* sexual reproduction

## 6.1 Introduction

The previous chapters focussed on the effects of humic substances, selenium, nitrate and other nutrients on *G. catenatum* vegetative cell growth and biomass production. However *G. catenatum*, like many other dinoflagellates, also undergoes sexual reproduction, where anisogamous sexually-compatible (heterothallic) gametes fuse to form resting cysts (hypnozygotes) (Blackburn *et al.* 1989). These resting cysts have a very short (< 2 week) dormancy period (endogenous inhibition of growth) and show no evidence of quiescence (inhibition of growth due to unfavourable environmental conditions) in culture (Blackburn *et al.* 1989, Bravo and Anderson 1994). This, and the rare occurrence of planomeiocytes (recently germinated cysts) in field samples (Bolch and Blackburn, unpublished data), suggests that cyst germination is not the major factor involved in Tasmanian *G. catenatum* bloom initiation. Instead regular excystment may provide a low level inoculum to maintain a perennial *G. catenatum* population in Tasmanian waters (Hallegraeff *et al.* 1995). Resting cyst production would also provide *G. catenatum* populations with a means of genetic recombination (Blackburn *et al.* 1989) and could act as potential vector for dispersal into new areas (Dale 1983).

Humic substances have been implicated in the induction of sexuality in both natural dinoflagellate populations and laboratory cultures. For example, Faust (1993) observed *Prorocentrum lima* gametes in tropical mangrove areas where there were high concentrations of dissolved humic and tannic compounds. Sexual stages (gametes, fusing gametes, planozygotes and hypnozygotes) of the same species were also observed in isolated cultures grown in Erdschreiber's seawater medium (Föyn 1934) supplemented with mangrove sediment extract (Faust 1993). Heil (1996) found that organic addition caused induction of meiosis in four other dinoflagellate species (*Alexandrium tamarense*, *Gyrodinium aureolum*, *Prorocentrum micans* and *Prorocentrum minimum*) when cultured with humic, fulvic and hydrophilic acids.

Dinoflagellate sexuality has been linked to other environmental factors such as irradiance, day length, temperature, nutrient depletion, and dissolved gases (see Pfiester and Anderson 1987 for review). Little is known about cues for sexuality in natural *G. catenatum* populations, however laboratory studies have shown that cysts are formed under both nutrient-replete and nutrient-deficient (low nitrate and phosphate

concentrations) conditions in the presence of soil extract, although the incidence of hypnozygotes is much greater when cultures are nutrient limited (Blackburn *et al.* 1989). Observations of planozygotes and resting cysts in humic Huon Estuary, Tasmania surface waters (M. Doblin, unpublished) gave rise to the hypothesis that these organic compounds stimulate sexual reproduction of *G. catenatum.*

The aim of this study was to investigate whether humic substances promote *G. catenatum* encystment in culture. Development of a *G. catenatum* bloom (> $10^4$ cells $l^{-1}$ with up to 770 µg PSP toxin / 100 g shellfish meat) in the Huon Estuary, Tasmania during December 1997 also enabled examination of the role of nutrients in sexual induction in a natural *G. catenatum* population.

## 6.2 Materials and Methods

### *6.2.1 The effect of humic additives on* G. catenatum *cyst production in culture*

Two compatible mating strains (GCDE08 and GCHU11), previously shown to produce large quantities of cysts (Blackburn *et al.* 1989, Blackburn *et al.* 1998) were obtained from the CSIRO Collection of Living Microalgae, Tasmania, Australia. Strain codes, isolation details and toxicity information are provided in Table 6.1. Equal volumes (2.5 ml) of exponential phase GCDE08 and GCHU11 cultures (grown for two weeks in enriched seawater medium (GSe/40; Table 2.1) were mixed in 50mm diameter petri dishes containing one of three types of media: (a) GSe/40, with 0.2 µm filter sterilised Huon River water (collected in October 1997 at Judbury; Fig. 1.4) replacing sterile Milli-Q® water; (b) GSe/40, with addition of standard aquatic Suwannee River humic acid (purchased from the International Humic Substances Society; final concentration = 1.62 mg $l^{-1}$); and (c) GSe/40 with no addition of humic substances (control). Cysts in cultures were counted every three days for approximately four weeks (until cysts began to germinate). Cyst production was used as an unambiguous index of sexual reproduction since gametes are morphologically indistinguishable from vegetative cells and fusing gametes are sometimes difficult to distinguish from dividing vegetative cells, although the dancing motion of gametes (von Stosch 1973) may be easily identified with experience.

Table 6.1: Isolation and toxicity information for the *Gymnodinium catenatum* strains used in the present study; CS-code is the accession code provided by the CSIRO Collection of Living Microalgae for purchasing strains

| Strain | CS-code | Location | Date of isolation | Isolator | Clonal | Toxic |
|---|---|---|---|---|---|---|
| GCDE08 | CS-301 | Derwent Estuary, Tasmania | 15 June 1987 | S. Blackburn | + (8-celled chain) | + (PSP) |
| GCHU11 | CS-302 | Huon Estuary, Tasmania | | C. Bolch | + | + (PSP) |

### *6.2.2 Bloom water collection*

An integrated (0-8m) bloom water sample was collected at Wheatleys, Huon Estuary, Tasmania (Fig. 1.4) on the 5th of December 1997, when *G. catenatum* cell density was approximately 3.27 x $10^5$ cells $l^{-1}$ and < 5% of the phytoplankton population was comprised of other species (P. Bonham, unpublished data). There were minimal numbers of zooplankton present, so samples were not filtered prior to enrichment.

### *6.2.3 The effect of nutrient enrichment on* G. catenatum *cyst production*

Ten ml subsamples of integrated bloom water were poured into clear sterile polystyrene well plates (six 35 mm diameter wells) before sterile nutrient enrichment (see Table 6.2). Enrichments were performed in triplicate and well plates were incubated at 180 µmol photons PAR $m^{-2}s^{-1}$ (cool white fluorescent tubes; 12:12 L:D cycle) for approximately two weeks. Cysts in wells were counted on days 7 and 14, until cysts began to germinate.

Procedures for statistical analysis are provided in Chapter 2.2.8.

Table 6.2: Concentration of nutrients used in enrichment bioassays for cyst production in a natural *Gymnodinium catenatum* population

| Nutrient enrichment | Abbreviation | Nutrient conc. (µM) |
|---|---|---|
| unenriched (control 1) | - | -[1] |
| Milli-Q® (control 2) | + Milli-Q | 50 µl |
| nitrate | $+NO_3$ | 50 |
| phosphate | $+PO_4$ | 50 |
| silicate | +Si | 50 |
| vitamins (thiamine, biotin, vitamin $B_{12}$) | +Vit | GSe/40 |
| metals (Fe, Zn, Co, Mn) | +Met | GSe/40 |
| chelator | +EDTA | 10 |
| selenite ($10^{-9}$ M) | +Se ($10^{-9}$ M) | 0.001 |
| selenite ($10^{-8}$ M) | +Se ($10^{-8}$ M) | 0.01 |
| selenite ($10^{-7}$ M) | +Se ($10^{-7}$ M) | 0.1 |
| selenite ($5 \times 10^{-7}$ M) | +Se ($5 \times 10^{-7}$ M) | 0.5 |
| all nutrients (nitrate, phosphate, vitamins, metals and selenium) | +ALL | GSe/40 |
| all nutrients (nitrate, phosphate, vitamins, metals, selenium and silicate) | +ALL+ Si | GSe/40 + 50 µM Si |
| all nutrients except nitrate (phosphate, vitamins, metals and selenium) | $+ALL-NO_3$ | GSe/40 |
| all nutrients except phosphate (nitrate, vitamins, metals and selenium) | $+ALL-PO_4$ | GSe/40 |
| all nutrients except vitamins (nitrate, phosphate, metals and selenium) | +ALL-Vit | GSe/40 |
| all nutrients except metals (nitrate, phosphate, metals and selenium) | +ALL-Met | GSe/40 |
| all nutrients exept selenium (nitrate, phosphate, vitamins and metals) | +ALL-Se | GSe/40 |

[1] Dissolved nitrate and phosphate in bloom water samples was ≤ 1.0 µM

## 6.3 Results

### *6.3.1 The effect of humic additives on* G. catenatum *cyst production in culture*

"Dancing gametes" (Von Stosch 1973), fusing gametes and planozygotes were abundant in all crossed strain cultures during the first week of incubation. Cysts were observed 24 h after *G. catenatum* strains were mixed, with cyst numbers steadily increasing until day 16 when they reached levels of approximately 200 cysts per 10 ml culture (Fig. 6.1). Control cultures (with no humic addition) had the greatest variability in cyst production and encystment was not significantly different between treatments ($n = 3$, $F = 0.61$, $p = 0.58$).

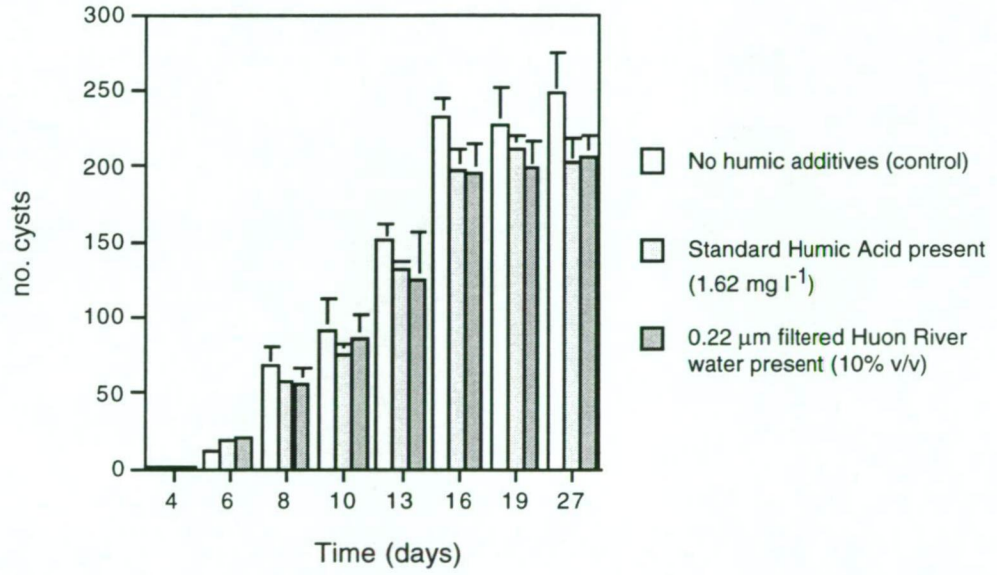


Fig. 6.1: Number of *Gymnodinium catenatum* cysts produced in culture crosses (strains GCDE08 and GCHU11) in GSe/40 culture medium with humic enrichment (standard aquatic Suwannee River humic acid = 1.62 mg $l^{-1}$; 0.22 µm filtered Huon River water = 10% v/v) compared to controls (no humic additives); n = 3; error bars = SE.

### *6.3.2 The effect of nutrient enrichment on* G. catenatum *cyst production*

Unenriched bloom samples, and samples to which EDTA, metals, vitamins and selenium were added, showed more evidence of sexual activity ("dancing gametes", fusing gametes and planozygotes) in the first week after enrichment than samples

enriched with all nutrients (nitrate, phosphate, metals, vitamins and selenium: ALL) and all nutrients including silicate (ALL+Si).

