Prymnesiophytes of New Zealand's coastal waters: taxonomy, physiology and ecology.

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Abstract

Prymnesiophytes are an important component of the marine phytoplankton of New Zealand coastal waters, but there is little knowledge of the taxonomy, physiology or ecology of local strains. The Class comprises four orders which contain putative families and genera of the microscopic organisms, the microalgae. The Prymnesiophytes are known for their ability to explode in population numbers into "blooms"; one of New Zealand's most common bloom-formers is the coccolithophore *Emiliania huxleyi* (Prymnesiophyceae), which dominated the extensive phytoplankton blooms observed around New Zealand's coastline during 1993 - 94. Aspects of the physiology of and factors contributing to bloom formation in *E.huxleyi* are investigated.

The phenomenon of seasonal blooms, a common occurrence amongst the microalgae, is reviewed. Studies of the blooms which occurred along the north-east coastline of New Zealand in 1992-93 showed that these were unusual events that were linked to the climatic conditions at that time, in particular cold sea temperatures associated with an "El-Niño" phase of the Southern Oscillation Index in the southern Pacific Ocean. The dominant microalgae were *Gephyrocapsa oceanica* (Prymnesiophyceae) and *Fibrocapsa japonica* (Raphidophyceae) and it is probable that the conditioning of the coastal waters by these microalgae had a role in the succeeding toxic event, in which human illnesses were definitively linked to shellfish toxins due to microalgae for the first time in New Zealand.

Allelopathic, or chemical, interactions between microalgae were investigated in this study and *Prymnesium parvum* and *P.patellifera* caused inhibition of the growth of species from several microalgal classes. Unfortunately *P.patellifera* rapidly lost its inhibitory activity *in vitro*, but *P.parvum* remained active for

several years in culture and was therefore selected as a positive control for the bioassays for ichthyotoxicity that were developed.

The class Prymnesiophyceae includes toxic species of three genera, namely *Chrysochromulina, Prymnesium* and *Phaeocystis.* Ichthyotoxin bioassays based on toxin sensitive microalgae (*Chattonella antiqua* and *Heterocapsa triquetra*), shellfish larvae (*Haliotis iris*), brine shrimps (*Artemia salina*) and salmon erythrocytes (*Oncorhynchus tshawytscha*), were developed or refined and evaluated to enable the rapid and inexpensive detection of the haemolytic and cytolytic prymnesiophyte toxin, prymnesin.

The novel quadriflagellate species *C.quadrikonta,* which bloomed in north-east New Zealand, May 1994, exhibited low levels of haemolytic activity in stationary phase cultures grown in standard nutrient medium (at 18^0C, 100 μmol $m^{-2}s^{-1}$) as determined by the erythrocyte assay; no other *Chrysochromulina* species tested was toxic.

Six locally occurring species were identified by electron microscopic examination of scales and light and electron microscopic observation of cultured isolates in this study. Seventeen of the nearly fifty named species of *Chrysochromulina* have now been identified in New Zealand. The average cell sizes and unmineralised spine scale lengths of the New Zealand isolates of *C.ericina, C.hirta* and *C.quadrikonta* were slightly larger than for their type species and the calcareous scales of *E.huxleyi* and *G.oceanica* were more heavily calcified than their northern hemisphere counterparts. No gradation of calcification with increased latitude was observed for the coccolithophores, as had been noted previously, and this might reflect the consistently lower sea temperatures prevailing, due to the unusually protracted "El-Niño" climatic conditions.

Fluorescent probes proved to be useful tools for the differentiation of some morphologically-like species under the light microscope: Calcofluor white helped distinguish between cells of *C.ericina* and *C.quadrikonta.* The differentiation of the genera *Prymnesium* and *Chrysochromulina* was enabled through the specific binding of fluorescently-tagged wheat germ lectin to *Chrysochromulina* species.

The growth characteristics and cultural idiosyncrasies of several southern hemisphere *Chrysochromulina* isolates have been described and compared with the toxic northern hemisphere relative, *C.polylepis*. *C.polylepis, C.ericina* and *C.hirta* fell into a temperate group on the basis of optimum growth rates (doublings d^{-1}), while *C.acantha, C.apheles, C.camella* and *C.quadrikonta* fell into a sub-tropical group. All but *C.acantha* grew equally well with potassium nitrate, urea or ammonium chloride; *C.acantha* grew significantly slower with urea as nitrogen source. Only *C.quadrikonta* had a selenium requirement for growth. Maximum growth rates (doublings d^{-1}) recorded *in vitro* were *C.acantha*, 1.2; *C.apheles*, 0.9; *C.camella*, 1.1; *C.ericina*, 1.5; *C.hirta*, 2.4 and *C.quadrikonta*, 1.4. The *Chrysochromulina* species and *E.huxleyi* and *G.oceanica* grew at low light intensities (25 μmol $m^{-2}s^{-1}$), which could give these prymnesiophytes a competitive advantage in bloom situations, where shading due to the phytoplankton biomass can occur.

The New Zealand isolate of *G.oceanica* grew optimally at salinities of 17 - 29º/oo, pH of 8.4 - 8.9 and temperatures of 20 - 25ºC; *E.huxleyi* grew optimally at 29º/oo, pH7.5 - 8.9 and 15 - 25ºC. *G.oceanica* grew equally well with ammonium chloride, urea or nitrate as nitrogen source; *E.huxleyi* grew optimally with ammonium chloride. Maximum growth rates recorded were 1.9 doublings d^{-1} for *E.huxleyi* and 1.4 doublings d^{-1} for *G.oceanica*.

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Preface

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❶ Rhodes,L.L., Haywood,A.J., Ballantine,W.J., MacKenzie,A.L. (1993) Algal blooms and climate anomalies in north-east New Zealand, August to December, 1992. *New Zealand journal of marine and freshwater research* **27**, 419-430.

❷ Rhodes,L.L., O'Kelly,C.J., Hall,J.A. (1994) Comparison of growth characteristics of New Zealand isolates of the prymnesiophytes *Chrysochromulina quadrikonta* and *C.camella* with those of the ichthyotoxic species *C.polylepis. Journal of plankton research* **16**, 69-82.

❸ Jones,J.B. and Rhodes,L.L. (1994) Suffocation of pilchards . (*Sardinops sagax*) by a green microalgal bloom, Wellington Harbour, New Zealand, December 1993. *New Zealand journal of marine and freshwater research*, **28**, 379-384.

❹ Rhodes,L.L., Edwards,A.R., Peake,B.M., MacKenzie,A.L., Marwick,S. (1994) Bloom and growth characteristics of the coccolithophores *Gephyrocapsa oceanica* and *Emiliania huxleyi* (Prymnesiophyceae) in New Zealand's coastal waters. *New Zealand journal of marine and freshwater research* (submitted November 1994).

Contents page

Abstract iii
Acknowledgments vi
Preface viii
Contents ix
Figures x
Tables xiii
Abbreviations xv

GENERAL INTRODUCTION 1

Chapter one: FLORISTICS: New Zealand's Prymnesiophytes 11

Chapter two: FLUORESCENT PROBES AS TAXONOMIC TOOLS 40

Chapter three: GROWTH CHARACTERISTICS OF PRYMNESIOPHYTES 55

Chapter four: PRYMNESIOPHYTE BLOOMS IN NEW ZEALAND 80

Chapter five: MICROALGAL TOXICITY INTERACTIONS
Section one: allelopathy 102
Section two: microalgal toxicity bioassays 117

CONCLUSION 133

REFERENCES 136

Figures **page**

1.1 Maps of New Zealand and Marlborough Sounds, showing sites from which prymnesiophytes were isolated. **15**

1.2 Unstained transmission electron micrographs of spine and plate scales of *Chrysochromulina acantha.* **19**

1.3 Transmission micrographs of scales of *Chrysochromulina apheles.* **19**

1.4 Transmission electron micrographs of scales of *Chrysochromulina camella.* **21**

1.5 Light micrograph of *Chrysochromulina ericina* with phagocytosed fluorescently labelled bead. **21**

1.6 Transmission electron micrographs of plate and spine scales of *Chrysochromulina ericina.* **23**

1.7 *Chrysochromulina hirta* scales (transmission electron micrographs). **25**

1.8 Light micrograph of *Chrysochromulina quadrikonta.* **28**

1.9 Transmission electron micrographs of *Chrysochromulina quadrikonta.* **29**

1.10 Scanning electron micrographs of *Emiliania huxleyi* isolated from Big Glory Bay, Stewart Island. **31**

1.11 Scanning electron micrographs of *Gephyrocapsa oceanica.* **32**

1.12 Light micrograph of *Pleurochrysis* sp. shedding scales and electron micrograph of scales. **34**

1.13 Light micrographs of *Syracosphaera* cf. *pirus* isolated from Northland and scanning electron micrograph of coccoliths of *Umbilicosphaera* sp. **34**

2.1 The spine scales of *Chrysochromulina ericina* and *C.quadrikonta* fluorescing under UV light following the addition of the fluorescent dye, Calcofluor White. **49**

2.2 The thecae of dinoflagellates with and without the addition. of FITC-tagged lectins, photographed under blue light excitation. **49**

3.1 Growth rates (doublings per day) of several *Chrysochromulina* species at different temperatures. **65**

3.2 Growth rates (doublings per day) of several *Chrysochromulina* species at different salinities. **67**

3.3 Growth rates (doublings per day) of several *Chrysochromulina* species at different pH. **68**

3.4 Growth rates of *Emiliania huxleyi, Gephyrocapsa oceanica* and *Pleurochrysis* sp. at different temperatures. **72**

3.5 Growth rates of *Emiliania huxleyi* and *Gephyrocapsa oceanica* at different salinities. **73**

3.6 Growth rates of *Emiliania huxleyi, Gephyrocapsa oceanica* and *Pleurochrysis* sp. at different pH. **74**

4.1 Maps showing sites of sampling and bloom events referred to in this study. **83-4**

4.2 Cell numbers of individual bloom species at Leigh, August 1992 to January 1993 and estimated cell volume of those species. **88**

4.3 Micrographs of the dominant species involved in the Northland bloom, spring-summer 1992. **92**

4.4 Light micrograph of *Gephyrocapsa oceanica* in scallop guts containing domoic acid from Rangaunu Bay, Northland and *Pseudonitzschia australis* from sea water samples. **92**

4.5 Distribution of *Emiliania huxleyi* within Big Glory Bay and Paterson Inlet, 26 November 1992. **93**

4.6 Maximum cell numbers of *Emiliania huxleyi* from onset to demise of a bloom at Big Glory Bay, Stewart Island, October-December 1992. **94**

4.7 Cell numbers of bloom species at Marsden Point, October to December 1993. **97**

5.1	U-tube apparatus used in the screening for allelochemicals in marine microalgae.	**108**
5.2	Results of dual culture experiments.	**111**
5.3	The effect of addition of *Chlamydomonas coccoides* to exponential phase cultures of *Chattonella antiqua.*	**112**
5.4	Extraction of blood from anaesthetised salmon (*Oncorhynchus tshawytscha*).	**125**
5.5	Paua larval bioassays.	**125**
5.6	Microalgal bioassays.	**129**
5.7	Haemolysis of salmon erythrocytes by microalgal culture extracts.	**128**
5.8	Salmon erythrocytes.	**129**

Tables **page**

1	Classification of Prymnesiophyta (Chrétiennot-Dinet *et al.*, 1993).	8
1.1	Published records of prymnesiophytes from New Zealand coastal waters, identified by scales using electron microscopy.	12
1.2	Differences in taxonomic characteristics between *Chrysochromulina ericina* (isolated from Marlborough Sounds, New Zealand), *C.ericina* and *C.quadrikonta* (isolated in Nelson, New Zealand).	22
1.3	Differences in taxonomic characteristics between *Chrysochromulina hirta* from the Marlborough Sounds, New Zealand, from south-western Australia and from the Galapagos Islands.	26
2.1	FITC-conjugated lectins used as probes.	44
2.2	Carbohydrates used to inhibit binding of FITC-labelled lectin.	44
2.3	FITC-conjugated lectins and Calcofluor used as fluorescent probes; their source and specificity.	47-8
2.4	Carbohydrate inhibition of FITC-labelled lectins.	51
3.1	Selenium as a limiting growth factor in *Chrysochromulina* species.	63
3.2	Growth rates (doublings per day) of *Chrysochromulina* species cultured with different nitrogen sources.	64
3.3	Growth rates (doublings per day) of *Chrysochromulina* species cultured at different light intensities.	69
3.4	Growth rates of coccolithophores cultured with different nitrogen sources.	71
4.1	Phytoplankton species present, and their abundance, at the height of the raphidophyte dominated bloom at Leigh, 8 October 1992.	89

4.2 Comparison of cell numbers of dominant species at sites throughout the north-eastern coastal region of New Zealand, 13 November 1992. **90**

4.3 Species isolated, cultured and tested for ichthyotoxicity by the *Artemia salina* bioassay during the raphidophyte-coccolithophore bloom of 1992. **90**

4.4 Nitrogen: phosphate (N:P) molar ratios calculated from water column analyses of samples from within Big Glory Bay, Stewart Island, 1988-1992. **100**

5.1 Microalgal species investigated for allelopathic interactions. **107**

5.2 Microalgal species tested in dual culture for allelopathic interactions. **110**

5.3 Results of *Artemia salina* bioassays for detection of microalgal toxicity. **124**

5.4 Results of *Haliotis iris* bioassays for detection of microalgal toxicity. **126**

5.5 Evaluation of *Chattonella antiqua* and *Heterocapsa triquetra* as bioassay organisms by testing with known toxic microalgal species. **127**