Cysts were formed in all incubated bloom samples, including those which received full nutrient enrichment (Fig. 6.2). Encystment on day 7 was low (< 75 cysts / 10 ml) in cultures enriched with all nutrients (ALL), all nutrients including silicate (ALL+Si), all nutrients excluding vitamins (ALL-Vit), all nutrients excluding metals (ALL-Met) and all nutrients excluding selenium (ALL-Se). In comparison, a relatively high number (>200 / 10 ml) of cysts were produced in ALL-$NO_3^-$ and ALL-$PO_4^{3-}$ treatments, control cultures (ie. unenriched and enriched with Milli-Q) and cultures enriched with only nitrate, phosphate and selenium. The ANOVA showed that encystment was different between treatments ($n = 3$, $F = 16.0$, $p < 0.0001$) and that control ($n = 3$, $F = 8.59$ , $p = 0.006$) and ALL- $NO_3^-$ and ALL- $PO_4^{3-}$ ($n = 3$, $F = 4.49$, $p = 0.042$) cultures had significantly greater numbers of resting cysts on day 7 (Fig. 6.2a). On day 14, cyst

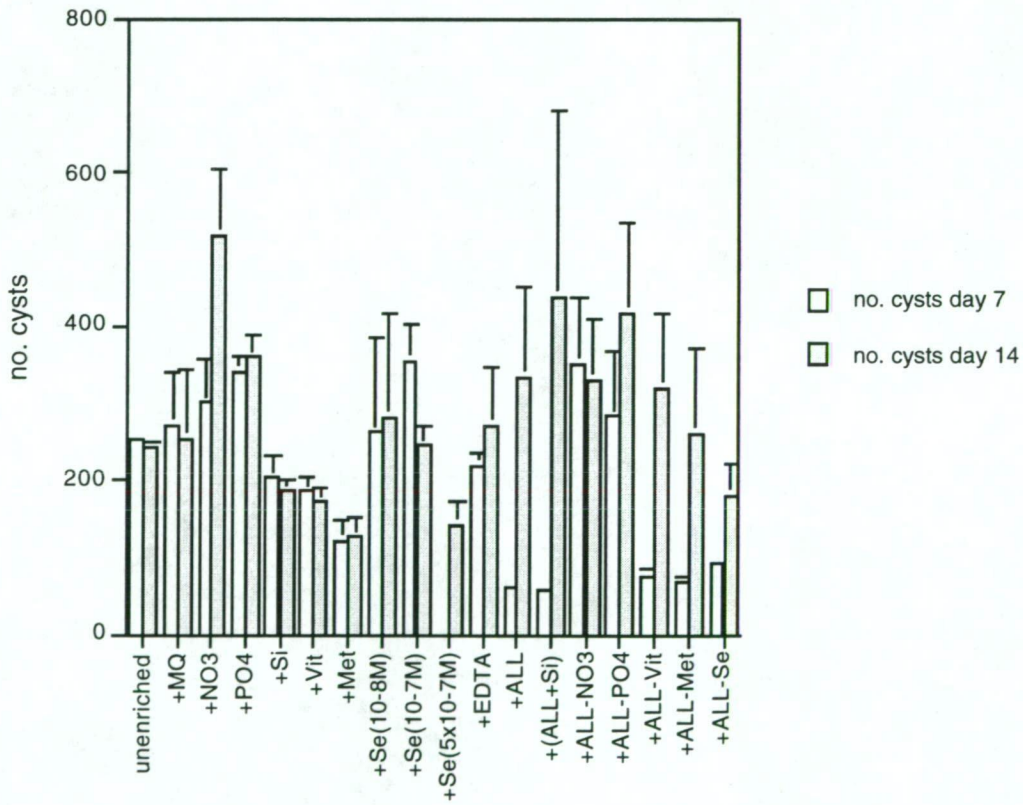


Fig. 6.2: Number of cysts produced on day 7 and 14 in a natural *G. catenatum* population enriched with nutrients: nitrate ($+NO_3$), phosphate ($+PO_4$), silicate (+Si), vitamins (+Vit), metals (+Met), ethylene diamine tetra-acetic acid (+EDTA), selenium (+Se $10^{-8}$, $10^{-7}$, 5 x $10^{-7}$ M), all nutrients (+ALL= $+NO_3$, $+PO_4$, +EDTA, +vitamins, +metals, +Se $10^{-8}$), ALL-$NO_3$, ALL-$PO_4$, ALL-metals, ALL-vitamins and ALL-Se.

production was greatest (approximately 500 cysts) in samples to which nitrate ($+NO_3^-$), all nutrients including silicate (ALL+Si) and all nutrients excluding phosphate (ALL- $PO_4^{3-}$) were added and lowest in samples enriched with silicate (+Si), vitamins (+Vit), metals (+Met), selenium (+Se($5x10^{-7}$M)) and all nutrients excluding selenium (ALL-Se) (100-200 cysts). Cyst numbers increased markedly during the second week in cultures enriched with all nutrients excluding vitamins (ALL-Vit), all nutrients excluding metals (ALL-Met), all nutrients excluding selenium (ALL-Se), all nutrients (ALL) and all nutrients including silicate (ALL+Si) (Fig. 6.1). Unenriched and Milli-Q® control samples and those enriched with $PO_4^{3-}$, Si, Vit, Met, Se (10-500 nM), EDTA, ALL- $NO_3^-$ and ALL- $PO_4^{3-}$ showed little or no increase in cyst numbers from day 7 to 14 (Fig. 6.2).

There was no significant difference in encystment in any of the enrichment cultures on day 14 ($n = 3$, $F = 1.55$, $p = 0.13$). However, comparison of the number of cysts produced when nutrients ($NO_3^-$, $PO_4^{3-}$, Se, Si, Vit and Met) were present or absent showed that there was a trend of greater cyst production when nitrate and selenium were present rather than absent. In contrast, there were a greater number of cysts produced in cultures when they weren't enriched with phosphate, silicate, vitamins or metals and approximately the same number of cysts were produced in cultures which had no or 10 µm EDTA (Fig. 6.3b). However, the ANOVA did not indicate that the presence or absence of any particular nutrient promoted encystment ($n = 3$, $F = 1.88$, $p = 0.13$).

Approximately two weeks after nutrient enrichment, cysts from incubated bloom samples began to excyst (Fig. 6.4). Empty (germinated) cysts were therefore counted on day 14 to examine the length of cyst dormancy in cultures under different nutrient conditions. Control cultures (unenriched or enriched with Milli-Q®) had the highest numbers of germinated cysts (30-50), followed by +Vit (30 cysts), +Se ($5 \times 10^{-7}$ M) (25 cysts) and + $PO_4^{3-}$ (20 cysts), and (+ALL-Vit), (ALL-Se), (+ALL) and (+ALL+Si) had the lowest levels of excystment (approximately 2 empty cysts per culture). Control cultures (unenriched and those enriched with Milli Q®) and vitamin-enriched cultures had the greatest proportion of germinated cysts (12-19%) compared to other cultures (<9%). The ANOVA confirmed that rates of excystment were different between treatments ($n = 3$, $F = 10.0$, $p < 0.0001$), with control treatments having significantly greater rates of germination ($n = 3$, $F = 79.9$, $p < 0.0001$).

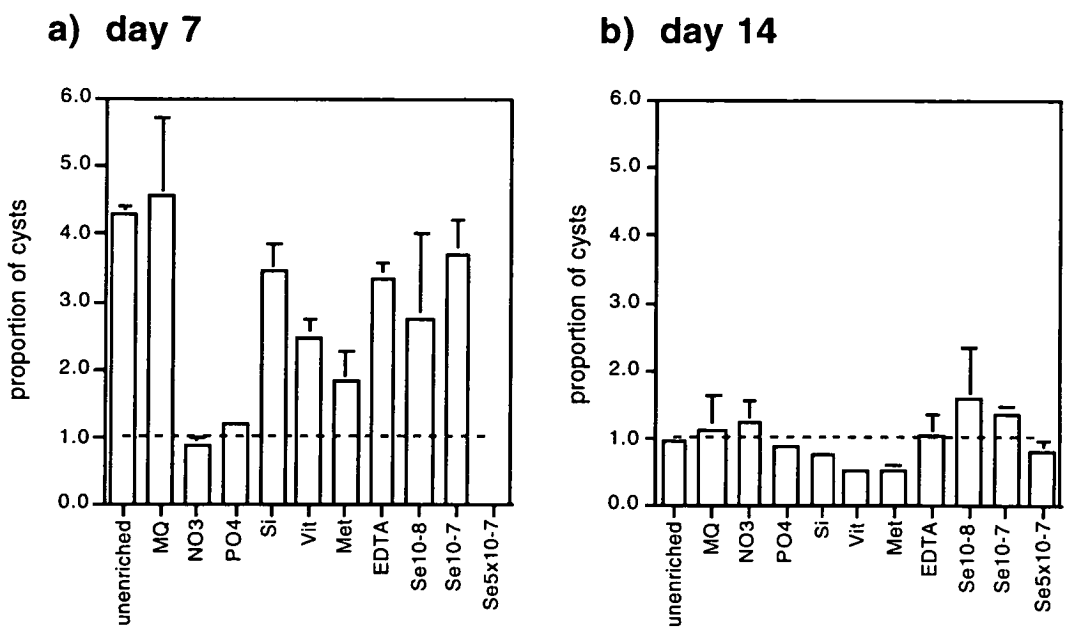


Fig. 6.3: Proportion of cysts produced in a natural *Gymnodinium catenatum* population when nutrients ($NO_3$, $PO_4$, Si, vitamins, metals, EDTA, Se ($10^{-8}$, $10^{-7}$, 5 x $10^{-7}$ M)) are present or absent (e.g. $NO_3$ = no. cysts produced in +$NO_3$ cultures / no. cysts produced in ALL- $NO_3$ cultures). Controls (unenriched and MQ = no. cysts produced in unenriched or MQ cultures / no. cysts produced in fully enriched (ALL) cultures). The horizontal line represents the situation where the same number of cysts are produced in the presence or absence of individual nutrients. Proportions greater than one represent the situation when more cysts are produced in the presence of individual nutrients, compared to the situation where there is greater encystment in the absence of individual nutrients (proportions less than one); n = 3; error bars = SE; day 7 (a), day 14 (b).

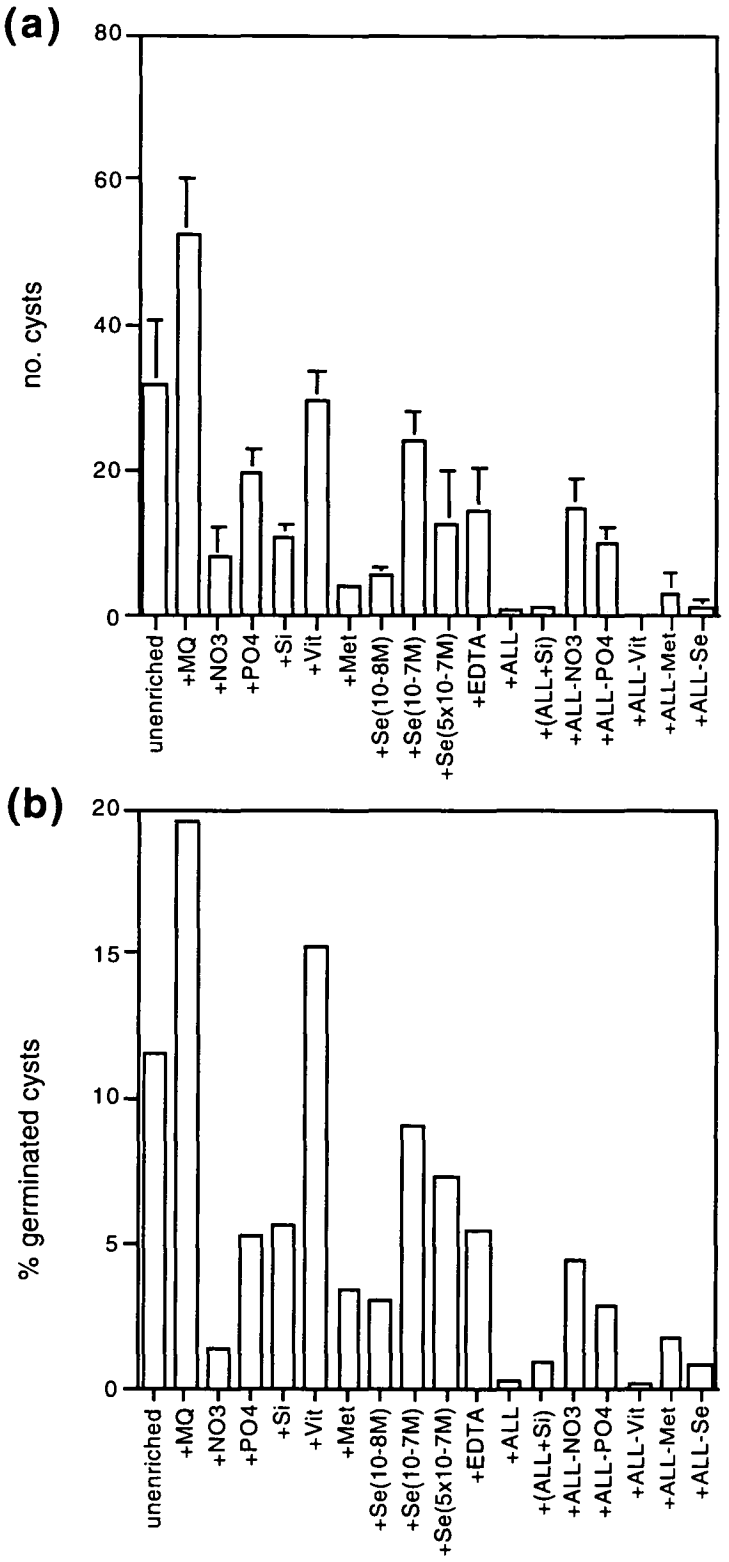


Fig. 6.4: (a) Number and (b) percentage of cysts which germinated 14 days after addition of nutrients in 10ml of a natural *Gymnodinium catenatum* population: $NO_3$, $PO_4$, Si, vitamins, metals, EDTA, Se ($10^{-8}$, $10^{-7}$, 5 x $10^{-7}$ M), ALL ($NO_3$, $PO_4$, EDTA, vitamins, metals), ALL-$NO_3$, ALL-$PO_4$, ALL-metals, ALL-vitamins and ALL-Se.

## 6.4 Discussion

### *6.4.1 The effect of humic additives on* G. catenatum *cyst production in culture*

The present study showed that addition of humic substances (in the form of standard aquatic humic acid or 0.2 µm filter sterilised Huon River water) had no effect on *G. catenatum* encystment, despite the potential of such organic compounds to stimulate vegetative growth and change nutrient availability for *G. catenatum* uptake (Doblin *et al.* 1998; Chapter 2). This is in contrast to similar studies which have found increased incidence of sexual dinoflagellate stages in the presence of humic, fulvic or hydrophilic acids (Heil 1996), mangrove sediment extract (Faust 1993) and other growth factors such as fetal bovine serum or platelet-derived growth factor (Costas *et al.* 1993). Riverine input of humic substances into south-east Tasmanian surface waters is important during *G. catenatum* bloom initiation (by provision of growth factors for vegetative division, Chapters 2 and 3). Although these compounds persist in surface waters during blooms (unpublished, CSIRO Huon Estuary Study), it is unlikely they form an encystment cue.

### *6.4.2 Induction of sexuality in* G. catenatum *populations*

Enrichment of a natural *G. catenatum* bloom population showed that nutrient availability plays a role in the induction of sexual reproduction in this dinoflagellate. Low nutrient concentrations (particularly nitrate and phosphate) promote encystment, while high nutrient concentrations inhibit cyst formation. This is consistent with previous observations of greater *G. catenatum* encystment in nitrate and phosphate deficient media (Blackburn *et al.* 1989), and supports the intuitive hypothesis that cysts are formed under adverse environmental conditions (Pfiester and Anderson 1987). Other species which have shown enhanced cyst production under nutrient limitation are *Peridinium cinctum* (Pfiester 1975), *Peridinium willei* (Pfiester 1976), *Alexandrium tamarense* = *Gonyaulax tamarensis* (Turpin *et al.* 1978, Anderson *et al.* 1984, Doucette *et al.* 1989), *Alexandrium monilatum* = *Gonyaulax monilata* (Walker and Steidinger 1979) and *Gyrodinium uncatenum* (Anderson *et al.* 1985).

While encystment of many phytoplankton species is induced by nitrate and / or phosphate deficiency, micronutrient limitation is also known to affect cyst production in some dinoflagellates. For example, Blanco (1995b) demonstrated that under Fe-deplete conditions, cyst production efficiency of the dinoflagellate *A. lusitanicum* increased. In this study, all trace elements apart from selenium were added together,

with little effect on *G. catenatum* encystment. Further investigation using ecological meaningful metal concentrations would provide further information on the role of such micronutrients in sexual reproduction.

After two weeks, encystment levels were the same in all treatments, suggesting that cells became nutrient deficient during the incubation and / or that other exogenous or endogenous factors apart from nutrients induce *G. catenatum* sexual reproduction. Although nutrient enrichments yielded external nutrient concentrations at least one order of magnitude greater than natural concentrations (Table 6.2), it is possible that *G. catenatum* cells were nutrient limited because of inadequate nutrient uptake (Anderson and Lindquist 1985). Cells could have been dividing at the expense of internal nutrient pools until they reached a threshold cell quota which triggered sexuality (Pfiester and Anderson 1987). Measurement of both external and internal nutrient concentrations, (e.g. Anderson *et al.* 1985) would allow proper evaluation of cellular nutrient status and a better assessment of the importance of nutrients in the induction of sexual reproduction.

An alternative explanation of these data is that induction of sexual reproduction was initiated before cells were captured and incubated. It is not clear how much time is required between induction of sexuality and cyst production, but if it is in the order of several days - weeks for *G. catenatum*, then encystment in cultures may not be related to nutrient concentration at all. Whether other factors can delay encystment once sexuality has been induced is also unclear. In this study, incubation of a natural *G. catenatum* population under fully enriched or nitrate and phosphate-replete conditions appeared to retard encystment. *G. catenatum* also produces temporary pellicular resting cysts (Blackburn *et al.* 1989) which suggests that the process of resting cyst (hypnozygote) formation may proceed in stages, which up until some point can be suspended or reversed. Further investigation is required to determine whether induction and encystment are regulated in the same manner and whether they can be decoupled by external or internal cues.

Although the life history cycle of *G. catenatum* has been well documented in culture (Blackburn *et al.* 1989), factors inducing sexuality in natural populations are little understood. Field observations of *G. catenatum* blooms show little correlation between the appearance of sexual stages and external macronutrient (nitrate and phosphate) concentrations (N. Parker, unpublished data, CSIRO Huon Estuary Study), suggesting that other factors are important in the induction of sexuality. Other studies have shown that temperature, light or external nutrient concentrations are important (alone or in combination) for encystment in various harmful agal bloom

species. Anderson *et al.* (1984) showed that encystment of the dinoflagellate *Alexandrium tamarense* (as *Gonyaulax tamarensis*) was influenced by temperature, with optimal cyst production occurring over a relatively narrow temperature range and inhibition of encystment at some temperatures that permitted growth. Hardeland (1994) found that encystment of *Gonyaulax polyedra* is photoperiodically controlled at 15 °C, but can also be induced by a decrease in temperature from 20 to 10 or 8 °C in the absence of photoperiodic signals. Imai (1989) reports that depletion of nutrients (especially nitrogen), low light intensity and the presence of solid substrates for adhesion are important factors for cyst formation in *Chattonella marina*, while Nakamura and Umemori (1991) show that encystment of *Chattonella antiqua* decreases exponentially with increasing irradiance. In addition, the dinoflagellate *Scrippsiella trochoidea* showed greater levels of encystment under N and P depletion with increased bicarbonate concentrations (Watanabe *et al.* 1982).

The importance of physical factors on sexual reproduction has also been investigated, since turbulence can inhibit asexual reproduction in some dinoflagellate species (e.g. *Gonyaulax polyedra*; Thomas *et al.* 1995). Blanco (1995b) agitated cultures of four neritic dinoflagellates to simulate turbulence and found that it had no or a negative effect on the efficiency of cyst production in *Scripsiella trochoidea*, *Ensiculifera* sp. and *Lingulodinium polyedra*, but encystment efficiency increased in *Alexandrium lusitanicum*.

Some phytoplankton species exhibit sexuality in the absence of any direct environmental changes. For example, the dinoflagellates *Gonyaulax spinifera* and *Polykrikos kofoidii* encysted spontaneously under batch culture (Taylor and Gaines 1989). In addition, Beam and Himes (1980) report sexuality in heterotrophic *Crypthecodinium cohnii* cultures without manipulation. Observation of greater *G. catenatum* encystment in water isolated from a *G. catenatum* bloom in the Huon Estuary compared to mixtures of Huon River water and seawater suggests that there are other factors (e.g. pheromones) influencing cell-cell interactions which promote sexual reproduction (N. Parker, unpublished data). For example, Blanco (1995b) found that when cultures of *Ensiculifera* sp. were exposed to culture filtrates of *G. polyedra* and *S. trochoidea*, cyst production efficiency increased. Such biological conditioning by phytoplankton species may also cause a negative effect on encystment of their own or other species. In the same study, *S. trochoidea* (non-toxic) filtrate caused a decrease in cyst efficiency of *G. polyedra*.

This study used cyst production as an unambiguous index of sexual reproduction since gametes are morphologically indistinguishable from vegetative cells and fusing

gametes are sometimes difficult to distinguish from dividing vegetative cells. However previous laboratory studies have shown that planozygotes can persist for up to 14 days before forming resting cysts (Blackburn *et al.* 1989), suggesting that cyst counts could underestimate the degree of sexuality within a cultivated or natural population. Groups of gametes and the presence of planozygotes could therefore be other useful life history stages with which to measure the induction of sexual reproduction. Subsequent assessment of cyst viability (by observation of cyst germination) would also provide a useful index of reproductive success.