Abbreviations

aff.	has an affinity with
BSA	bovine serum albumin
cf.	compares with
CHRY	*Chrysochromulina*
ConA	Concanavalin A
d	day
DAPI	4'6-diamidino-2-phenylindole
DMS	dimethylsulphide
DTT	dithiothreitol
ECA	*Erythrina cristagalli* A (coral tree) lectin
EDTA	ethylenediamine tetraacetic acid
FITC	Fluorescein isothiocyanate
GC	gas chromatography
GP	general purpose
GR	growth rate
h	hour
HPA	*Helix pomatia* A (snail) lectin
HPLC	high performance liquid chromatography
MAF ORPP	Ministry of Agriculture and Fisheries operational research phytoplankton programme
N:P	nitrogen:phosphate
Na; NaOH	sodium; sodium hydroxide
PAHBAH	para hydroxy-benzoic acid hydrazide
PEA	*Pisum sativum* (pea) lectin
PHA	*Phaseolus limensis* (lima bean) lectin
PWM	*Phytolacca americana* (pokeweed) lectin
SBA	*Glycine max* (soy bean) lectin
Se	selenium
SEM	scanning electron microscopy
sp.	species
SST	sea surface temperature
tD_{50}	time until 50% of bioassay organisms are dead
tM_{50}	time until 50% of bioassay organisms are morbid
TEM	transmission electron microscopy
TES	(N-tris[hydroxymethyl]-methyl-2-aminoethane-sulfonic acid)
UEA	*Ulex europaeus* (gorse) lectin
UV	ultraviolet
v/v	volume per volume
WGA	*Triticum vulgaris* (wheat germ) lectin
$^{0}/_{00}$	salinity (grams salt per kilogram seawater) in parts per thousand

The Prymnesiophytes: general introduction

In 1988 a golden brown marine microalga, *Chrysochromulina polylepis,* attained notoriety as a fish killer (Dahl *et al.*, 1989; Lindahl and Dahl, 1990). *C.polylepis* (Prymnesiophyceae) was thought to be benign prior to the deaths of caged salmon in Scandinavian waters during a toxic bloom of that species (Leadbeater, 1972a,b). Over the next 5 years toxic blooms of other *Chrysochromulina* species, including *C.leadbeateri,* were responsible for more, sometimes massive, fish kills in the Scandinavian region (Tangen, 1991; Knipschildt, 1992).
The genus *Chrysochromulina* is also found in New Zealand coastal waters, and the need to understand the triggers for bloom development and the taxonomy, growth characteristics and toxicity of prymnesiophytes occurring in New Zealand waters led to the studies described in this thesis.

Phytoplankton blooms

Phytoplankton blooms cause yellow foams, green slimes and red or brown slicks in the ocean when a particular microalgal species increases rapidly in numbers. Such blooms have occurred for aeons and the term "red tide" has been documented since biblical times. The white cliffs of Dover were formed over millennia from the constant deposition of the calcite scales of the most abundant and ubiquitous of microalgae, the coccolithophores (Prymnesiophyceae; Coccolithophorales). Coccolithophore blooms have been observed extending thousands of km^2, colouring the water a milky-green (Balch *et al.*, 1991); in Australasian waters, *G.oceanica* and *E.huxleyi* are the most abundant of the coccolithophore species recorded from the Coral Sea to Bass Strait (Hallegraeff, 1984), with *G.oceanica* dominating in the north and *E.huxleyi* in the south. The billions of individual cells sequester carbon (with bicarbonate as the major inorganic carbon source) in their calcareous scales, or coccoliths (Dong *et al.*,

1993), and the high reflectivity of these coccoliths has enabled the tracking of coccolithophore blooms with the aid of satellite imagery (Brown and Yoder, 1993; Holligan *et al.,* 1983). Coccolithophores also emit dimethyl sulphide (DMS) into the atmosphere, the DMS being produced with acrylic acid by enzymatic cleavage of dimethylsulfonium propionate, which is formed within the living cells (Keller *et al.,* 1989; Matrai and Keller, 1993; Vairavamurthy *et al.*, 1985). Both sequestering of carbon and production of DMS have implications for our atmospheric environment, by affecting the levels of carbon dioxide in the atmosphere (Williamson and Gribbin, 1991) and by providing the precursors of cloud condensation nuclei over the oceans (Charlson *et al.*, 1987; Kelly and Smith, 1990).

Multiple interacting physical, chemical and biotic factors have been cited as important variables in bloom formation. Seed beds of dormant cysts can germinate to form motile, vegetative populations in the water column (Bolch and Hallegraeff, 1990). Zooplankton can exert grazing pressure on microalgal stocks and alter bloom composition by preferentially grazing non-toxic species or selecting microalgae in a particular size-range (Tilman *et al*., 1982); growth of the tintinnid *Favella ehrenbergii* was inhibited by addition, *in vitro,* of the toxic bloom former *C.polylepis* (Carlsson *et al.*, 1990). The physiological and ecological characteristics of the species present help determine whether or not a body of water becomes "bloom sensitive". Even the chemical excretions of one microalgal species can affect the growth of another species, an interaction termed allelopathy (Rice, 1984).

Analysis of studies of blooms suggests that an increase in population size, suitable support factors (nutrients, salinity, water column stability, temperature, light and growth factors) and also the maintenance and transport of blooms by winds and currents are all aspects of bloom development and stability (Paerl, 1988). Increased light intensity, for example, has been shown to augment growth of *Prymnesium parvum* cultures (Shilo and Aschner, 1953).

Human impact on the ocean margins, in the form of increased nitrates in nutrient run-off and human effluent discharge, has been linked to the increasing numbers of large blooms (Cherfas, 1990). Acid rain, another offshoot of human activity,

was proposed as a key factor in the massive *C.polylepis* bloom that decimated life along the Norwegian coast in 1988, by releasing cobalt from soils (Anon, 1988). That blooms are becoming more frequent, as opposed to being more rigorously investigated, is supported by studies of the China coastline (Zhijie, 1990) and the Seto Sea in Japan. Red tides along Japanese coastlines reputedly increased 7-fold during the 1960s, then decreased during the 70s following run-off control by the regulatory authorities (Cherfas, 1990).

Prediction of the identity of the dominant species in an algal bloom is difficult, as bloom composition depends on so many complex, interactive factors, but the prediction of bloom development *per se* is now firmly linked to sea water temperatures, increasing day length and intense rainfall (leading to run-off and the formation of salinity gradients) followed by periods of sunny, calm weather.

In New Zealand coastal waters non-toxic blooms have occurred both as large-scale seasonal events, as occur regularly off the open coastline along the west coast of the South Island (Bradford and Chang, 1987; Chang, 1988), and as localised, unexpected events. The photosynthetic ciliate *Myrionectra rubrum* (=*Mesodinium rubrum*) has regularly caused purple-red blooms in north-eastern coastal waters and in the Marlborough Sounds (Challis, 1990; personal observation, 1994). These blooms are regularly reported because of their distinctive colouration, which generally alarms aquaculturalists who relate "red tides" to toxic dinoflagellate blooms. Blooms of surf diatoms (Diatomophyceae), such as the brown, "foamy" *Gonioceros armatum* (=*Chaetoceros armatum)* and *Aulacodiscus* cf. *kittonii* bloom which formed along the south-west coast of the North Island, March 1992 (C.O'Kelly, personal communication), form a vital food supply for tuatua (*Paphies subtriangulata*) and toheroa (*Paphies ventricosa*) (Cassie and Cassie, 1960).

The coccolithophore *G.oceanica* was a co-dominant species in a spring-1992 bloom which extended more than 200 km along the north-east coast of the North Island, from Leigh down to the Hauraki Gulf (Haywood, 1993; Rhodes *et al.*, 1993; Rhodes and Haywood, 1993), an event which was repeated in 1993 (refer Chapter four, Discussion). The 1992 bloom immediately preceded a major

toxic shellfish event in summer 1993, during which the whole of New Zealand's multi-million dollar shellfish industry was closed to harvesters. The latter event was attributed to the toxic dinoflagellates *Gymnodinium* cf. *breve* and *Alexandrium minutum* (Dinophyceae) (MacKenzie and Rhodes, 1993; Smith *et al.*, 1993; Bradford-Grieve *et al.*, 1993).

Other toxic events in New Zealand have included a bloom of *Heterosigma akashiwo* (Raphidophyceae) in Big Glory Bay, Stewart Island (Figure 4.1c), which was responsible for a nearly $20 million loss of quinnat salmon to New Zealand's farmed finfish industry in 1989 (Chang *et al.*, 1990).

A smaller event involving pilchard deaths occurred in Wellington Harbour on 10 December, 1993. This was the first wild fish kill documented in New Zealand in which microalgae and fish deaths were examined simultaneously. The deaths were believed to be due to asphyxiation by clogging of gills by the green flagellate *Tetraselmis* sp. (Prasinophyceae; Jones and Rhodes, 1994).

A strong common feature between the north-east coast blooms of 1992 - 94 and previous persistent blooms along both coasts, has been the presence of abnormally cold water. This was due either to large-scale climatic fluctuations as indicated by the Southern Oscillation Index (the anomaly of air temperature difference between Darwin and Tahiti) (Heath, 1985; Ballantine, 1992), or localised upwelling. Along the north-east coast in 1992, in a negative Southern Oscillation Index, the sea temperatures were lowered overall, the weather was calmer than usual and high rainfall occurred in short duration bursts. These conditions were widespread and long-lasting, and might be useful as bloom indicators in the future.

Toxic prymnesiophytes

The 1988 *C.polylepis* bloom in the Scandinavian fiords and the subsequent toxic prymnesiophyte blooms in those waters were responsible for sometimes massive kills of fish and other biota (Granmo *et al.,* 1988; Skjoldal and Dundas, 1989; Tangen, 1991; Knipschildt, 1992). Increased toxicity in prymnesiophyte blooms has been attributed to increased nitrogen:phosphate molar ratios. Phosphate

limited cultures of *Prymnesium parvum* produce more toxin than phosphate replete cultures *in vitro* (Shilo, 1971).

The toxic prymnesiophytes produce the ichthyotoxin prymnesin, actually a suite of toxins which act as neurotoxins, cytotoxins and haemolysins (Edvardsen *et al.*, 1990; Meldahl and Fonnum, 1993; Igarishi *et al.*, 1994). Prior to the infamous Scandinavian bloom, prymnesin-producing species had been thought to be confined to the genus *Prymnesium*, with *P.parvum* having a well-documented history as a fish-killer (Reich and Aschner, 1947; Shilo, 1967).

The apparently sudden expression of toxicity in the genus *Chrysochromulina* fuelled the debate as to whether ichthyotoxic blooms are more evident due to increased surveillance and the increases in caged, and therefore vulnerable, fish world-wide, or whether toxic blooms are actually increasing dramatically in number and extent (Cherfas, 1990). Certainly, nuisance microalgal blooms are now a major concern and have caused economic losses through the deaths of shellfish and fin-fish and through the poisoning of consumers of contaminated seafood (Taylor, 1990).

The Scandinavian experiences led to the development of a "toxic algal bloom" project, supported by the Danish Ministry for the Environment, to investigate all aspects of toxic blooms in Danish coastal waters (Ravn, 1991). It also led to the development of a global *Emiliania* modelling programme (Westbroek *et al.*, 1993) aimed at the development of a system for the rapid and intensive study of all aspects of individual species.

Toxic and nuisance prymnesiophyte blooms have also occurred in New Zealand coastal waters. *Phaeocystis pouchetii* was linked to lowered fish catches in Tasman Bay, New Zealand, in 1981 (Chang, 1983), mainly due to the copious amounts of mucilage which it produced in its non-motile colonial form. *P.pouchetii* is known to produce the antibiotic acrylic acid in conjunction with dimethylsulphide (Sieburth, 1960) and this could have had an effect on the health of fish, although in the Tasman Bay "slime" event toxin production was not investigated.

In the summer of 1983 fish and shellfish died at Bream Bay, north of Leigh,

during a bloom dominated by the non-toxic diatom *Cerataulina pelagica*. The fish deaths were attributed to anoxia (Taylor *et al.,* 1985), although the previously undescribed prymnesiophyte *Prymnesium calathiferum*, a sub-dominant during the bloom, was also implicated (Chang and Ryan, 1985). This species was ichthyotoxic to *Gambusia* sp. (a small species of fish) (Chang and Ryan, 1985).

Characteristics and Classification of the Prymnesiophyta

The prymnesiophytes are a group of yellow-brown marine microalgae most of which bear a unique organelle, the haptonema. This distinctive structure varies in length between species, from a rudimentary bud (sometimes even lacking in the non-motile stages of many coccolithophores), to a fine whip-like structure of over 150 *μ*m in some *Chrysochromulina* species. Prymnesiophyte cells typically contain chlorophyll *a* and two or three chlorophyll *c*s in usually two chloroplasts (Jeffrey, 1990). Cells of most prymnesiophytes are covered with carbohydrate scales, and also, in the coccolithophores, calcareous coccoliths.
Early studies of the Prymnesiophyceae concentrated on taxonomic descriptions of the 50-plus genera and ≈500 species and included sterling work by several groups of scientists in England and Europe (e.g. Carter, 1937; Parke *et al.*, 1956, 1958; Leadbeater, 1972a; Hällfors and Niemi, 1974; Green *et al.*, 1989). The type species of the genus *Chrysochromulina, C.parva* Lackey (a freshwater inhabitant), was initially cast into the ranks of the Chrysophyta (Lackey, 1939). The development of the electron microscope enabled the discrimination of the characteristic haptonema from the flagella (Parke *et al.*, 1962; Hibberd, 1976); prymnesiophytes bear two, rarely four, flagella plus a haptonema (Parke *et al.*, 1955). The new class was named the Haptophyceae, because of this distinctive organelle, and the taxon was formally established by Christensen (1962; Boney, 1970). Hibberd (1976) later proposed that the typified name Prymnesiophyceae be used in preference to Haptophyceae, based on the priority of the genus *Prymnesium*.