### *6.4.3 Dormancy and excystment of* G. catenatum *resting cysts*

Consistent with the findings of Blackburn *et al.* (1989) and Bravo and Anderson (1994), this study showed that cysts formed in enrichment cultures of a natural *G. catenatum* population have a short dormancy period (<14 days) and germinate under a wide range of external nutrient conditions. The lower rates of excystment under conditions of high nutrient availability (e.g. ALL and ALL+Si treatments) and higher rates of excystment under nutrient deficient conditions (control treatments which were either unenriched or enriched with Milli-Q), are probably a reflection of faster cyst production rates in these treatments rather than any specific excystment cues.

Cues implicated in excystment of other phytoplankton species include changes in temperature (e.g. *Gonyaulax tamarensis* = *Alexandrium tamarense*; Anderson and Morel 1979), DO concentrations in bottom waters (e.g. *Scrippsiella* spp.; Ishikawa and Taniguchi 1994) and light (e.g. *Gonyaulax polyedra*; Anderson *et al.* 1987). Some species such as the marine dinoflagellate *Scrippsiella trochoidea* do not require a shift in temperature or nutrient conditions for cyst germination, although there is a large decrease in germination rate at low temperatures (Binder and Anderson 1987). The relatively short dormancy period of *S. trochoidea* (25 days), combined with the absence of requirement for a large change in environmental conditions facilitates rapid cycling between resting and vegetative stages in natural populations, while the simultaneous reduction in germination rate at low temperatures also permits cells to overwinter. *G. catenatum* also has a short dormancy period (Blackburn *et al.* 1989) and decreased rates of germination in the dark and at temperatures < 16 °C (Bravo and Anderson 1994). The rare occurrence of planomeiocytes (recently germinated cysts) in field samples at the beginning of summer when water temperatures are > 15 °C (Bolch and Blackburn, unpublished data) supports the hypothesis that *G. catenatum* blooms are unlikely to be initiated from a synchronised mass germination event and that regular germination throughout the year would help to maintain a perennial motile vegetative

population (Hallegraeff *et al.* 1995). Further discussion of the importance of cysts in *G. catenatum* bloom dynamics is found in the following Chapter.

This study shows that humic additives (in the form of standard aquatic humic acid and natural dissolved organic matter in Huon River water) play a minor role in the induction of *G. catenatum* sexual reproduction in culture. However encystment of natural *G. catenatum* populations is dependent on external nutrient concentrations. Greater numbers of cysts were produced in bloom samples deficient in nitrate and phosphate, with low levels of encystment in fully enriched samples. Because cysts were counted rather than fusing gametes or planozygotes, the nutrient dynamics associated with induction of sexual reproduction remain obscure. Detailed monitoring of nutrient pools and life cycle stages is required to resolve the timing of sexual induction in this species. The absence of any stimulation of *G. catenatum* sexuality in cultures enriched with humic substances indicates that the results of previous experiments described in Chapter 2 are not complicated by possible effects on sexual as well as asexual (vegetative) reproduction.

# Chapter 7: Discussion of *Gymnodinium catenatum* bloom development in south-east Tasmania

## 7.1 Introduction

The previous chapters have examined some of the physiological responses of the toxic dinoflagellate *G. catenatum* to the individual and combined effects of dissolved organic substances, the trace element selenium and the macronutrient nitrate. Diel vertical migration experiments in stratified laboratory columns extended these nutritional studies to include physical, biological and chemical factors involved in bloom development. Although field observations suggest that these factors are all important during bloom initiation and maintenance, development of a *G. catenatum* bloom in the Huon Estuary during December 1997 allowed an assessment of their relative importance in the ecological success of this organism. The discussion below addresses each of the different phases of *G. catenatum* bloom development (initiation, maintenance and decline) and outlines the critical processes leading to population dominance during monospecific blooms.

## 7.2 Bloom initiation

*G. catenatum* blooms in the Huon Estuary, Tasmania have an autochtonous origin, developing during a seasonal temperature window following a rainfall "trigger" (Hallegraeff *et al.* 1995). There are two potential mechanisms for bloom initiation: accumulation of biomass (> 1 x $10^4$ cells $l^{-1}$) by (i) coordinated germination of resting cysts from sediments; and / or (ii) an over-wintering vegetative population during an extended period of enhanced growth. The relative importance of these two bloom initiation mechanisms in the development of *G. catenatum* blooms in south-east Tasmania is discussed below.

### *7.2.1 The cyst germination hypothesis*

The (i) short dormancy period of cultivated cysts (several weeks - months); (ii) wide range of germination conditions (Blackburn *et al.* 1989, Bravo and Anderson 1994); and (iii) rare occurrence of planomeiocytes (recently germinated cysts) in field samples (Bolch and Blackburn, unpublished data) led Hallegraeff *et al.* (1995) to conclude that cyst germination wasn't important in seasonal Tasmanian *G. catenatum* bloom formation.

*G. catenatum* cysts are well distributed throughout the middle and lower regions of the Huon Estuary, from Brabazon Point to Hideaway Bay (Fig. 7.1) including Deep Bay, Port Cygnet (Bolch and Hallegraeff 1990; Bolch, unpublished data). Laboratory studies have shown that while cysts have a short (approximately 2 weeks) dormancy period (Blackburn *et al.* 1989; Chapter 6), germination is enhanced by light and temperature (>16 °C), suggesting that these may be key factors regulating *G. catenatum* cyst populations and their dynamics in sediments (Bravo and Anderson 1994). Sediment resuspension (caused by strong winds or storm events), with consequent exposure to higher irradiance and dissolved oxygen concentrations, may therefore induce *G. catenatum* cyst germination and could have initiated the 1997/8 bloom in the upper-mid estuary (as shown by the high river flow event preceding the

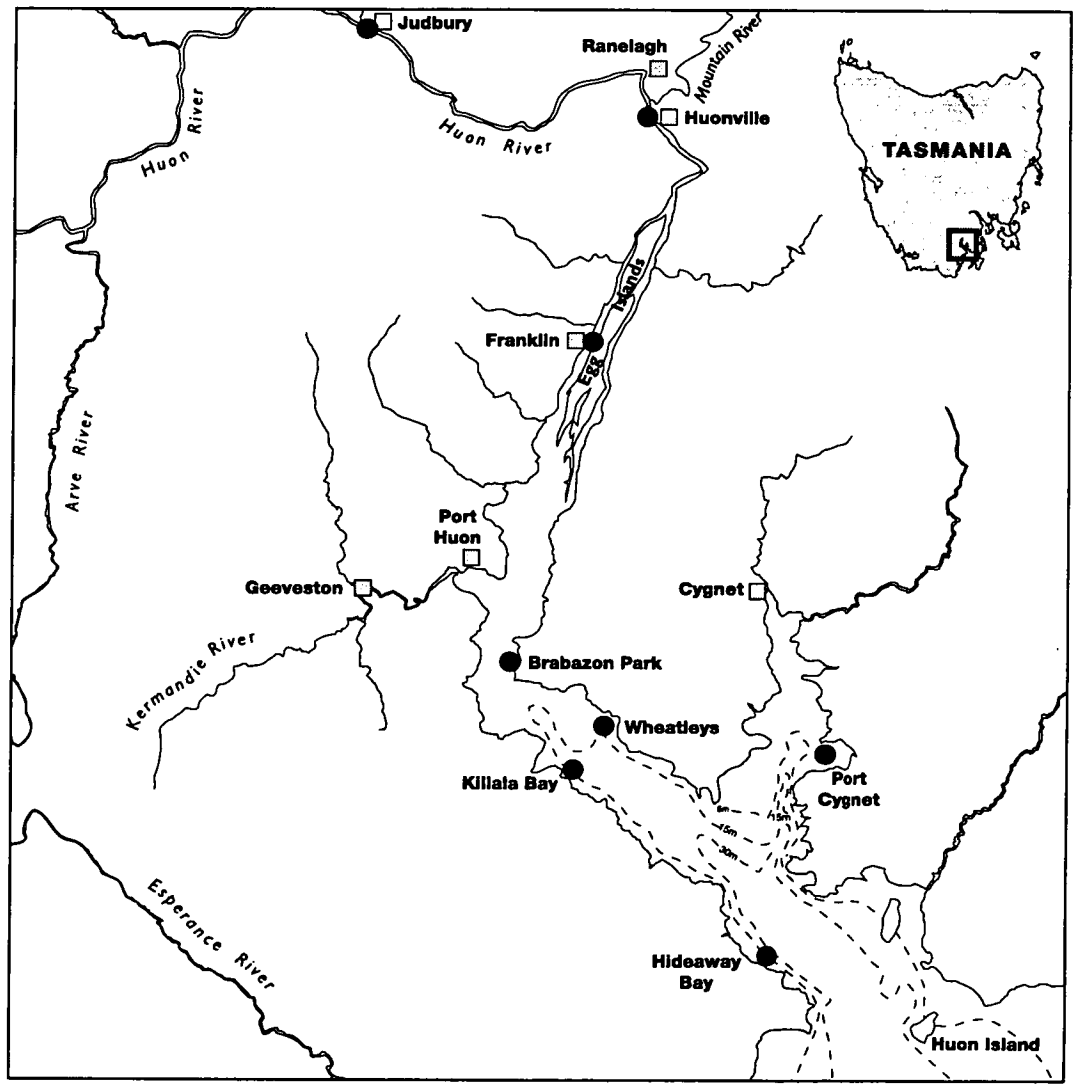


Fig. 7.1: The Huon Estuary, showing phytoplankton monitoring stations and water collection sites. Figure originally designed by M. Bessell and reproduced with the permission of CSIRO Division of Marine Research.

increase in cell density; Fig. 7.2). However, *G. catenatum* sexual stages (either planomeiocytes (recently germinated cysts) or planozygotes (recently formed cysts), "dancing" gametes, paired cells and resting cysts) were observed in net samples during all stages of the 1997/8 bloom with relatively constant frequency (N. Parker and M. Doblin, unpublished data). This suggests that *G. catenatum* cyst germination (and formation) occurs regularly and is not coordinated in a single excystment (or encystment) event such as bloom initiation in the toxic dinoflagellate *Alexandrium tamarense* (Anderson and Wall 1978, Anderson and Morel 1979). Deployment of a cyst germination trap (Ishikawa *et al.* 1995) is planned for future outbreaks, allowing real-time *in situ* quantification of *G. catenatum* excystment and a more thorough evaluation of the role of cysts in bloom dynamics (N. Parker, in progress).

### *7.2.2 The growth hypothesis*

Rapid cellular growth of dinoflagellates (> 1 division per day) has been shown to be a mechanism which could trigger blooms (Smayda 1995). The maximum growth rate of Tasmanian *G. catenatum* is approximately 0.33 $day^{-1}$ (equivalent to one division every 3 days; Blackburn *et al.* 1989), which means that under optimal conditions, it would take approximately three weeks for a bloom (>$10^4$ cells $l^{-1}$) to form with an initial cell concentration of $10^2$ cells $l^{-1}$. On 11 November 1997, *G. catenatum* cell density at Brabazon Park in the Huon Estuary (Fig. 7.1), was 1.8 x $10^4$ cells $l^{-1}$, and by 3 December 1997, it had increased to approximately 1.6 x $10^5$ cells $l^{-1}$ (Fig. 7.3). This is a rise of 1.4 x $10^5$ cells $l^{-1}$ in 2 weeks, indicating that *G. catenatum* biomass at Brabazon Park could have been formed *in situ* if growth rates approached 0.33 $day^{-1}$ (and biomass losses due to horizontal advection or sedimentation were minimal).