Much debate has followed; principle 3 of the International Code of Botanical Nomenclature (Greuter *et al.*, 1988) states clearly that "each taxonomic group with a particular circumscription, position and rank can bear only one correct name, the earliest that is in accordance with the Rules except in specified cases", but adds (article 11.4) that the "principle of priority is not mandatory for names above the rank of family". Recommendation 16B.1 states that "the name of a division is taken either from distinctive characters of the division (descriptive names) or from a name of an included genus", clarifying that the use of the typified name is not a requirement. However the typified taxon names Prymnesiophyta and Prymnesiophyceae are currently favoured internationally and will be used in this thesis. They may yet be deemed in accordance with the Rules by exception, but the usage is with the acknowledgment that Haptophyta and Haptophyceae are correct in the terms of the "Code". The first international symposium on "The biology of the Prymnesiophyta" , University of Plymouth, England, 29 March -1 April 1993 (organised by the Systematics Association and the British Phycological Society) comprised 43 papers and 12 poster papers, with only one author preferring to use Haptophyta (Cavalier-Smith, 1993).

Tappan (1980) suggested that the classification of the prymnesiophyte taxon is the least certain of the algal divisions because of the heteromorphic life histories of its species (Thomsen *et al.*, 1991). There has been a tendency to classify species on the basis of coccoliths or unmineralised scales alone, without substantiation by comparison with living cells. In fact calcification processes in the coccolithophores have been intensively analysed and the processes of both calcified and non-mineralised scale development within intracellular vesicles and their consequent movement to the surface of the cell have been elucidated with the aid of electron microscopy (Manton and Parke, 1962; Romanovicz, 1981). In this thesis species identification has been made following observation of both scales and living cells in most instances and the recent classification system from class to genus published by Chrétiennot-Dinet *et al.* (1993) has been followed (Table 1). Thus, for example, the order name Coccolithophorales has been used rather than Coccosphaerales, the name

Table 1 Classification of Prymnesiophyta (Chrétiennot-Dinet *et al.*, 1993)

Class: Prymnesiophyceae Hibberd 1976

Order: Prymnesiales Papenfuss 1955

Family: Prymnesiaceae Conrad 1926
Genus: *Chrysochromulina* Lackey 1939
Prymnesium Massart ex Conrad 1926

Family: Phaeocystaceae Lagerheim 1896
Genus: *Phaeocystis* Lagerheim 1893

Order: Isochrysidales Pascher 1910

Family: Isochrysidaceae Pascher 1910
Genus: *Isochrysis* Parke 1949

Order: Pavlovales Green 1976

Family: Pavlovaceae Green 1976
Genus: *Pavlova* Butcher 1952

Order: Coccolithophorales Schiller 1926

Family: Braarudosphaeraceae Deflandre 1947
Calciosoleniaceae Kamptner 1937
Calyptrosphaeraceae Boudreaux et Hay 1969
Ceratolithaceae Norris 1965

Coccolithaceae Kampner 1928
Genus: *Emiliania* Hay et Mohler in Hay *et al.*, 1967
Gephyrocapsa Kamptner 1943

Family: Deutschlandiaceae Kamptner 1928
Halopappaceae Kamptner 1928
Heliosphaeraceae Black 1971
Hymenomonadaceae Senn in Engler & Prantl 1900
Papposphaeraceae Jordan et Young 1990

Pleurochrysidaceae Fresnel et Billard 1991
Genus: *Pleurochrysis* Pringsheim emend. Gayral et Fresnel 1983

Family: Pontosphaeraceae Lemmermann in Brandt & Apstein 1908
Rhabdosphaeraceae Lemmermann *sensu* Norris 1984

Syracosphaeraceae Kamptner 1958
Genus: *Syracosphaera* Lohmann emend. Gaarder in Gaarder & Heimdal 1977

preferred by Parke and Green (Parke and Dixon, 1976).

The definition of "species" is a further problem (Taylor, 1993). Many of the hypotheses argued in this thesis are based on observations and analyses of single populations comprising the genetic offspring of cells that survived the *in vitro* conditions provided. It is reasonable to presume that a naturally occurring field population will contain a mixture of genotypes and that during the different stages of a bloom, or when a population is isolated into culture, different genotypes will dominate depending on the particular conditions at the time. Morphology can be useful for the identification of microalgae, but plasticity is a common feature of plants and must be taken into account. For example, there is a remarkable diversity of form in cells derived from clonal cultures of *Gymnodinium* cf. *breve* isolated from New Zealand coastal waters, (Allison Haywood, personal communication). Heteromorphic life histories further compound the species problem. In this respect recognition techniques based on molecular genetic or immunocytochemical features should prove useful when they are refined.

While taxonomic studies have dominated prymnesiophyte research in the past, more recent studies have had a broader scope. Studies of feeding behaviour and physiology have established that some *Chrysochromulina* species are mixotrophic, being at times autotrophic, heterotrophic (Pintner and Provasoli, 1968) and/or phagotrophic (Jones *et al.*, 1993). There is now evidence that the haptonema can act as a food collecting device and assist in surface gliding by attaching to surfaces (Kawachi *et al.*, 1991). A form of remote parasitism, in which chemicals excreted by nutrient starved *Chrysochromulina* cells cause leakage of cell membranes in prey organisms has even been hypothesised (Estep and MacIntyre, 1989). Optimal growth conditions in culture have been determined for a few species of coccolithophore (Klaveness and Paasche, 1979) and a requirement for specific growth regulators, secreted by bacteria, has been identified (Maestrini and Graneli, 1991). Studies of oceanography (Aure and Rey, 1992) and biogeography have shed light on the distribution of blooms (in particular coccolithophores) in the world's oceans (Honjo, 1977; Nishida, 1979). Recently, two prymnesiophyte ichthytoxins, prymnesin-1 and -2 from

Prymnesium parvum, have been purified and their chemical and biological properties have been described (Igarishi *et al.*, 1994). The photosynthetic pigment biochemistry of 29 species of prymnesiophyte has been elucidated (Jeffrey and Wright, 1993) and the phylogenetic relationships with other microalgal groups are being determined by Rubisco gene characterisation (Fujiwara *et al.*, 1993).

In this thesis, locally (New Zealand) occurring species of the ubiquitous group, the Prymnesiophyta, have been identified by electron microscopic examination of scales and light microscopic observation of cultured isolates. A new quadriflagellate species was isolated from Nelson Harbour in 1991, and it has been described in detail. A Japanese strain of this species was isolated in 1986 and, with consultation, the species was named *C.quadrikonta* (Kawachi and Inouye, 1993) and the growth characteristics of the New Zealand isolate elucidated (Rhodes, 1993; Rhodes *et al.*, 1994b).

The growth characteristics and cultural idiosyncrasies of five other southern hemisphere *Chrysochromulina* isolates have also been described and compared with their northern hemisphere relatives.

The role of fluorescent probes in the identification of morphologically-like species and in the differentiation of genera has been assessed. Species of the genera *Chrysochromulina* and *Prymnesium* were tested, and also members of the Dinophyceae and Diatomophyceae.

The key ecological factors contributing to the development of the recent blooms in New Zealand's coastal waters are discussed (Rhodes *et al.*, 1993) as are aspects of the ecology and physiology of the coccolithophores *Gephyrocapsa oceanica* and *E.huxleyi;* coccolithophore occurrence and distribution throughout 1992-93 has been investigated (Rhodes *et al.*, 1994a).

The toxicity status of all species isolated has been determined by bioassays developed or refined for that purpose and these bioassays have been evaluated.

Overall the objective of this thesis has been to draw together current knowledge of the prymnesiophyte taxon in New Zealand and to contribute to the growing body of knowledge about this important group worldwide.

Chapter one: FLORISTICS: New Zealand's prymnesiophytes

Introduction

The Prymnesiophyceae form one of 17 classes of marine phytoplankton (Chrétiennot-Dinet *et al.*, 1993). In 1991 the number of genera within the Prymnesiophyceae was seventy eight, comprised of approximately 270 species. These figures are an estimate only, but indicate that the prymnesiophytes are an important component of the marine phytoplankton, comprising ≈16% of genera and ≈7% of the total number of species (Sournia *et al.*, 1991).

Actual figures are difficult to compile as new species are found each year and some previously separate species have been merged as their heteromorphic life histories have become known. *Chrysochromulina polylepis*, for example, has been found to have two morphologically distinct, scale-covered flagellate forms in its life history (Paasche *et al.,* 1990; Edvardsen and Paasche, 1992). Several coccolithophores previously considered to be autonomous species have been shown to be part of life histories combining hetero- and holococcolithophorid forms, sometimes with intermediate forms having the calcareous scales, or coccoliths, of the different stages appearing on the one cell (Thomsen *et al.,* 1991). (Holococcoliths are comprised of individual calcite elements, visible under the electron microscope; in heterococcoliths minute crystals join to form diverse plate-like elements (Green, 1986)).

The fossil record indicates that the coccolithophore *E.huxleyi* was present in New Zealand waters some 270 000 years ago; coccoliths of that age were found in drillholes on the eastern continental slopes of the South Island (Nelson, 1988). *Gephyrocapsa oceanica* is recorded from the sediments laid down in the Holocene, particularly in northern and western New Zealand (Burns, 1975; Edwards, 1992). Recent New Zealand isolates of these coccolithophores have

been shown to have heavier calcification of their coccoliths than do their northern hemisphere relatives (Burns, 1977; Young and Westbroek, 1991).

The first records of living prymnesiophytes in New Zealand waters included the coccolithophores *Calyptrosphaera quadridentata* and *Syracosphaera coronata*, from Foveaux Strait and the Chatham Islands respectively (Cassie, 1961). The first records of *Chrysochromulina* species (two species in the vicinity of the Kermadec Islands and three in Wellington Harbour) and further observations of coccolithophores (31 species) were made during a Pacific cruise in 1958 (Norris, 1961; 1964). All these observations were by light microscopy and so in several instances only resemblances to known species could be recorded. However in 1974 O.Moestrup examined seawater samples under the transmission electron microscope and was able to name New Zealand prymnesiophyte isolates on the basis of scale morphology (Moestrup, 1979) (Table 1.1).

Table 1.1 Published records of prymnesiophytes from New Zealand coastal waters, identified by scales using electron microscopy.

Species	***Reference***	***Species***	***Reference***
Prymnesiales			
Chrysochromulina alifera	Moestrup, 1979	*C.apheles*	Moestrup & Thomsen, 1986
C.brevifilum	" "	*C.camella*	Moestrup, 1979
C.chiton	" "	*C.ephippium*	" "
C.ericina	" "	*C.*aff.*fragilis*	" "
C.mactra	" "	*C.novae-zelandiae*	" "
C.parkeae	" "	*C.quadrikonta*	Rhodes *et al.*, 1994
C.spinifera	" "	*Corymbellus aureus*	Moestrup, 1979
Phaeocystis pouchetii	" "	*P.scrobiculata*	" "
Prymnesium calathiferum	Chang & Ryan, 1985		
Coccolithophorales			
Emiliania huxleyi	Moestrup, 1979	*Gephyrocapsa oceanica*	Burns, 1977

Prymnesium calathiferum was first discovered in New Zealand coastal waters (Chang and Ryan, 1985) and was shown to be ichthyotoxic (Chang, 1985). *Chrysochromulina pringsheimii* was isolated from Big Glory Bay, Stewart Island in 1990 during a mixed *C.pringsheimii/ E.huxleyi* bloom (L.MacKenzie, personal communication) and definitively identified by transmission electron microscopy (C.O'Kelly, personal communication).

In the present study, seawater samples from around New Zealand's coastline were investigated for prymnesiophytes and, where present, cells were isolated, cultured and identified by light and electron (scanning and transmission) microscopy. Cultures were compared with the literature describing northern hemisphere isolates and differences between geographically separated strains noted.

A previously undescribed, quadriflagellate *Chrysochromulina* species was isolated from Nelson Harbour in autumn 1991 and the key taxonomic features of this species are presented. A morphologically identical microalga was isolated from Tokyo Bay in 1986 and named *Chrysochromulina quadrikonta* sp. nov. (Kawachi and Inouye, 1993). A non-toxic bloom of a strain of this organism occurred in Kesen-num Bay, Japan in 1991, causing the colouration of oysters. *C.quadrikonta* was also isolated from Australian waters in 1991 (D.Hill, personal communication).

The prymnesiophytes appear to be a ubiquitous group and it is not surprising that species previously unknown in these waters are being identified.

Methods

Microalgae

New Zealand prymnesiophyte isolates were obtained from seawater samples collected either by vertical haul with a 10 μm or 17 μm mesh net from a depth of up to 10 m or with a Van Dorn water bottle sampler at various discrete depths.