Favourable growth conditions for *G. catenatum* may be related to organic or other growth factors contained in river water (e.g. DOM or selenium), which in this study, have been shown to increase growth and cell yields by 10-20%. Measurement of *in situ* growth rates (by $^{14}C$ incorporation or other means) is necessary to confirm whether natural *G. catenatum* populations can achieve growth rates of 0.33 $day^{-1}$. Laboratory studies have shown that maximum growth rates for Tasmanian strains occur between 15 - 18 °C (Blackburn *et al.* 1989), indicating the importance of a seasonal temperature window for *G. catenatum* bloom development. *G. catenatum* bloom initiation is therefore most likely caused by a temperature dependent increase in vegetative growth (Hallegraeff and Fraga 1998), supplemented by growth factors such as DOM and selenium (this study), with biomass accumulation being facilitated by calm weather conditions and favourable hydrological conditions.

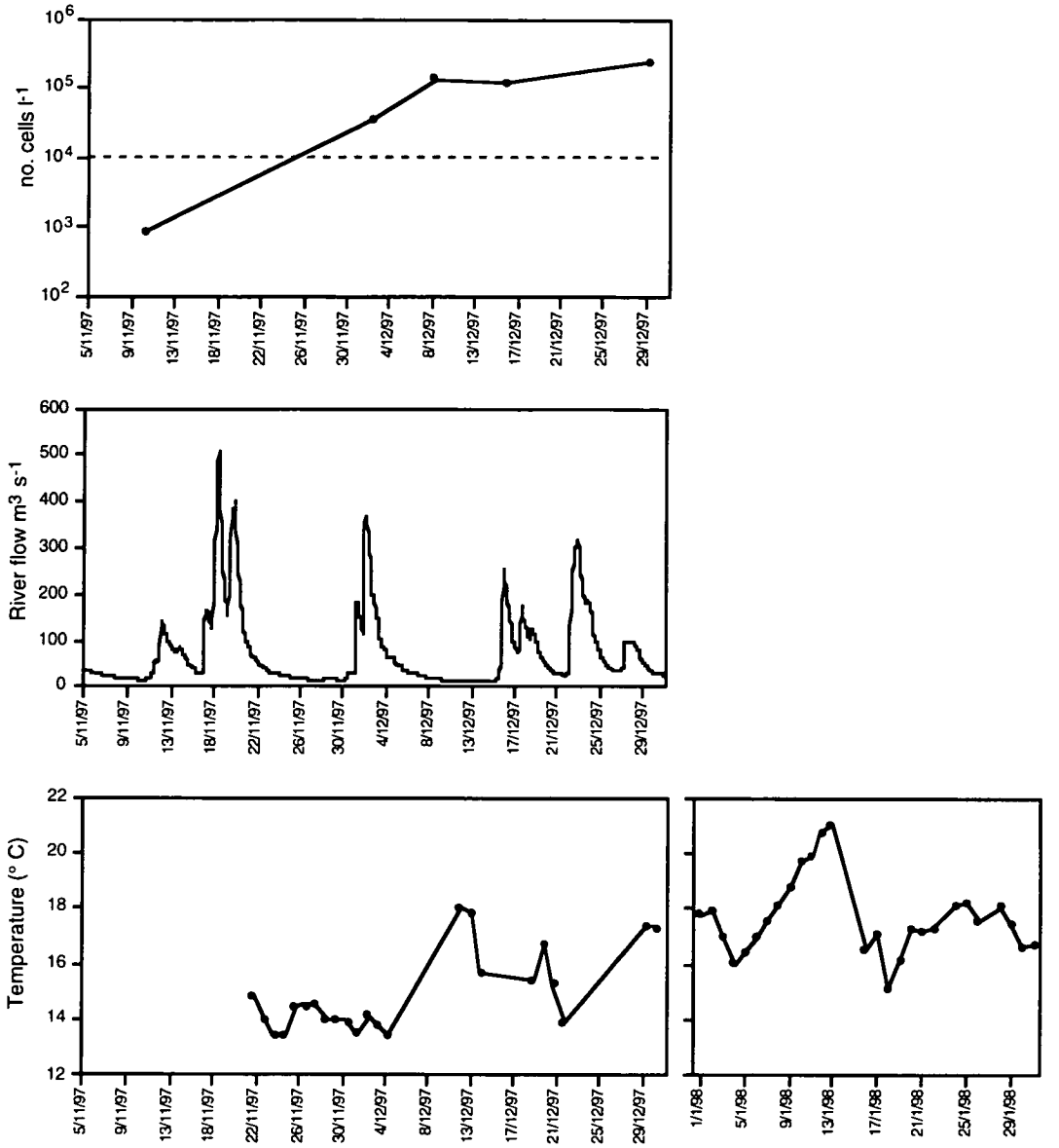


Fig. 7.2: *G. catenatum* cell density at Killala Bay (a), Huon River flow measured at Frying Pan Creek (b), and average daily surface water temperature at Killala Bay (c) from November - December 1997, showing rapid decrease on 16 January (arrow) following an intrusion of oceanic water into the Huon Estuary. Data were obtained from the DPIF and M. Sherlock (CSIRO Huon Estuary Study) and reproduced with the permission of CSIRO Division of Marine Research.

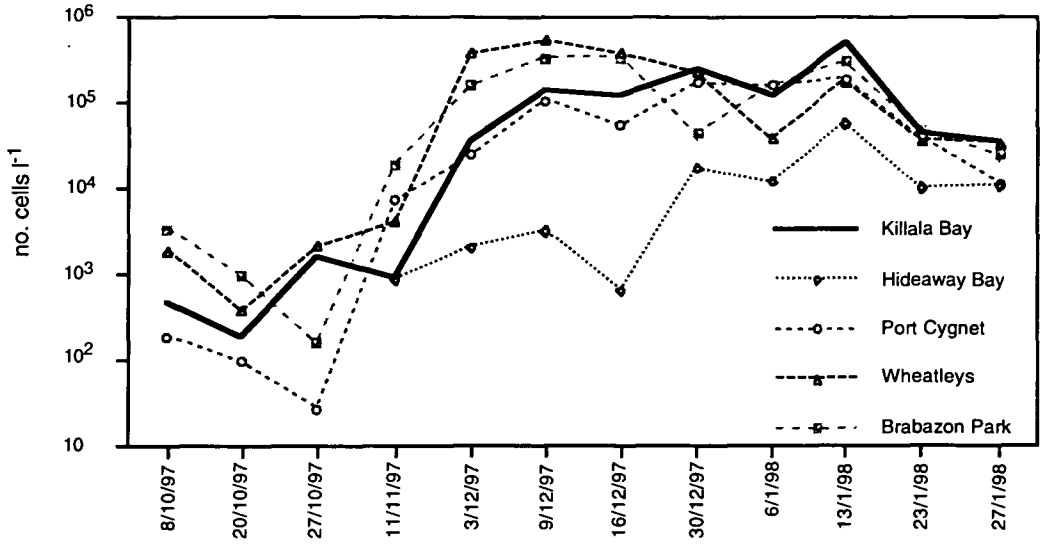


Fig. 7.3: *G. catenatum* cell density in the Huon Estuary from October 1997 - January 1998. Data were obtained from P. Bonham (CSIRO Huon Estuary Study) and reproduced with the permission of CSIRO Division of Marine Research.

## 7.3 Bloom maintenance

Since algae don't normally grow at their temperature dependent maximum growth rate even during bloom development (Riegman *et al.* 1993), and phytoplankton species succession can only be partly explained by seasonal changes in temperature and the optimal temperature for growth of the individual species (e.g. Karentz and Smayda 1984), it is likely that *G. catenatum* biomass during blooms is accumulated by additional mechanisms. *G. catenatum* is a classic K-strategist (MacArthur and Wilson 1967) directing proportionally more resources into non-reproductive activies (e.g. vertical migration) to maximise the exploitation of available resources (Kilham and Kilham 1980). *G. catenatum* is therefore slow growing relative to other phytoplankton (even at maximum growth rates) and achieves high biomass levels by being energy efficient (Hallegraeff 1998). This involves a competitive nutrient uptake strategy involving vertical migration (Chapter 5), facilitated by favourable hydrological conditions (vertical stratification) which support growth and biomass accumulation (Hallegraeff *et al.* 1995). It may also involve biological interactions such as production of toxins which could act as grazing deterrents and / or life cycle transitions from motile vegetative cells to benthic resting cysts (and vice versa) (Riegman 1998).

Riegman *et al.* (1996) in their competition experiments under N-, P- and light-limited growth conditions with mixtures of dinoflagellates, prymnesiophytes and diatoms

showed that smaller algae are better competitors for nutrients than larger ones. However under conditions where light and nutrients are vertically separated, larger species (such as *G. catenatum*) which are capable of vertical migration, may have a competitive advantage over smaller, non-migratory species (e.g. diatoms) that cannot access nutrients at depth (Cullen and MacIntyre 1998). The sensitivity of *G. catenatum* to disturbance in culture (Flynn *et al.* 1996, M. Doblin, unpublished data) and the fact that DVM of *G. catenatum* in the Huon Estuary has only been observed under vertically-stratified conditions (Fig. 7.4) suggests that water column stability is a necessary pre-requisite for a competitive nutrient uptake strategy by this dinoflagellate. In addition, the dense accumulation of biomass at the surface and the pycnocline during the DVM cycle (Chapter 5) may promote *G. catenatum* dominance by the physical exclusion of other phytoplankton species for access to light and nutrients, thus providing a self-perpetuating mechanism which allows persistance in space and time.

Although the function of toxins is still under debate (e.g. Wyatt and Jenkinson 1997) it has been suggested that toxic vegetative cells and resting cysts of harmful algae are poorly edible and this contributes to their low mortality rates during blooms and overall ecological success (Riegman 1998). Chain formation of *G. catenatum* vegetative cells could be an additional impediment to grazing because of faster swimming speeds (Fraga *et al.* 1990) and increased prey size. This has not been substantiated for *G. catenatum*, but low grazing pressure (release of top-down regulation) of water column and benthic biomass may be part of this species successful ecological strategy during outbreaks (see further discussion below). The short dormancy of *G. catenatum* resting cysts and the relatively constant rate of excystment/encystment during the 1997/8 bloom suggests that *G. catenatum* life cycle transitions between the benthos and water column provide a constant inoculum and help to maintain a perennial motile population (Hallegraeff *et al.* 1995).

## 7.4 Bloom decline

Observations of previous *G. catenatum* and other phytoplankton blooms suggest that the factors of (i) horizontal advection; (ii) grazing; (iii) encystment; and (iv) turbulence (with the concomitant input of nutrients to surface layers), acting alone or in combination, regulate *G. catenatum* abundance in the water column and may be responsible for bloom decline.