Sampling was facilitated by the weekly monitoring carried out by Regal Salmon Ltd., New Zealand in the Foveaux Strait/Stewart Island region and in the Marlborough Sounds, and also by the sampling carried out by Nelson Marlborough Health Services for the Marlborough Shellfish Quality Programme (Figure 1.1). Samples were received at Cawthron Institute, Nelson, for analysis. From August 1993 until June 1994 samples from a further 17 sites in the north-east of the North Island were collected by local health services personnel and analysed at Cawthron Institute for the Ministry of Agriculture and Fisheries phytoplankton research programme.

Unialgal cultures were obtained by picking out individual cells with a fine tipped Pasteur pipette (drawn out under flame) and transferring them to culture media or by setting up dilution series in Multiwell™ tissue culture plates (Becton Dickinson).

Culture Conditions

Chrysochromulina species were cultured in a general purpose enrichment medium (GP) (Loeblich and Smith, 1968), f2 medium (Guillard, 1975) and *Chrysochromulina* (CHRY) medium (Andersen *et al.*, 1991). Chemicals were analytical grade. Soil extract, a component of GP and CHRY media, was prepared from garden topsoil, untreated with herbicides or pesticides, as follows: friable, dark brown silty loam, was placed (5 cm deep) in a 10 l Erlenmeyer flask with distilled water (5 l) and sterilised (121ºC; 30 minutes). After standing for 1 day the supernatant was decanted and frozen until required. Media were modified by the addition of 0.02 μM selenium (sodium selenate) and double the normal addition of vitamins. Media were sterilised at 121^{0}C for 15 min, except for the phosphate and vitamin additions, which were filter sterilised (0.2 μm nuclepore) separately and added to the media after autoclaving. Filtered oceanic sea water (salinity 34$^{0}/_{00}$) was filtered (0.2 μm nuclepore filters), adjusted to pH8.0 with 1 M sodium hydroxide and used for maintenance media. Cultures were incubated at a light intensity of 100 μmol m^{-2} s^{-1}, with a

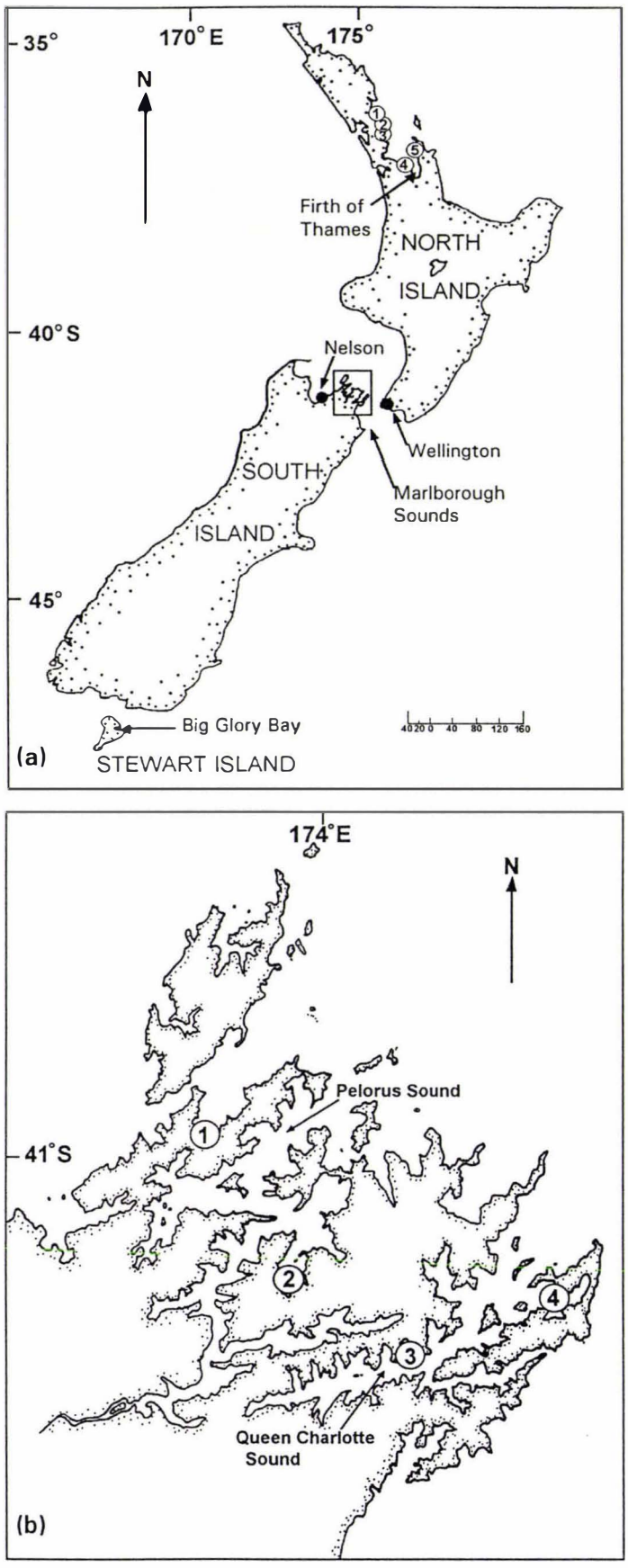


Fig. 1.1 Map of New Zealand (a) and the Marlborough Sounds (b) showing sites from which prymnesiophytes were isolated. (a) 1, Pakari Beach; 2, Leigh marine reserve; 3, Omaha Bay; 4, Waimangu Point; 5, Coromandel. (b) 1, Admiralty Bay; 2, Elie Bay; 3, Ruakaka Bay; 4, East Bay.

Cultures were incubated at a light intensity of 100 μmol m^{-2} s^{-1}, with a light/dark cycle of 14:10 h and temperatures of 18^{0}C (± 1^{0}C).

Non-motile coccolithophores were obtained from sea water by adding samples to growth media as above, but with germanium dioxide (0.5 mg l^{-1} final concentration) included to inhibit the growth of diatoms. Coccolithophore cells multiplied rapidly and unialgal cultures were obtained by picking out groups of cells with a Pasteur pipette and transferring to fresh medium every three or four days.

Microscopy

Light Microscopy: An inverted microscope was used for examining live cultures either directly in culture bottles or in Utermöhl settling chambers. An AHBT New Vanox Olympus photomicrographic microscope was used for epifluorescence microscopy and photography. Brilliant cresyl blue stain was used for highlighting the unmineralised scales and Janus green stain highlighted mucocysts.

Electron Microscopy: Cultures of the *Chrysochromulina* species were gently filtered under vacuum on to 0.2 μm nuclepore filters and salt crystals removed by rinsing with filtered deionised water. (For whole cell mounts glutaraldehyde was added (final conc. 1%).) The sample was resuspended in a drop of water and mounted on 400 and 200 mesh copper grids precoated with Formvar (0.4% in dichloroethane). Some samples were positively stained with uranyl acetate (30 seconds), then washed with ethanol (50%) followed by deionised water. Others were directionally shadow cast with platinum. Whole cells and individual scales were then examined under a Philips 201c transmission electron microscope (TEM) and photographed.

Coccolithophores were filtered and washed as for TEM, but drops of sample were transferred to pieces of glass cover slip glued (with silver paint) to metal stubs and air-dried prior to gold-coating. In the cases where scales required fixing prior to metal coating, the cells were treated with glutaraldehyde (1% v/v), passed through a seawater/distilled water dilution series up to 100%

distilled water and then a water/ethanol series up to 100% ethanol. Cells were then dried with liquid carbon dioxide in a critical point drier.
Coccolithophores were identified by their coccoliths after examination under a Cambridge Stereoscan 250 Mark 3 scanning electron microscope (SEM; EM Unit, Hort Research, Palmerston North).

Results

Prymnesiales

Chrysochromulina acantha

Several different species of *Chrysochromulina* were observed in seawater samples taken from Pakari Beach, in the Hauraki Gulf, February 1994. These included the biflagellate *C.acantha*, which was cultured in GP medium. Cell size averaged 8.2 μm length and 7.0 μm breadth, with a range of from 7.2 x4.8 μm - 9.6 x7.2 μm. The equal flagella averaged 15 μm in length and the haptonema 27 μm (range 20 - 34 μm). *C.acantha* was identified, using TEM, by its distinctive spine scales (Leadbeater and Manton, 1971; Hallegraeff, 1983; Figure 1.2). The culture was lost before more detailed observations of its taxonomy could be made.

Chrysochromulina apheles

The biflagellate *C.apheles* was also isolated from Pakari Beach. The cells were extremely small, averaging 5.5 μm in length (range 3.6 - 7.2 μm) and 4.6 μm in breadth (range 2.4 - 7.2 μm). The majority of cells were round, but approximately one third were elongated with a concavity on one side, appearing bean-shaped. The flagella were unequal in length, with the longer flagellum averaging 15.4 μm (range 12.0 - 21.6 μm) and the shorter averaging 12.2 μm (range 9.6 - 16.8 μm). The haptonema was long in relation to the cell size,

averaging 47 μm (range 30 - 70 μm). No coiling of the haptonema was observed.

The unmineralised scales observed under TEM bore 23 - 25 ridges on one surface. These ridges radiated out from a central cross (which consistently comprised three equal arms and one longer arm) to a slightly raised outer rim (Figure 1.3a). The obverse side of the scale bore concentric rings (Figure 1.3b). Scales were either concentric (0.33 - 0.43 μm) or slightly ellipsoid (0.27 - 0.42 μm x0.23 - 0.39 μm), and were slightly larger than the scales described for the type species (Moestrup and Thomsen, 1986). The central cross measured from 0.06 x0.09 μm in the smallest scales to 0.17 - 0.19 μm in the largest.

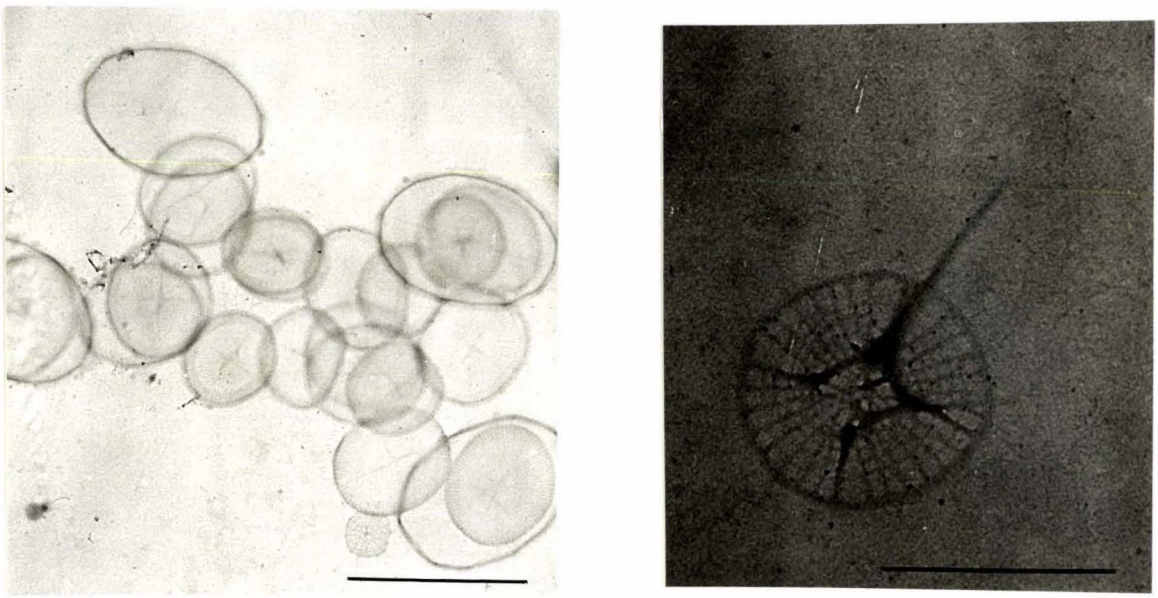
The cells fitted the type description of *C.apheles* except for the lack of central holes in the unmineralised scales, but this lack was noted in scales previously observed in New Zealand waters and also described as *C.apheles* (Moestrup and Thomsen, 1986).

Chrysochromulina camella

Chrysochromulina camella grew in GP medium inoculated with a seawater sample obtained from Ruakaka Bay, Queen Charlotte Sound in March 1992.

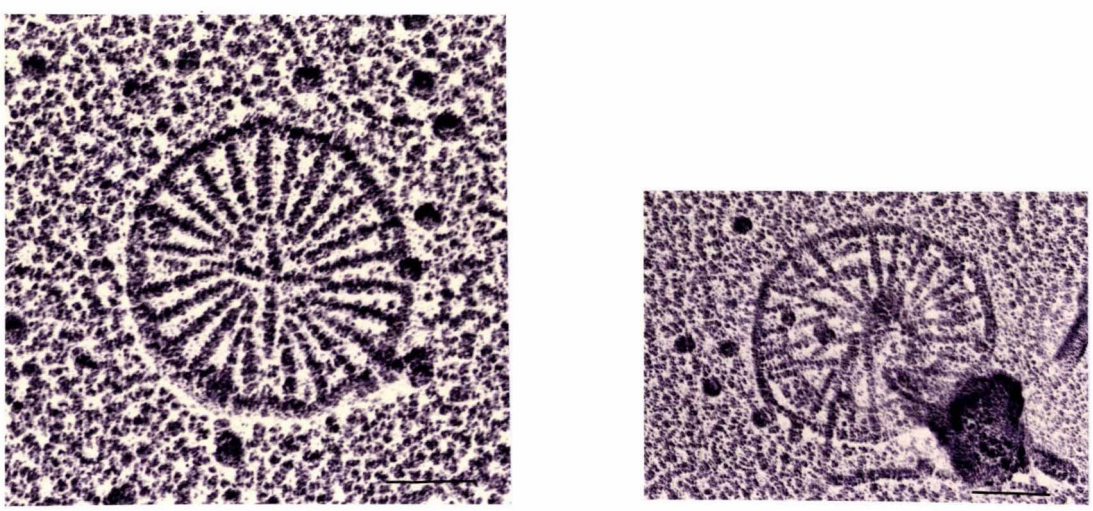
C.camella initially grew in both GP and CHRY media. However, after several weeks of subculturing, cultures failed to thrive until grown for several generations in GP with urea as nitrogen source. This species would grow in a synthetic medium only if the filtered seawater was gradually replaced with synthetic seawater over several subculturings.

Light microscope examination of this isolate suggested that the microalga was one of the three members of the *C.strobilus* affinity (Leadbeater and Manton, 1969). It possessed a saddle shaped cell (16 x13 μm), two centrally attached homodynamic flagella (25 μm in length) and a long haptonema ($>$50 μm). Cell movements were of the "Catherine wheel", or spinning, type; no flagella autofluorescence was noted. TEM of scales confirmed the identification of this species as *C.camella* (Leadbeater and Manton, 1969; Figure 1.4) .



(a) (b)

Fig. 1.2 Unstained transmission electron micrographs of scales of *Chrysochromulina acantha:* (a) plate and (b) spine scales. Bar = 1μm.



(a) (b)

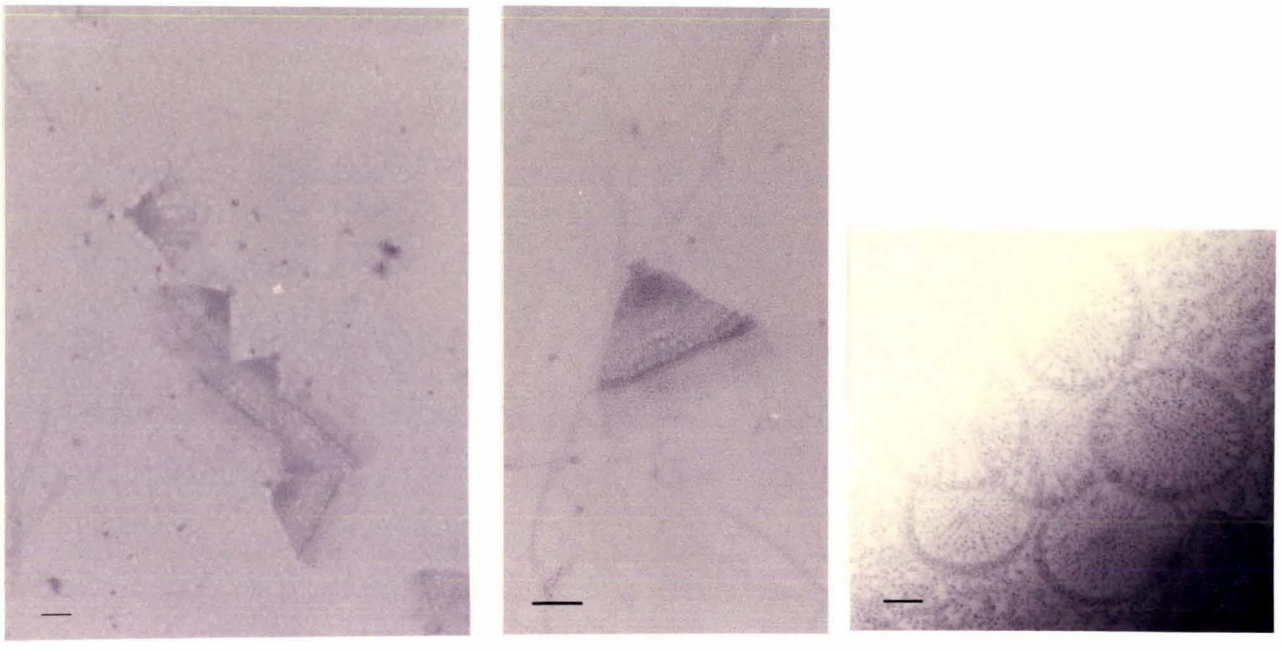
Fig. 1.3 Transmission electron micrographs of scales of *Chrysochromulina apheles:* (a) ridges and central cross; (b) concentric rings showing through from obverse side. Bar =0.1 μm.

Chrysochromulina ericina

C.ericina arose when CHRY medium was added to sea water samples collected from Ruakaka Bay, Queen Charlotte Sound, New Zealand, February 1992 (Figure 1.1). The biflagellate cells were identified by light microscopy as *C.ericina,* based on spine scales (Parke *et al.*, 1956) and autofluorescence of one of the paired flagella. (Flagellar autofluorescence was reported for a Japanese isolate (Kawai and Inouye, 1989).) Phagocytosis of fluorescently labelled beads (0.8 μm) was observed microscopically in *C.ericina* under blue excitation (Figure 1.5). Cell length ranged from 7 - 12 μm and cell breadth from 5 - 10 μm. Flagella length ranged from 12 - 24 μm and the length of the non-coiling haptonema from 12 - 28 μm. Cells in stationary phase cultures often developed an amoeboid form. Identification was confirmed by TEM examination of the spine and plate scales. Spine scales measured 9.7 - 16.4 μm in length and 0.13 - 0.33 μm in breadth; plate scales bore radiating fibrils on their proximal surface and measured 0.7 - 0.9 μm x0.9 - 1.2 μm (Figure 1.6; Table 1.2).

Chrysochromulina hirta

C.hirta was isolated from Elie Bay, Pelorus Sound, March 1992 and in March 1994 from seaweed bearing mussel spat (during analysis for toxic microalgal cysts). The weed was being transported from the far north of New Zealand to the Marlborough Sounds. Cells were similar to *C.ericina* under the light microscope, but lacked a fluorescing flagellum. Under TEM scales differed markedly from *C.ericina*. The largest spine scales, measuring 11 μm, tapered to a fine point with delicate struts running from the periphery of the basal plate to join the centre spine 2 μm up the shaft. Shorter spine scales were also present, measuring between 2.4 μm and 4.7 μm. The largest of these had extremely delicate struts and the smallest lacked struts altogether. Plate scales



(a) (b) (c)

Fig. 1.4 Transmission electron micrographs of scales of *Chrysochromulina camella*: (a,b) cup scales and (c) plate scales, shadow cast with platinum. Bar = 0.1μm.

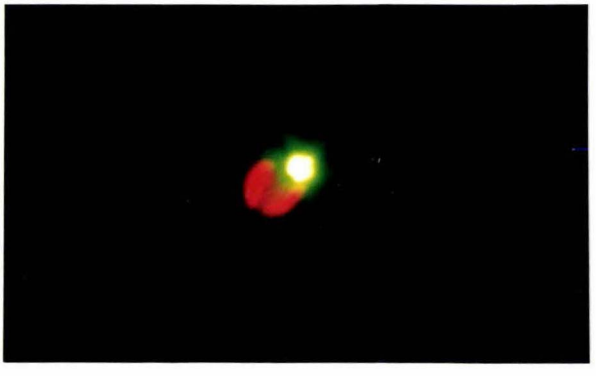


Fig. 1.5 Light micrograph of *Chrysochromulina ericina* with phagocytosed fluorescently labelled bead (0.8 μm).

Table 1.2 Differences in taxonomic characteristics between live cells *Chrysochromulina ericina*[a] (isolated from Marlborough Sounds, New Zealand), *C.ericina*[b] and *C.quadrikonta*[a] (isolated in Nelson, New Zealand). Measurements are in μm.

Characteristic	*C.ericina* (NZ)	*C.ericina*[b]	*C.quadrikonta*
Cell length	7-12	5-12	6-25
Cell breadth	5-10	4-10	4.5-17.5
Haptonema length	12-28	20-60	9-20
Flagella length	12-24	10-30	10-25
Plate scales			
length x breadth	0.7-0.9 x0.9-1.2	0.6-0.9 x0.5-0.7	1.7-2.1 x1.0-1.8
Spine scales			
length x breadth	9.7-16.4 x0.1-0.3	9-15 x0.2-0.3	3.2-5.2 x0.2-0.4
base diameter	1.4-1.7	1-1.4	1.2-1.8
Flagella number	2	2	4
Flagella autofluorescence[c]	one flagellum	one flagellum	one flagellum
Coiling ability of haptonema	not observed	yes	not observed
Number of spine scales	12-16	28-30	38-48
Phagotrophy	yes	yes	not observed

[a] Results for New Zealand isolates are ranges from a minimum of 50 measurements taken from cultures at different phases of growth;
[b] Reference: Parke *et al.*, 1956;
[c] Reference: Kawai and Inouye, 1989.
Batch cultures were grown in GP medium, incubated at 20^0C, 100 μmol m^{-2} s^{-1} (14:10 h day/night cycle).

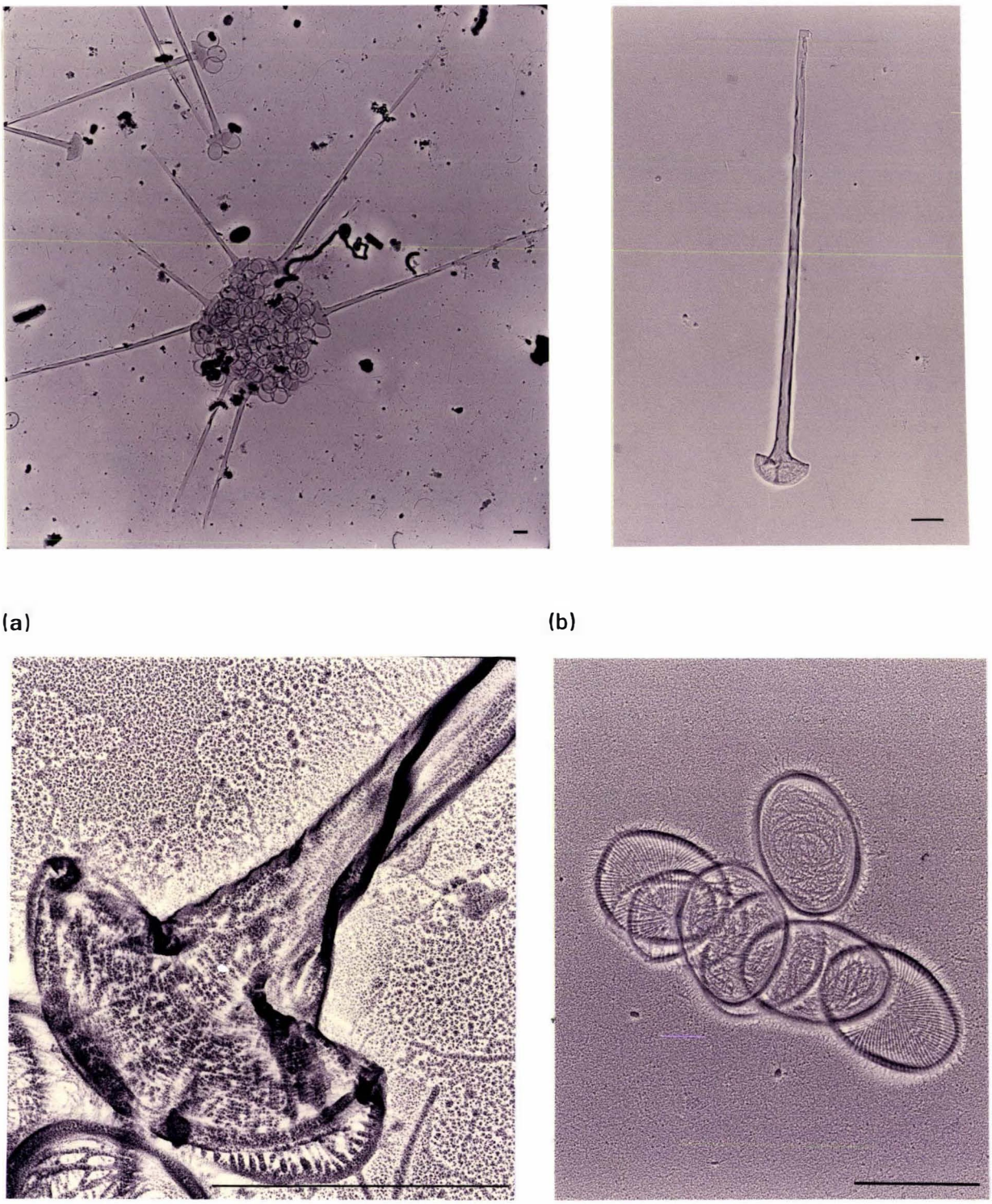


Fig. 1.6 Transmission electron micrographs of scales of *Chrysochromulina ericina:* (a) cell with attached scales (haptonema partially coiled), (b) spine scale, (c) base of spine scale, (d) proximal (l and r) and distal (centre) faces of plate scales. (Scales shadow coated with platinum; bar = 1μm).