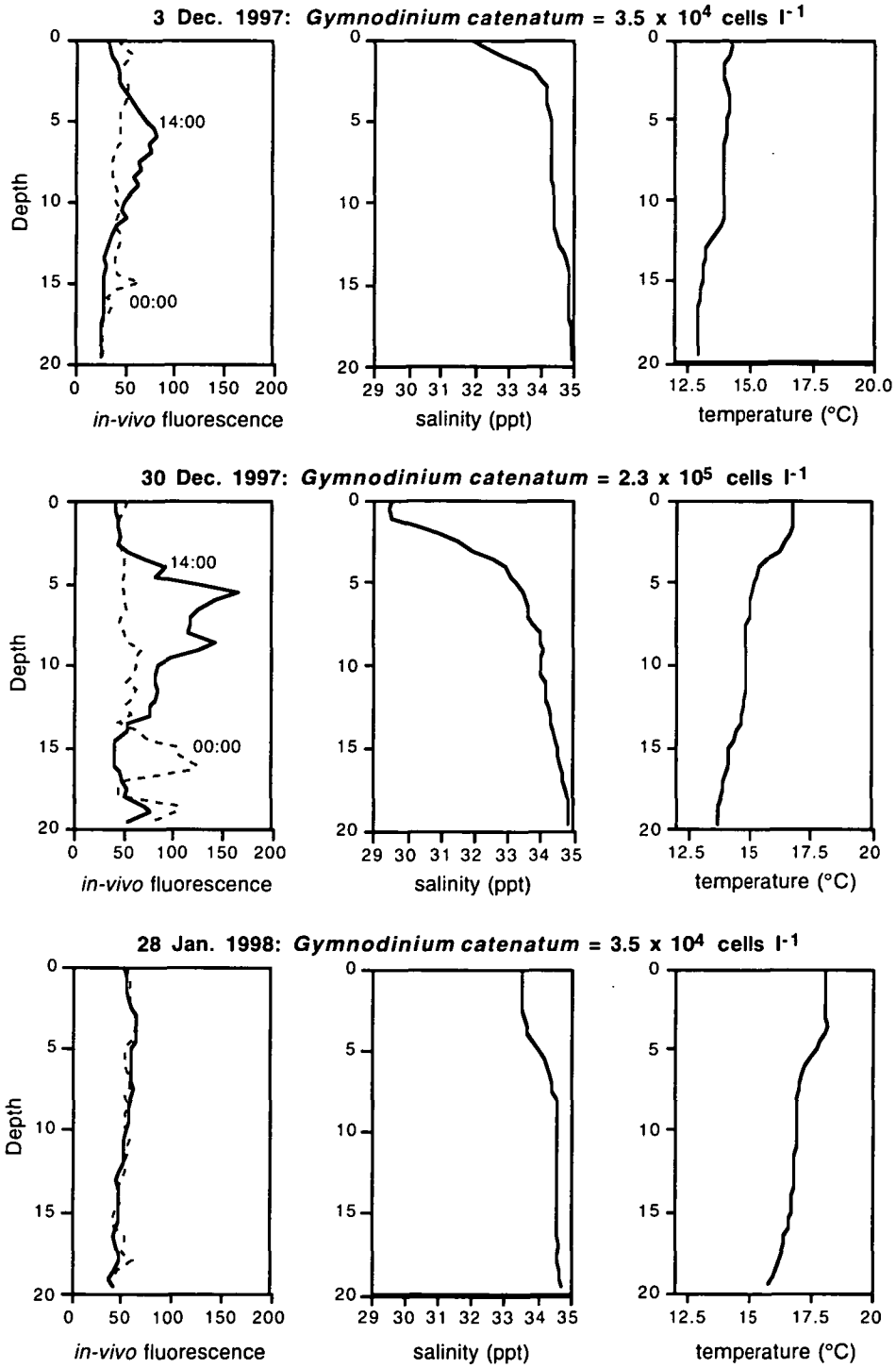


Fig. 7.4: Fluorescence, salinity and temperature profiles of the water column at Killala Bay on 3 December (early bloom), 30 December (mid bloom) and 28 Jan (end bloom), showing moderate, pronounced and slight degrees of vertical stratification. Times of fluorescence profiles are shown in 24-hour mode and indicate vertical migration of the phytoplankton biomass at night and its accumulation at a sub-surface maximum during the day. Data were obtained from M. Sherlock (CSIRO Huon Estuary Study) and reproduced with the permission of CSIRO Division of Marine Research.

### *7.4.1 Horizontal advection*

The Huon Estuary is a classic salt wedge estuary, with an upper freshwater layer and a lower seawater layer. Horizontal advection of *G. catenatum* biomass could therefore occur in surface waters as a result of high river flow, or as a result of tidal (or other) currents in bottom waters. Observations of Huon Estuary net samples (20 μm) throughout the year confirm that after heavy rainfall (resulting in high river flow), the phytoplankton population is less diverse and has a lower biomass, due to "washout" and the high concentration of suspended sediment in the water column. During low flow periods (e.g. 3 -14 December 1997; Fig. 7.2) , when the water column is stratified (Fig. 7.4), dinoflagellates become more abundant (Jameson 1993) and in warmer months (December - April), toxic *G. catenatum* biomass can rapidly accumulate (Fig. 7.3). In contrast, moderate river flow can also form a dispersal mechanism for vegetative *G. catenatum*. During the 1997/8 bloom, an increase in river flow (Fig. 7.2) caused movement of freshwater across the Estuary to form a brown coloured surface "slick", spreading *G. catenatum* vegetative cells from the eastern to the western shore (ie. from Brabazon Point and Wheatleys across to Killala Bay; Fig. 7.1).

Diurnal tides may also affect the spatial distribution of cells in the bottom layer of the Estuary, aiding both upstream and downstream dispersal. However the tidal range is relatively low (<1m) in most areas of the Huon Estuary and the tidal excursion is only a few kilometres long (up to Brabazon Point from the mouth of the Estuary; P. Morgan pers. comm.), suggesting that there may be minimal net biomass loss from the middle of the Estuary (where *G. catenatum* blooms are concentrated). The ability of *G. catenatum* to regulate its position in the water column by diel vertical migration (DVM) may also prevent large advective losses, similar to the dinoflagellates *Alexandrium tamarense* and *Heterocapsa triquetra* (Anderson and Stolzenback 1985) and the phototrophic ciliate *Mesodinium rubrum* (Crawford and Purdie 1992, see Chapter 5).

### *7.4.2 Grazing*

Little is known about grazing on *G. catenatum* in Tasmanian waters, but observations during the 1997/8 bloom indicate that the number of micro-invertebrate grazers (e.g. tintinnids) and heterotrophic algae (e.g. *Polykrikos schwartzii*) increase during bloom decline. These organisms can feed on live *G. catenatum* single cells and short chains at natural predator:prey densities (Sampayo 1997, M. Doblin and N. Parker, unpublished data). However the diversity of ingested tinitinnid prey in Huon Estuary samples (Fig. 7.5) suggests that they are generalist grazers, exerting top-down control

of *G. catenatum* abundance only when cell density increases during blooms. This is in contrast to grazing by the heterotrophic dinoflagellate *Polykrikos kofoidii* which caused rapid decline of a *G. catenatum* bloom on the Lisbon coast in Portugal (Sampayo 1997). The absence of any specialist grazers on *G. catenatum* in Tasmania may be one factor leading to the ecological success of this species, however large scale quantitative *in situ* studies are required to determine the impact of grazing on Tasmanian bloom dynamics.

### *7.4.3 Encystment*

Incubation of a natural *G. catenatum* bloom population under different nutrient conditions showed that encystment was higher in samples which were nitrate or phosphate deficient, with low levels of encystment in fully enriched samples (Chapter 6). This suggests that cyst production may be important in late bloom stages when external nutrient concentrations in surface and bottom layers are depleted. However observations of net samples during the 1997/8 *G. catenatum* bloom suggest that encystment rates were relatively constant, with no increase in cyst abundance during bloom decline (N. Parker and M. Doblin, unpublished data). Estimates of vertical biomass flux (including cysts) from the deployment of two sediments traps during the 1997/8 bloom will provide additional information to evaluate the importance of encystment in bloom dynamics.

### *7.4.4 Deterioration in growth conditions and increased diatom competition*

The decrease in *G. catenatum* biomass during the 1997/8 bloom was correlated with an increase in the abundance and diversity of diatoms, and a deterioration of the vertical stratification (characterised by a decrease in surface water temperatures and a decline in the vertical salinity gradient; Fig. 7.4). The changes in the physical structure of the Estuary are significant, not only because they are associated with an increase in faster-growing diatoms, but also because they generate turbulence and lead to surface nutrient input.

*G. catenatum*, like many other dinoflagellates, is extremely sensitive to disturbance, in both laboratory culture (Flynn *et al.* 1996, Thomas and Gibson 1990, Doblin unpublished data) and in nature (Hallegraeff *et al.* 1995). Double peaks of shellfish toxicity during most previous *G. catenatum* blooms (see Fig. 1.5), suggests that the persistence of this species in the water column is strongly affected by the degree of

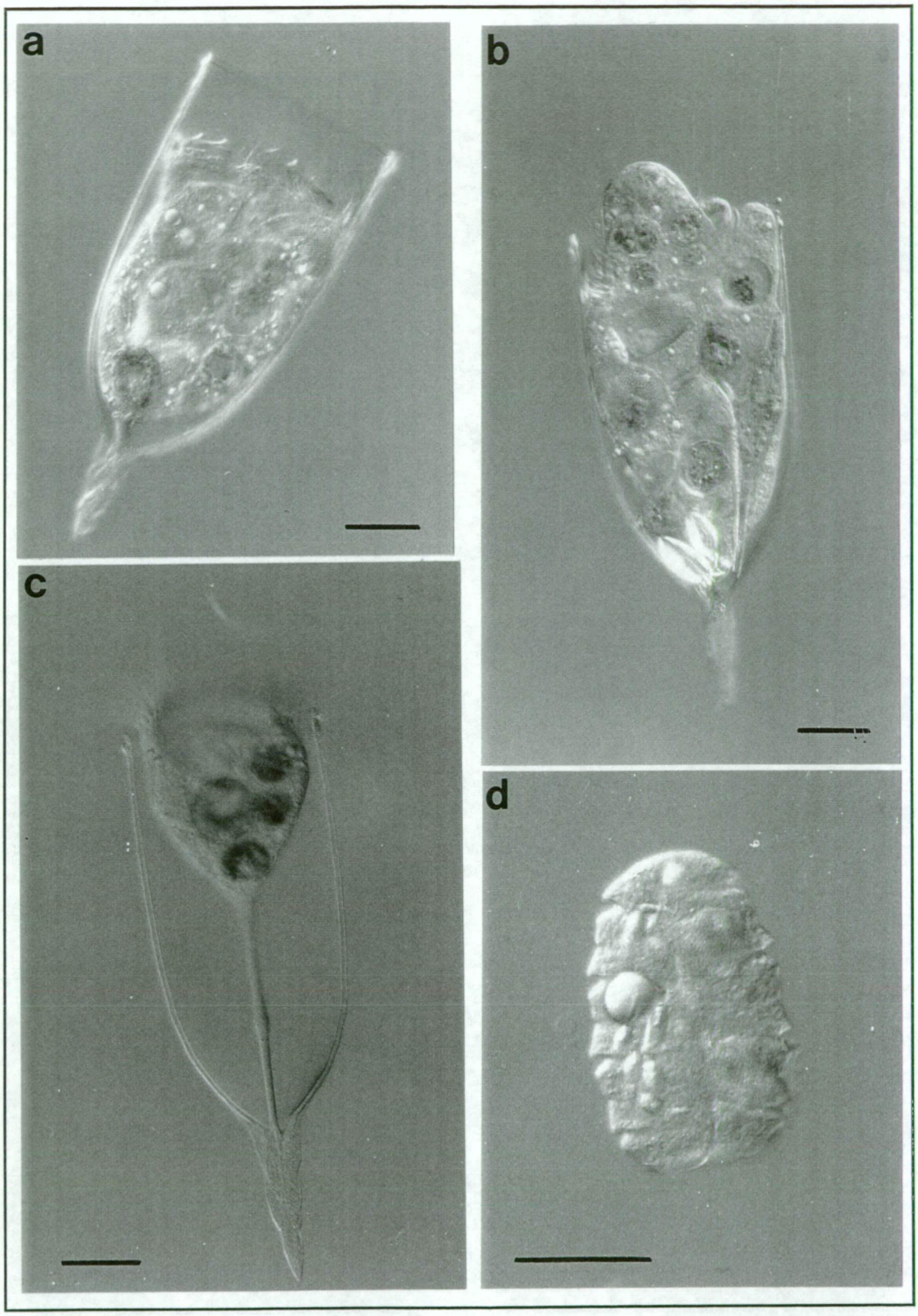


Fig. 7.5: LM. Generalist tintinnid grazers observed in 20 µm net samples collected in the Huon Estuary (Port Cygnet) on 13 January 1998, showing diversity of ingested prey organisms (a, b) and mode of feeding (c). Potential *G. catenatum* grazer *Polykrikos schwartzii* Bütschli, a heterotrophic flagellate (d). Scale bars = 40 µm.