Oates, 1983) from coastal waters near Sydney, Australia, *C.latilepis*, from the Galapagos Islands (Manton, 1982), and *C.hirta* (Manton, 1978; Table 1.3). The species was confirmed as *C.hirta* (O.Moestrup, personal communication).

Chrysochromulina pringsheimii

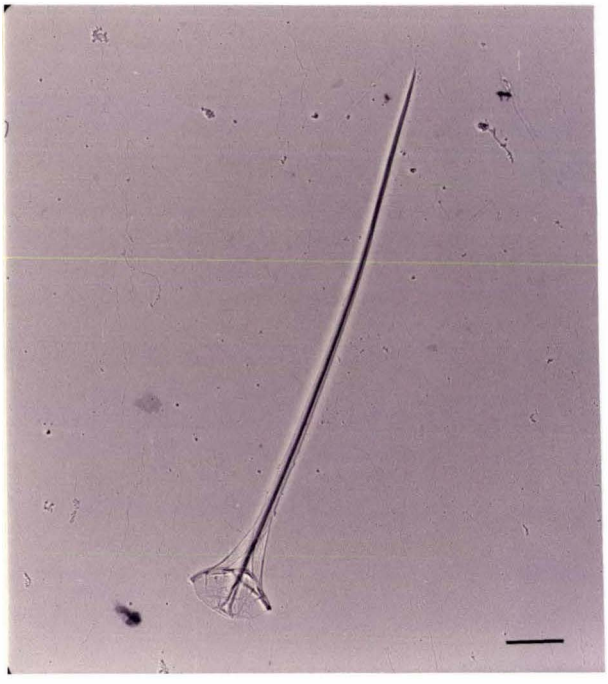
A *Chrysochromulina* species bearing long spines was observed using light microscopy on several occasions during 1992-94, in samples from around the South Island coastline and from the Bay of Plenty, and was identified as *C.pringsheimii* (Parke and Manton, 1962). Only individual cells were observed and TEM confirmation was not possible on those occasions. However *C.pringsheimii* was identified by TEM of whole cells during a mixed *Emiliania huxleyi/C.pringsheimii* bloom in Big Glory Bay, Stewart Island, November 1990 (C.O'Kelly, personal communication).

Chrysochromulina quadrikonta

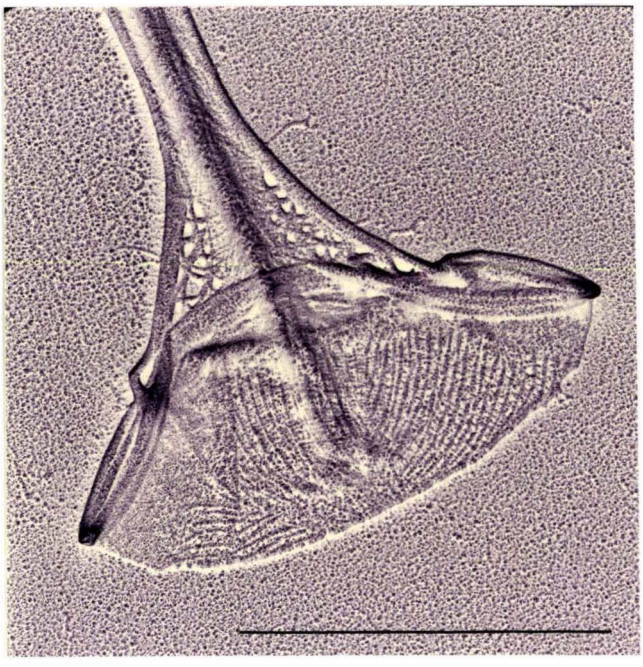
The quadriflagellate species of *Chrysochromulina, C.quadrikonta*, was isolated in April 1991 from a sea water sample obtained from Nelson. The culture died in 1993, but *C.quadrikonta* was again isolated, from seawater samples taken at Waimangu Point, in the Firth of Thames, in May 1994 (cells numbered 60 $x10^3$ l^{-1} at the surface; MAF Regulatory Authority operational research phytoplankton programme).

C.quadrikonta was established in GP medium, although initially subcultures that survived often did so in an amoeboid form on the bottom of the culture vessel.

Light microscopic examination of the Nelson Harbour isolate showed a tear-shaped cell with four homodynamic flagella and a haptonema of equal length inserted at the polar end and surrounded by a cluster of spine scales (Figures 1.8; 2.1). The haptonema was observed coiled only when cells were treated



(a)



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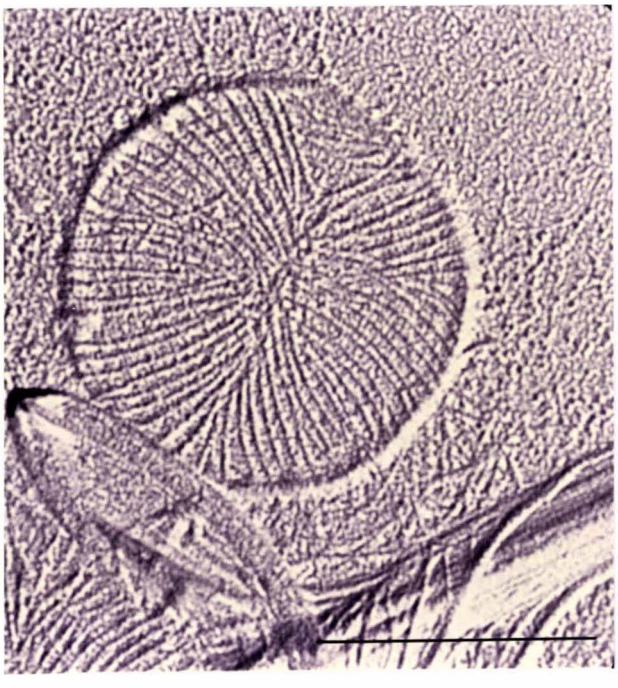

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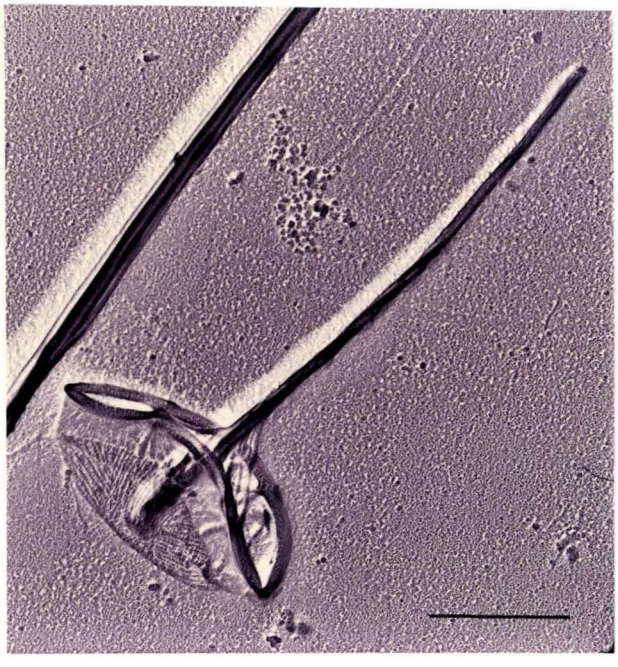


(d)

Fig. 1.7 ***Chrysochromulina hirta*** **scales (transmission electron micrographs):** (a) large spine scales with struts, (b) base of spine scale, (c) proximal surface of plate scale and (d) small spine scale. Bar = 1μm.

Characteristic	*C.*sp. *NZ*	*C.latilepis*[a]	*C.latilepis*[b]	*C.hirta*[c]
Cell breadth	4 - 9	a	6	≈6-12
Haptonema length	>30	a	≥40	>20
Flagella number	2	a	2	2
Flagella length	≈20	a	20:≥10 (unequal)	≈20
Tapered spine scales				
small	2.4 - 4.7	5	<6	<5
large	11	NF	NF	20-30
Plate scales				
small, oval	0.6 x0.9	0.5 x0.7	0.5 x0.7	
large	1.0 x1.5	1.0 x1.0 - 1.3 x1.3	3.0 x2.5	≈1.3 x1.6

[a] scales only were observed; Reference: Hallegraeff, 1983;
[b] Reference: Manton, 1982.
[c] Reference: Manton, 1978.
NF: not found.
Results for the New Zealand isolate are ranges from a minimum of 50 measurements taken from cultures at different phases of growth;
Batch cultures were grown in GP medium, incubated at 20^{0}C, 100 μmol m^{-2} s^{-1} (14:10 h day/night cycle).

with fixative. Muciferous bodies were noted on occasions, but were only rarely seen to eject mucilaginous threads. Chloroplasts, easily discernible under UV excitation, varied from 2 to 4 in number and were usually bifid towards the non-flagellar pole. A 1 μm diameter yellow autofluorescing body occurred in the "tail" of most of the *C.quadrikonta* cells, in axenic and non-axenic cultures, whether phosphate starved or replete. Autofluorescence was also observed in one only of the four flagella.

Cells were completely covered in a hedgehog-like coat of spine scales. These were highlighted with cresyl blue stain but were shed when cells were fixed with Lugol's iodine. Under the stress of fixation, cells were occasionally observed withdrawing from the spiny coat, leaving it empty but intact.

TEM examination of the cells revealed dimorphic scales (Figure 1.9). A comparison between *C.quadrikonta* and the biflagellate species *C.ericina* is presented in Table 1.2.

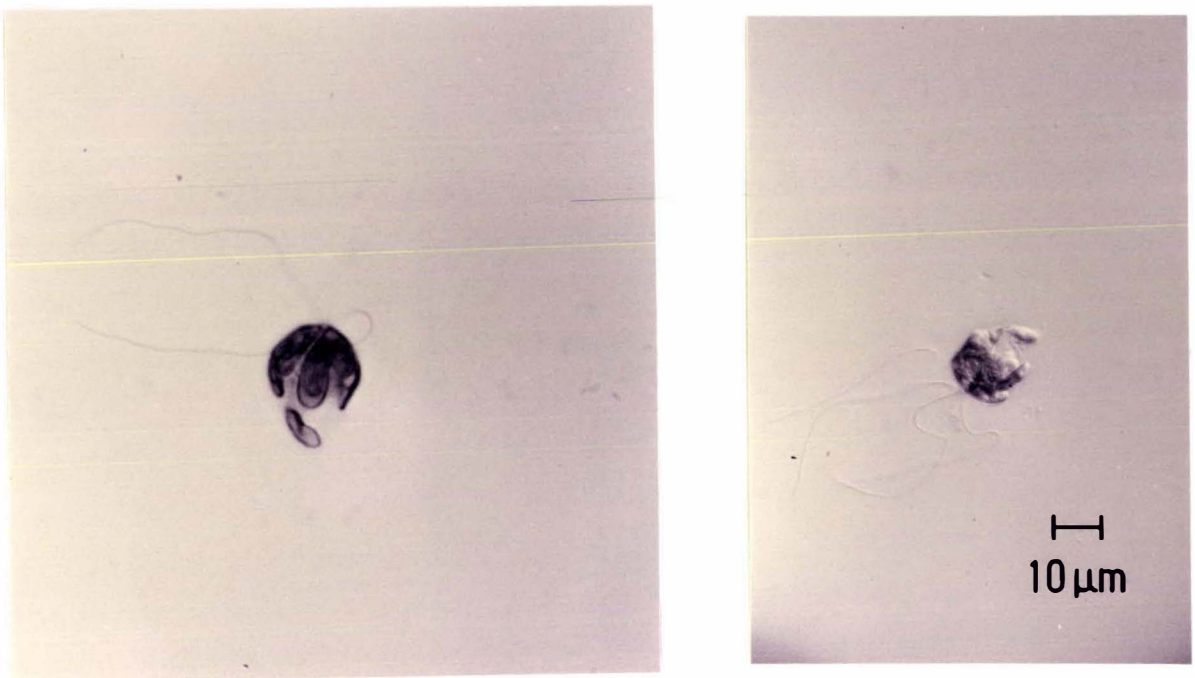
Chrysochromulina strobilus; Chrysochromulina spp.

C.strobilus (Leadbeater and Manton, 1969) was isolated with *C.camella* from Ruakaka Bay, Marlborough Sounds, March 1992, and the cup scales identified by TEM. This species did not, however, survive in culture.
Individual cells of several species of *Chrysochromulina* were observed during the course of the study, but did not grow in culture media and were not identified by electron microscopy, for example a round motile prymnesiophyte (15 μm) with a long haptonema (120 μm) was observed with *Syracosphaera* sp. cells in seawater samples from Admiralty Bay, Pelorus Sound, March 1993.