turbulence or horizontal advection (Hallegraeff *et al.* 1995). This turbulence, caused by changes in wind direction/speed, tidal currents, river flow or large scale estuarine circulation, can result in growth inhibition. For example growth of the dinoflagellate *Gonyaulax polyedra* was inhibited during simulation of light winds near the sea surface (by incubation in the gap between rotating outer and fixed inner concentric cylinders) (Thomas *et al.* 1995).

However it is not only the physical action of disturbance which may inhibit *G. catenatum* growth. The resulting environmental conditions of decreased vertical stratification, input of nutrients to surface layers and greater diatom abundance may also be critical because slower growing organisms such as *G. catenatum* are unable to compete effectively under such conditions. Margalef (1978), in his studies of phytoplankton succession and species dominance, formulated a Mandala (Fig. 7.6) to predict the dominance of functional life forms according to prevailing environmental conditions: turbulence (coefficient of vertical eddy diffusivity) and nutrient concentration. Under turbulent conditions with relatively high concentrations of

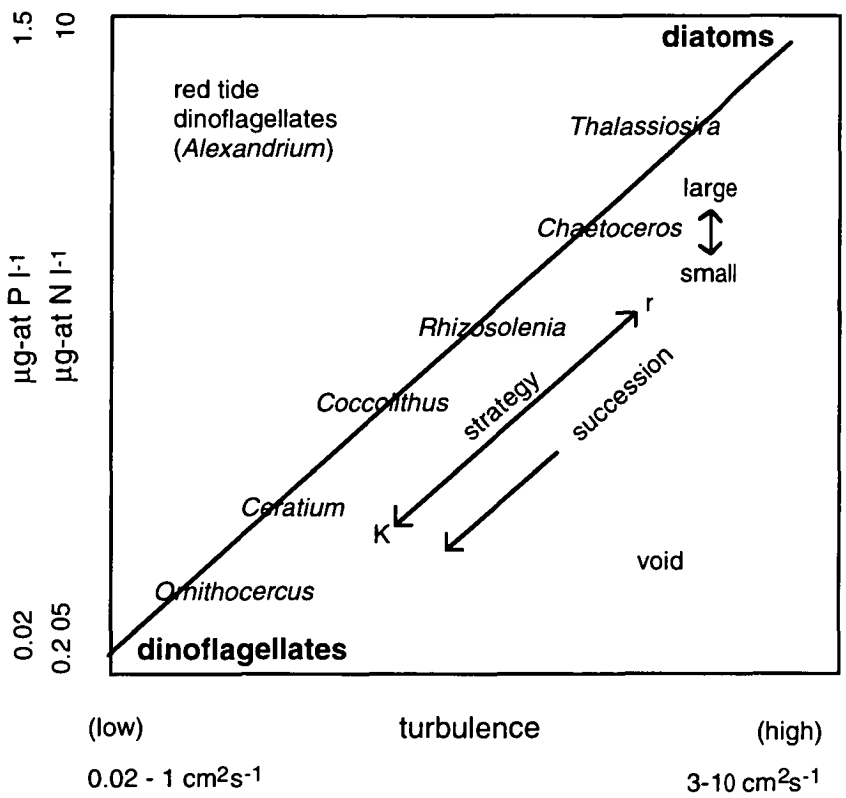


Fig. 7.6: Margalef's Mandala showing the principal phytoplankton life-forms in an ecological space defined by the concentrations of nutrients and by the coefficient of vertical eddy diffusivity. Source: Margalef 1978.

nutrients, diatoms such as *Thalassiosira* and *Chaetoceros* dominate the phytoplankton, and under calm conditions with relatively low nutrient concentrations, dinoflagellates such as *Ceratium* are dominant (Fig. 7.6). Under intermediate turbulence and nutrient concentration, diatoms such as *Rhizosolenia* and coccolithophorids are dominant. The degradation of vertical stratification in the Huon Estuary at the end of January 1998 (Fig. 7.4) coincided with an increase in surface nitrate concentration (Fig. 7.7) and an increase in the abundance of diatoms such as *Rhizosolenia imbricata* and *Chaetoceros socialis* (N. Parker, unpublished data), indicating a shift in species dominance towards r-selection (Fig. 7.6). This change in species dominance occurred very rapidly (< 2 days) with an intrusion of cold water into the Estuary on the 16 January (Fig. 7.2). The maintenance of such conditions and the sustained diatom growth within the Estuary probably played a significant role in *G. catenatum* bloom decline. This provides strong evidence of physical control of *G. catenatum* dominance in the Huon Estuary and supports the hypothesis that blooms can only form under prolonged periods of low turbulence (Hallegraeff *et al.* 1995). Nevertheless, intermittant periods of river runoff may be necessary to maintain a sufficient degree of water column stratification to separate light and nutrients to promote vertically migrating phytoplankton such as *G. catenatum*. Under moderately stratified conditions, when vertical nutrient and salinity gradients are smaller, other more stenohaline dinoflagellates which are not capable of true DVM but regulate their position in the water column (e.g. *Ceratium* spp.), may become dominant (Cullen and MacIntyre 1998). It is only after large perturbations which cause significant breakdown of vertical stratification that r-selected diatoms may become established. However, observation of *G. catenatum* cells in net samples under no apparent stratification (N. Parker, personal communication) suggests that there are other factors that determine phytoplankton species dominance and succession which remain to be elucidated.

In summary, it appears that the disruption of physical water column stability and the reduction in vertical nutrient gradients are the principal large scale factors leading to *G. catenatum* bloom decline. Biomass losses due to grazing and horizontal advection out of the Estuary may also contribute to a decrease in *G. catenatum* abundance, while the absence of a large increase in cyst production during late bloom stages suggests that encystment isn't important.

A pictorial representation of the principal factors involved in Tasmanian *G. catenatum* bloom initiation, maintenance and decline is provided in Fig. 7.8.

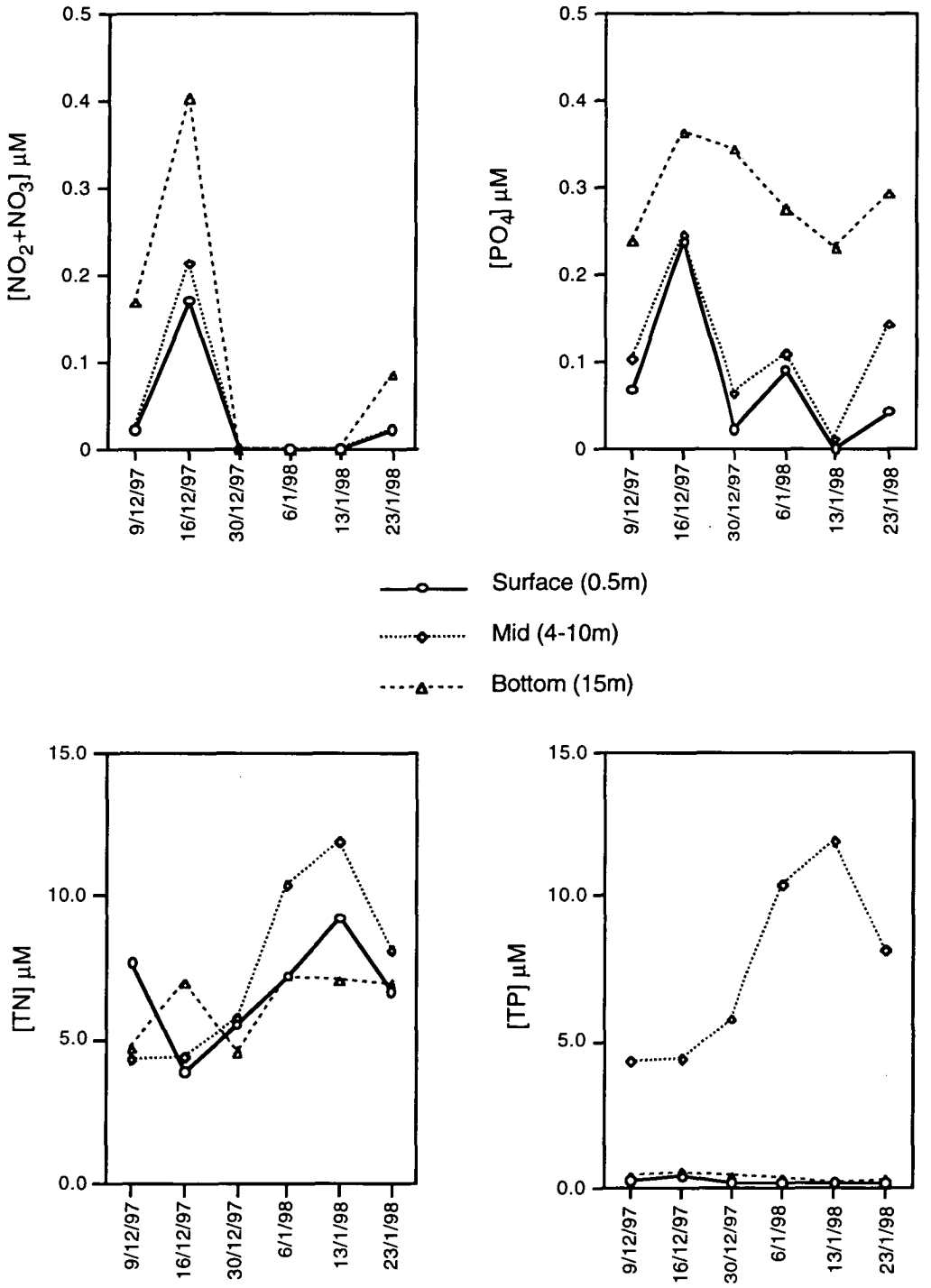


Fig. 7.7: Concentration of nitrite + nitrate (a), phosphate (b), total nitrogen (c) and total phosphorus (d) at Killala Bay during December 1997 - January 1998. Data were obtained from V. Latham (CSIRO Huon Estuary Study) and reproduced with the permission of CSIRO Division of Marine Research.