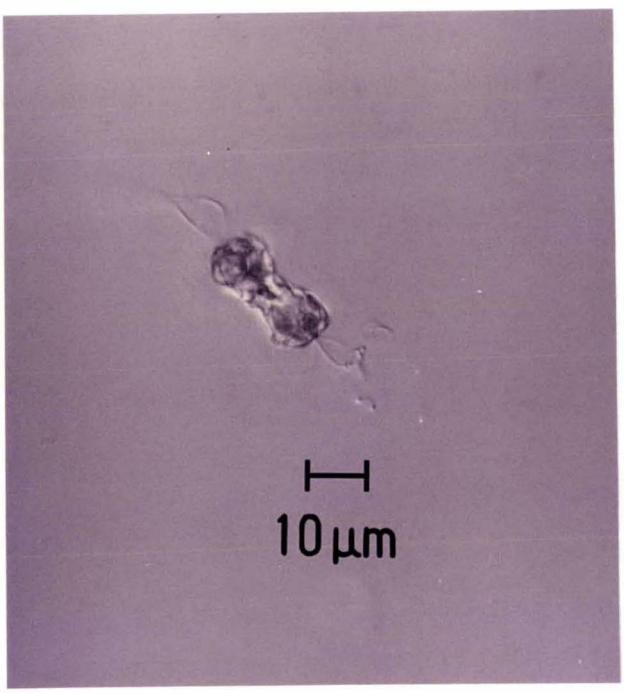
Coccolithophorales

Emiliania huxleyi

E.huxleyi was isolated from sea water samples obtained from Big Glory Bay, Stewart Island, November 1992, during a bloom of this species, and from various sites in the Marlborough Sounds, February, 1993. *E.huxleyi* was identified by scanning electron microscopy (SEM) (Figure 1.10a) (Tappan, 1980). Isolates readily changed between a flagellated motile form (lacking coccoliths) and a non-motile coccolith bearing C form, as described by Klaveness (1972), in laboratory cultures. No particular trigger for the interchange was identified and subcultures from the same source presented different forms at any one time. However, as the number of subculturings increased so did the proportion of cultures entering the motile stage of the life-cycle. SEMs of *E.huxleyi* from Northland (Haywood, 1993), Marlborough



(a) (b)



(c)

Fig. 1.8 Light micrographs of *Chrysochromulina quadrikonta:* (a,b) cells showing four flagella (c) cell dividing.

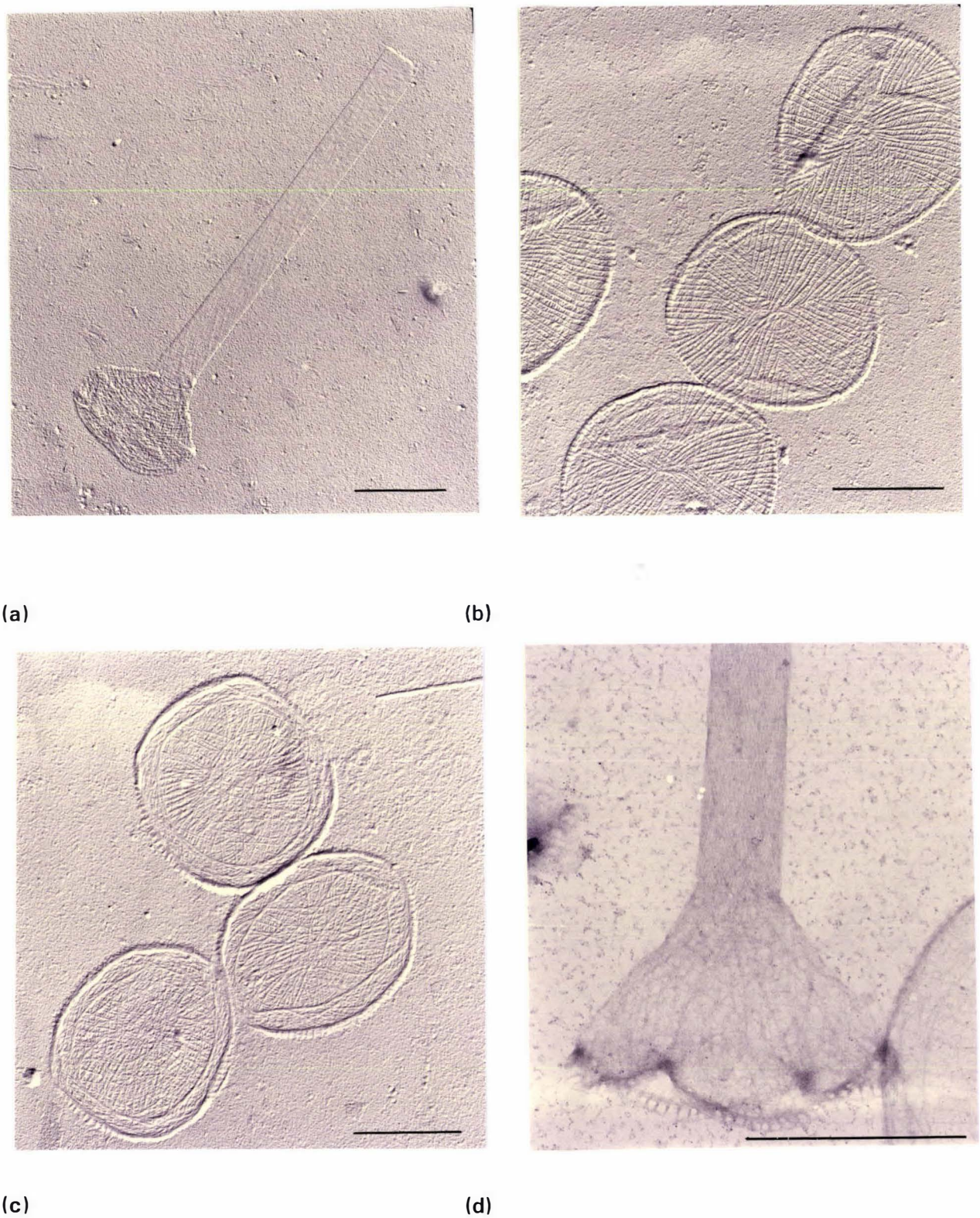


Fig. 1.9 Transmission electron micrographs of *Chrysochromulina quadrikonta*: (a) spine scales and (b) proximal and (c) distal faces of plate scales, shadow coated with platinum. (d) Base of spine scale positively stained with uranyl acetate. Bar = 1μm.

Sounds and Big Glory Bay showed that all cells bore coccoliths with thickened calcite elements (Figure 1.10b-c). No substantial difference was noted between calcite thickening of elements of coccoliths from northern New Zealand isolates (latitude 36^0S) and those from Big Glory Bay (latitude 47^0S). Dissolution of the coccoliths of *E.huxleyi* was observed after a year in culture (Figure 1.10e).

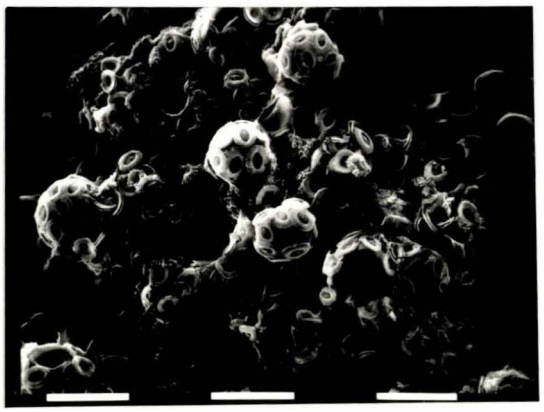
Gephyrocapsa oceanica

Spherical, biflagellate microalgal cells (10 μm diameter) bearing haptonema dominated water samples collected from East Bay in the Marlborough Sounds on 23 March 1992. The haptonema and flagella were all approximately 15 μm. This motile prymnesiophyte did not persist in culture, but was replaced by non-flagellate coccolith-bearing cells (10 μm), which divided rapidly in CHRY medium. A thallus form was observed in one of the early cultures but did not recur. There was no reversion to the motile form, despite growing the cells under a range of environmental and nutrient regimes. These coccolithophores proved to be *Gephyrocapsa oceanica* (Bréherét, 1978; Hallegraeff, 1984) under SEM examination (Figure 1.11a,b). Non-motile coccolithophores isolated from Leigh, October 1992, and Coromandel, January 1993, also proved to be *G.oceanica*. Some of these coccoliths showed signs of dissolution (Figure 1.11,c).

G.oceanica has been divided into three forms, based primarily on variations in the bridge and collar formations of the coccoliths (Okada and McIntyre 1977). The New Zealand isolates conformed to "Form 2", having a large central area, a moderately thick bridge and a well-developed central collar (Figure 1.11d-f).

Pleurochrysis sp.

Pleurochrysis sp. was first observed in Admiralty Bay, Pelorus Sound, in March 1993. The surface sea water temperature was 15.8^0C and salinity was $35.4^0/_{00}$. Cell numbers reached 3.0 x10^4 l^{-1}, but gradually decreased over the next three months and had disappeared by early July. Identification was by light



(a)

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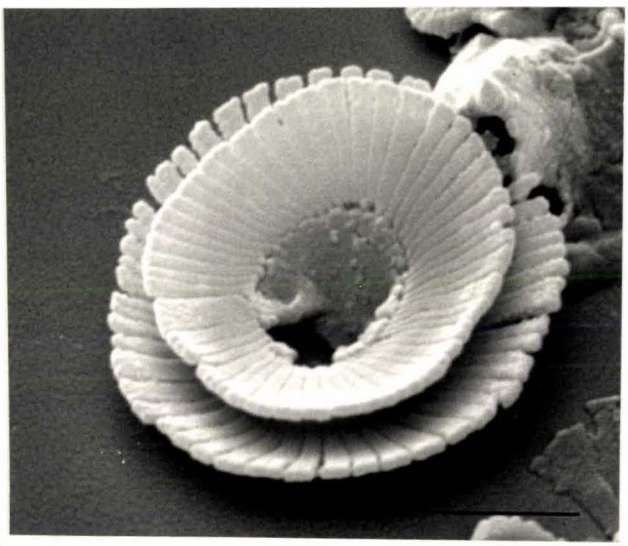
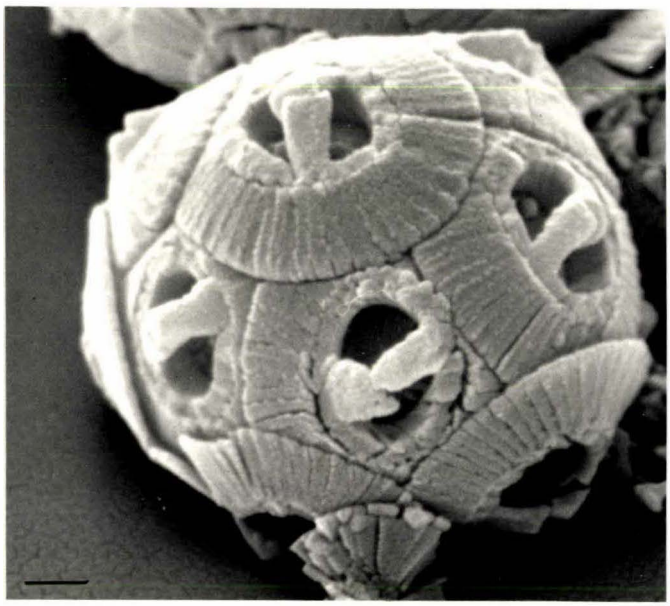


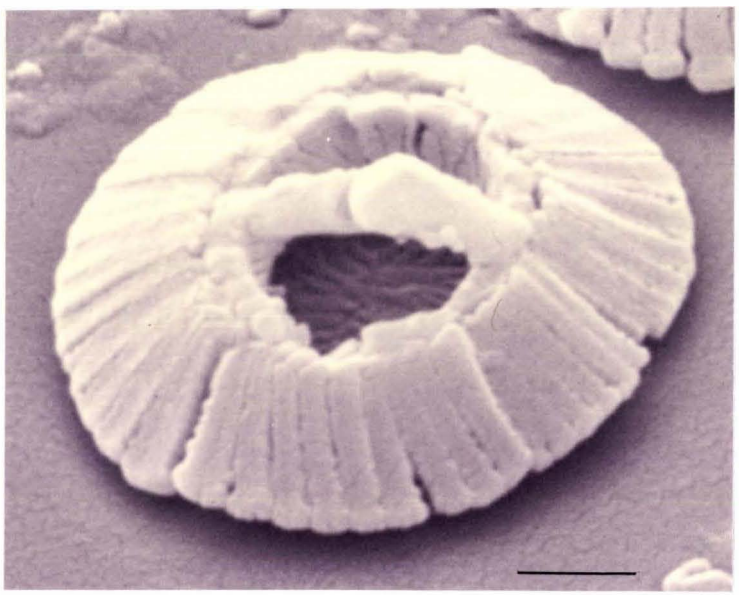
(d)

(e)

Fig. 1.10 Scanning electron micrographs of *Emiliania huxleyi* isolated from Big Glory Bay, Stewart Island: (a,b) whole cells bearing coccoliths (bar = 10 μm); (c) secondary calcite thickening on distal surface and (d) underside of coccolith; (e) dissolution of coccoliths (bar = 1μm).



(a)



(b) **(c)**

Fig 1.11 Scanning electron micrographs of *Gephyrocapsa oceanica*: (a) isolate from the Marlborough Sounds; (b) cell showing dissolution of coccoliths; (c) distal view of secondarily calcified coccolith. Bar = 1μm.

microscopy of the motile cell and SEM of the scales (Figure 1.12a). The scales appeared to be unmineralised and collapsed during early attempts toprepare them for SEM. They were finally prepared by fixation in an alcohol series followed by critical point drying. By observing the scales under a 2.5 kv electron beam, which produced an image at the surface of the scale, and thenprogressively increasing the beam to 15 kv, which produced an image beneath the surface, it could be deduced that the scales formed a cylinder with no decoration apart from etching on the surface rim (Figure 1.12b-c). No cells bearing calcified scales were observed.