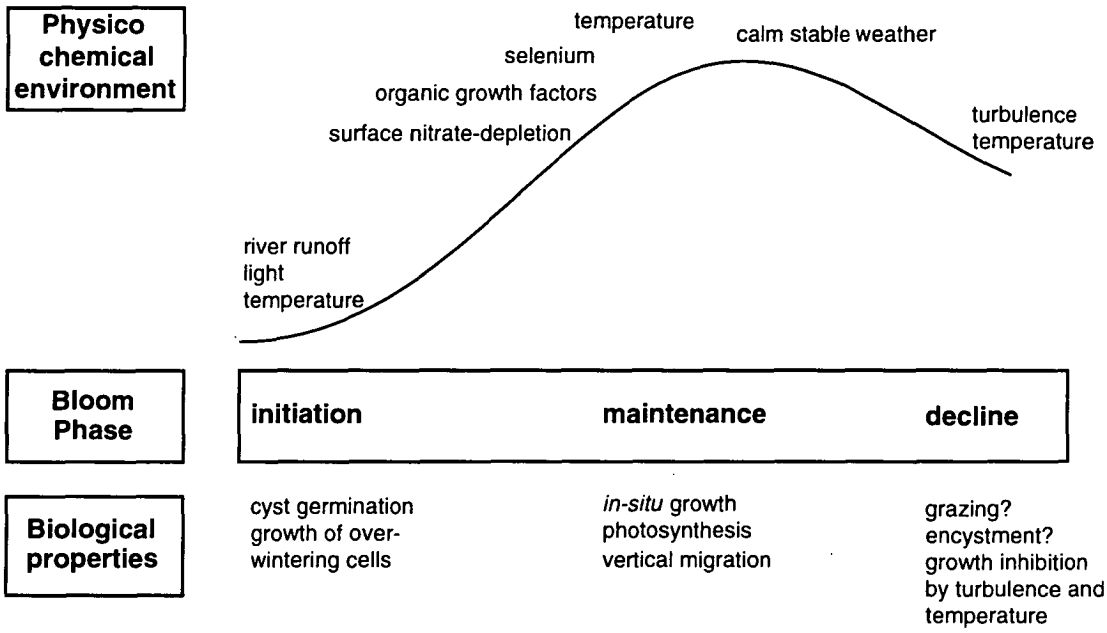


Fig. 7.8: Pictorial representation of the principal factors involved in Tasmanian *Gymnodinium catenatum* bloom development.

# Chapter 8: Further development of a conceptual model for *Gymnodinium catenatum* bloom formation in south-east Tasmanian waters

## 8.1 The role of river runoff in *Gymnodinium catenatum* bloom development

This study examined the hypothesis that river runoff and the concomitant input of DOM is important in *G. catenatum* bloom development. Use of small scale batch cultures (< 0.2 l) and large scale (approx. 9 l) laboratory columns allowed investigation of chemical, physical and biological variables associated with river runoff. Field data provided additional information against which to evaluate the role of river runoff in *G. catenatum* bloom development and place it into the framework of an existing conceptual model (see Hallegraeff *et al.* 1995).

In summary, this study has confirmed that river runoff is an important pre-requisite for *G. catenatum* blooms in south-east Tasmanian waters. Riverine input of freshwater and allochthonous DOM causes:

- stimulation of *G. catenatum* growth and biomass production (Chapter 2); and a
- shift in nutrient requirements (particularly selenium) for *G. catenatum* uptake (Chapter 2);

And from empirical field observations (Chapter 7), freshwater flow causes:

- vertical water column stratification, leading to the separation of light and nutrients which promotes vertically migrating phytoplankton species such as *G. catenatum.*

This study has also found that:

- *G. catenatum* has an obligate requirement for selenium, like other bloom forming PSP dinoflagellates *Alexandrium minutum* and *Pyrodinium bahamense* (Chapter 3);
- the selenium requirement of *G. catenatum* is different between strains isolated from different regions (Chapter 4);
- DOM and SHA play no role in the induction of *G. catenatum* sexual reproduction in culture (Chapter 6); and
- low external nitrate and phosphate concentrations promote *G. catenatum* encystment (Chapter 6).

## 8.2 The relative importance of dissolved organic matter and vertical stratification in *Gymnodinium catenatum* bloom development

The present study showed that addition of dissolved organic substances in the form of natural dissolved organic matter (DOM) and standard aquatic humic acid (SHA), caused a 15% and 30-60% increase in *G. catenatum* growth and biomass yields, respectively. DOM may stimulate growth by altering nutrient availability (e.g. through metal chelation or interactions with inorganic selenium) or by providing other growth factors (e.g. nitrogen), and could be a critical factor in *G. catenatum* bloom initiation. However, given the sensitivity of this species to disturbance (demonstrated in both laboratory culture and the natural environment), and the importance of vertical water column stratification in a competitive nutrient uptake strategy for *G. catenatum*, the stimulation of growth and biomass production by DOM requires concomitant stable physical conditions to allow bloom development.

## 8.3 The importance of selenium and nitrate in *Gymnodinium catenatum* bloom development

Selenium and nitrate are important in Tasmanian *G. catenatum* bloom development, since both of these nutrients affect *G. catenatum* vegetative cell growth (Chapters 2-5) and nitrate depletion may be involved in induction of sexual reproduction (Chapter 6). The relative importance of these nutrients in *G. catenatum* bloom development is further discussed below.

Laboratory bioassays with five *G. catenatum* strains demonstrated a variable requirement for selenium, with a direct effect on growth and biomass production at selenite concentrations of 1- 100 nM. Total selenium concentrations in the Huon Estuary are generally < 1 nM, indicating that at certain times of year and / or in certain parts of the estuary, selenium may be limiting for *G. catenatum* biomass production (and possibly also for growth). However experiments with unialgal *G. catenatum* cultures showed that in the presence of DOM and SHA, selenium was not the limiting nutrient for biomass production - nitrate is. In addition, vertical migration activity of *G. catenatum* was related to the nitrogen status of cells (but other factors are probably involved) and field data indicated that during summer, when surface nitrate concentrations are depleted and the water column is vertically stratified, *G. catenatum* is abundant (CSIRO Huon Estuary Study). This suggests that nitrate (or other forms of inorganic nitrogen) is the principal nutrient controlling the distribution of *G. catenatum* in these waters, with selenium availability also being an important regulating factor.

A greater understanding of the importance of nitrogen in bloom dynamics could be achieved with *in situ* $^{15}N$ incubations and measurement of internal nitrogen pools in natural *G. catenatum* populations (Dortch 1982, Dortch *et al.* 1985). Use of large volume enclosures to incubate natural *G. catenatum* populations (with different relative abundances of other dinoflagellates and diatoms) at different depths in the water column and manipulated to reproduce the vertical migration behaviour of *G. catenatum* may also provide useful information about the importance of DVM in allowing access to nitrate in bottom layers and in determining the outcome of phytoplankton species succession.

The experiments described in this study demonstrate that nitrogen, humic substances and selenium all have an affect on the yield of *G. catenatum*, with effects on growth being considerably smaller. These nutrients, rather than controlling growth limitation, are therefore more important in determining biomass yields, although continuous cultures (e.g. chemostats) could be used to test the hypothesis that these nutrients are also involved in *G. catenatum* growth limitation.

## 8.4 Bloom prediction and management

Ideally, bloom management should take an active (ie. prediction) rather than a reactive (ie. monitoring and detection) approach. While our knowledge about the factors determining phytoplankton species dominance and succession is growing, and modelling of the relationship between nutrient loading and algal biomass is improving (e.g. Vollenweider and Kerekes 1980), prediction of algal blooms at the species level is still limited. Furthermore, observations of *G. catenatum* blooms in other areas (e.g. Spain, Portugal, Mexico) and the strain-specific differences in nutrient requirements demonstrated in this study, suggest that predictive models need to be at least partly site-specific (Hallegraeff *et al.* 1995).

The ecophysiological and population characteristics of *G. catenatum* demonstrated in this study contribute significantly to our knowledge and predictive capability of blooms. This combined with current research and monitoring efforts will improve bloom prediction in south-east Tasmania. This research and monitoring includes:

- completion of an estuarine sediment survey to identify cyst beds and potential "hot spots" for bloom formation (N. Parker, in progress);
- real-time *in situ* measurements of excystment to determine the rate of resting cyst germination under different environmental conditions (N. Parker, in progress);

- laboratory studies investigating excystment/encystment cues of wild *G. catenatum* cysts/vegetative cells (respectively) (N. Parker unpublished);
- knowledge of the genetic diversity within *G. catenatum* populations (C. Bolch, in progress) and how this relates to cellular nutrient requirements;
- development of a monitoring buoy to measure local meteorological conditions such as sea surface wind speeds (Hallegraeff *et al.* unpublished data);

- measurement of real-time vertical profiles of salinity, temperature, dissolved oxygen and fluorescence, which will allow monitoring of the physical structure of the Estuary (e.g. extent of vertical stratification), and the vertical distribution of phytoplankton (including *G. catenatum*) (Huon Estuary Study, CSIRO); and
- faster data retrieval from monitoring buoys and platforms using telemetry (Huon Estuary Study, CSIRO).

Also needed is:

- a better understanding of phytoplankton dynamics in the Huon Estuary by testing the hypotheses relating turbulence and nutrient concentrations (and light) to phytoplankton dominance and succession in laboratory water columns or large field enclosures.

For water managers and users there are a number of already defined critical factors for bloom development:

- Water temperatures > 15 °C (December - June);
- Calm weather (high insolation, low wind speed);
- Low surface nutrients (promoting the existence of vertically migrating phytoplankton which are able to access nutrients at depth); and
- Low surface salinity (which excludes more stenohaline, depth regulating dinoflagellates and other non-migratory phytoplankton such as diatoms)

Monitoring of local weather conditions and regular examination of water and net samples for phytoplankton composition (carried out by Department of Health and Community Services staff) will allow aquaculturists to take appropriate precautions (e.g. early harvesting or translocation of stocks) when *G. catenatum* abundance increases, minimising the impact such blooms can have on their shellfish produce.

Unfortunately, once a bloom has been initiated, there is limited opportunity to halt its development. If it is detected promptly, it may be possible to contain the bloom or restrict its dispersal into other parts of the Estuary (e.g. by use of impermeable "curtains") but this may not be practical over large spatial scales. Alternatively, farmers may translocate cages or rafts or consider offshore aquaculture developments, although they may risk contaminating new sites by providing a vector for vegetative cell and resting cyst dispersal. Strategies to inhibit vegetative cell growth may include reducing irradiance (PAR) levels (e.g. floating shade cloth) and increasing turbulence (e.g. use of large bubblers/agitators). Reduction of diffuse and point source nutrient discharges, while limiting the proliferation of fast-growing phytoplankton such as diatoms, may do little to reduce the risk of *G. catenatum* blooms, since this dinoflagellate can migrate vertically to access nutrients at depth.

This study has shown that river runoff is an important pre-requisite for the initiation and development of Tasmanian *G. catenatum* blooms by providing suitable chemical and physical conditions for growth and biomass accumulation. It has also highlighted the diversity in ecophysiological responses within and between *G. catenatum* populations, suggesting that blooms of this toxic dinoflagellate can develop under different conditions in different regions. Understanding, modelling and predicting such blooms therefore requires both a site-specific as well as generic approach.

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