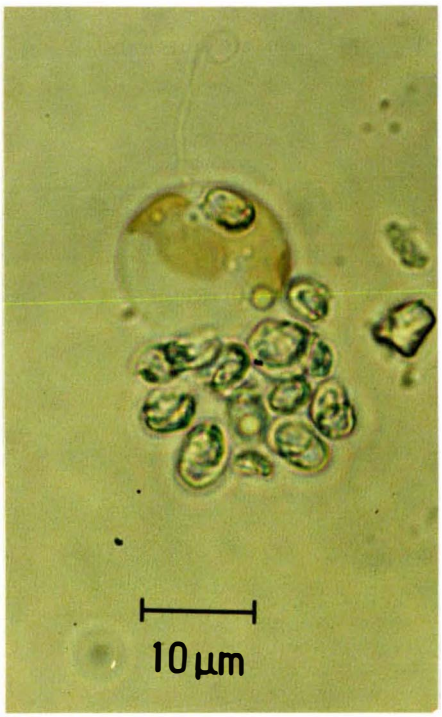
Syracosphaera cf. *pirus; Umbilicosphaera* sp.

A species of the genus *Syracosphaera* was dominant in sea water samples taken from Omaha and Pakari Beaches, north of Auckland (refer Chapter 4, results), October 1993. Cells most closely resembled *S.pirus* under the light microscope (Hallegraeff, 1984; Figure 1.13a,b), but did not survive in culture.
Coccoliths of *Umbilicosphaera* sp. (Okada and McIntyre, 1977) were observed in seawater samples from Big Glory Bay, Stewart Island, November 1992, in which *Emiliania huxleyi* dominated (Figure 1.13c).

Discussion

Prymnesiales

The novel species *C.quadrikonta* was isolated in autumn 1991 and again in autumn 1994. Transmission electron micrographs (TEM) of the scales of the New Zealand isolate proved to be identical to those of a Japanese isolate (Kawachi and Inouye, 1993). *C.quadrikonta* has many morphological similarities to *C.ericina*, which raises the question of whether the former is a polyploid form of the latter. Both species exhibit autofluorescence in one flagellum and both develop an amoeboid form on the bottom of culture flasks in stationary phase cultures. Plate and spine scales of *C.quadrikonta* closely fit descriptions of the biflagellate *C.ericina,* in which spine scales form an open-



(a)

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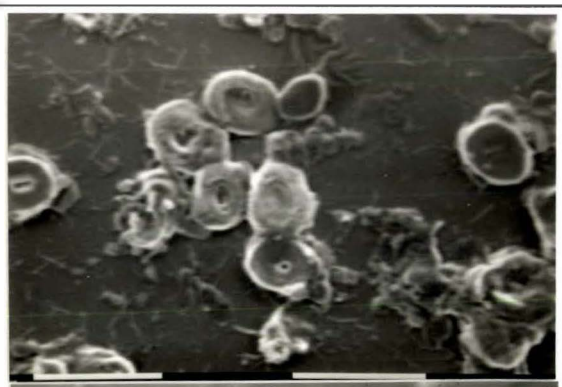
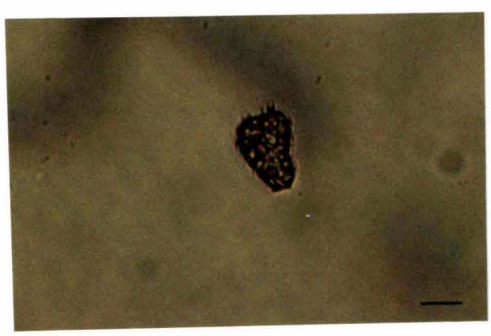


(c)

Fig. 1.12 ***Pleurochrysis*** sp.: (a) Light micrograph of cell shedding scales and (b,c) scanning electron micrographs of scales. (Bars = (b) 10 μm and (c) 1 μm).

(a)



(b)

(c)

Fig. 1.13 ***Syracosphaera*** **cf.** ***pirus*** **and** ***Umbilicosphaera*** **sp.:** (a,b) *S.*cf.*pirus* (light micrographs). Bar = 10 μm; (c) coccoliths of *Umbilicosphaera* sp. (scanning electron micrograph). Bar = 1μm.

ended tube, probably by hypertrophy of the distal surface of the scale (Manton and Leedale, 1961; Parke et al., 1956). However, differences in both the size and the number of spine scales were noted between the bi- and quadriflagellate forms and ultrastructural studies carried out at the University of Tsukuba (Kawachi and Inouye, 1993) support the contention that the quadriflagellate form should be accorded species status and not be designated *Chrysochromulina* aff. *ericina.*

Spine scales which closely fit the description for *C.quadrikonta* were observed by TEM in seawater samples taken from the English Channel nearly a decade ago (C.Course, personal communication). Those scales were believed to belong to a form of *C.ericina* at that time, which highlights the need for identifications to include both observations of live cells and electron microscopy of scales whenever possible.

C.ericina isolated from New Zealand waters differs in some respects from descriptions of northern hemisphere strains of that species (Parke *et al.*, 1956). The cell bodies are of comparable size and flagella lengths fall within the same size range. The plate scales of the New Zealand isolate are comparable with previous descriptions of scales in both New Zealand (Moestrup, 1979) and Norwegian (Leadbeater, 1972) waters, but tend to be larger than the type description (Parke *et al.*, 1956). Spine scales also tend to be longer and fewer in the New Zealand strain, the majority having no more than half the number per cell.

Other "spiny" *Chrysochromulina* species identified in this study are *C.acantha, C.pringsheimii* and *C.hirta,* none of which has been recorded previously in New Zealand waters. *C.hirta* has also been found the cool temperate waters of South Africa, Europe, South Alaska and the N.W. Passage. (Manton, 1978) and in Japanese waters (Kawachi et al., 1991). The New Zealand strain, which has shorter spine scales than the type species, but has scales of similar length to the Japanese strain, also has similarities to *C.vexillifera* (=*C.latilepis.* aff.; Hallegraeff, 1983), *C.latilepis* and *C.mantoniae* (Manton and Leadbeater, 1974). Both *C.hirta* and *C.mantoniae* have equal flagella, a relatively short haptonema and rimmed scales. However the New Zealand isolate identified as *C.hirta*

differs from *C.mantoniae* in that no small plate scales have been observed, no tilting of the spine scales in relation to the base plate have been noted, and only the spine scales, not the plate scales, of the New Zealand isolate exhibit rims. The struts of the New Zealand isolate are more delicate than in *C.mantoniae* and the base scales are concave rather than convex, both characteristics which fit the descriptions of the Galapagos isolate of *C.latilepis* as well as *C.hirta.* Finally, the New Zealand isolate has spine scales clearly scattered over the periplast, not attached at the poles as in *C.mantoniae* (Manton, 1982). (The distribution of spine scales of *C.latilepis* is not recorded.)

Of the non-spiny species observed in this study, both *C.apheles* and *C.camella* have been identified by electron microscopy in New Zealand waters previously, but neither has been described in relation to living cells (Moestrup, 1979; Moestrup and Thomsen, 1986). *C.apheles* appears to have a greater size range than its northern hemisphere counterpart, reaching slightly larger cell sizes and longer flagella and haptonema lengths. The scale form closely fitts the earlier New Zealand description, although scales are slightly larger (Moestrup and Thomsen, 1986).

The haptonema, the unique organelle of the prymnesiophytes, differs between geographically separate strains of some species. The haptonema of northern hemisphere isolates of *C.ericina* (20 - 60 μm) is generally observed coiled during rapid backwards movements and have been reported as being twice the length of the flagella (Parke et al., 1956). However the haptonema of the New Zealand strain is shorter, or at most falls within the first quartile of the size range for northern hemisphere isolates. Possibly due to the shorter haptonemal length, coiling has not been observed in the New Zealand strain and in this regard it more closely resembles *C.quadrikonta.* The New Zealand strain of *C.hirta* also has a shorter haptonema (≈30 μm). Japanese strains of the species have haptonemata of up to 55 μm (Kawachi et al., 1991).

The haptonema of *C.quadrikonta* (≈20 μm) has not been observed coiling, although on rare occasions bending to one side has been noted near the tip. The haptonema of the local isolate is typically shorter than the flagella and is directed anteriorly, even during the frequent collisions and entanglements of cells that

occur in stationary phase cultures. This is akin to *C.polylepis*, which is described as having coiling haptonemata (Leadbeater, 1971), but which is generally observed with its 12 μm organelle pointing anteriorly (personal observation). Although approximately four times the cell length, the haptonema of the New Zealand isolate of *C.apheles* has not been observed coiling.

The haptonema can facilitate gliding movements in some prymnesiophytes by adhering to surfaces, and has been shown to be a food-capturing device in *C.hirta* (Kawachi et al., 1991). The long haptonema (approximately 120 μm) of the local isolate of *C.camella* fits closely with previous descriptions of this species (Leadbeater, 1971), coiling rapidly and often. Uncoiling takes about 3 seconds and is usually in three jerking stages, beginning at the basal end.

Food capture has not been observed in any of the New Zealand *Chrysochromulina* isolates, but all have been observed with the haptonema attached to the surface of the culture vessel. It has been postulated that the same processes are involved in the attachment to, and subsequent translocation of food particles by the 40 - 55 μm coiling and bending haptonemata of *C.hirta* (Kawachi *et al.*, 1991), as are involved in surface adhesion. Further studies using high speed video will be needed to determine whether haptonema mediated food capturing occurs in any of the New Zealand species.

During this study members of the genus *Chrysochromulina* were most commonly observed in coastal waters during the early summer and late autumn, presumably when water temperatures and day length are most favourable to growth.

Coccolithophorales

The ubiquitous coccolithophores, *Emiliania huxleyi* and *Gephyrocapsa oceanica,* appear to be as common in New Zealand coastal waters as they are in other parts of the world's oceans. The motile form of *E.huxleyi* occurred in laboratory cultures with no particular trigger evident for the change in form from non-motile to motile. A heteromorphic life history is suggested for *G.oceanica* by the observations made during this study, but this requires confirmation.

Heteromorphic life histories have been recorded for a number of coccolithophores, including *E.huxleyi,* and several taxa previously considered as autonomous species are now known to be alternate phases of the life history of other coccolithophore species (Green *et al.*, 1989; Thomsen *et al.,* 1991). Information about motile forms is sparse and classification is at present based solely on coccolith descriptions. Clocchiatti (1971) reported coccoliths of both the *E.huxleyi* and *G.oceanica* forms on the same cell. No such combination has been noted in southern hemisphere isolates and the results of the growth studies carried out in this study demonstrate clear physiological differences between the two species (refer Chapter three, Results).

Morphological differentiation has been described for coccoliths of cells of *E.huxleyi* isolated from subtropical as opposed to subantarctic waters, the difference being due to increased calcite deposition in the coccospheres from the colder southern waters (Burns, 1977; Nishida, 1979). In this study secondary calcification, leading to fusion of the outer T-shaped coccolith elements and to thickening of the elements of the central grill, was confirmed on New Zealand isolates. No substantial difference was observed between the calcite elements of coccoliths from northern New Zealand (latitude 36^{o}S) and those from Big Glory Bay (latitude 47^{o}S); all had noticeable calcite thickening. The generally cooler sea temperatures around New Zealand, due to the El Niño phase of the Southern Oscillation (Heath, 1985), might be the controlling factor accounting for the lack of the classical T-shaped elements of northern hemisphere isolates (Burns, 1977).

Calcite deposition in *E.huxleyi* requires the initial laying down of a polysaccharide matrix material within the cell (Wilbur and Watabe, 1963). If the cell is subjected to nitrogen starvation the organic molecule synthesis and transport involved in coccolith formation is jeopardised. The marked dissolution observed in stationary phase cultures of both *E.huxleyi* and *G.oceanica* might well be associated with just such nitrogen depletion, rather than being strain specific to cold water phenotypes as has been suggested (Burns, 1977).

The organic scales of *Pleurochrysis scherffelii,* in its non-calcifying phase, consist of radial and concentric cellulosic fibrils (Romanovicz, 1981). The

Pleurochrysis species isolated in this study also bore organic scales. By altering the strength of the electron beam during microscopic investigation it was clear that, apart from minor etching on the outermost surface, the scales were simple unmineralised cylinders lacking ornamentation.

E.huxleyi dominated in the South Island waters and *G.oceanica* in Northland waters. Apart from minor blooms of *Pleurochrysis* sp. and *Syracosphaera* cf. *pirus,* and despite extensive sampling, few other species of coccolithophore were observed during the course of the study.

The marine prymnesiophytes of New Zealand's waters are, as elsewhere in the world, a notable component of the phytoplankton. Already 17 species of the 55 described in the genus *Chrysochromulina* have been identified in New Zealand coastal waters. Several prymnesiophyte species not yet recorded in New Zealand have been observed in the east Australian current (Hallegraeff, 1983) and it is likely that, even if they are not already in New Zealand waters, they will be observed here over the next few years. *C.hirta* was successfully transported ≈750 km with mussel spat from the north of New Zealand to the Marlborough Sounds. The ability of many of the prymnesiophytes to develop resting forms could further assist in their dispersal via ship's ballast water (Hallegraeff and Bolch, 1992).

Chapter two: FLUORESCENT PROBES AS TAXONOMIC TOOLS

Introduction

The prymnesiophytes, nanoplankton in the size range 2 - 20 μm, usually require electron microscope examination of their scales for confirmation of their identification. Fluorescent probes have been used to circumvent this need for other classes of microalgae, for example the Dinophyceae, where only light microscopy was available.

Calcofluor White M2R is a fluorescent dye, or fluorochrome, which binds to cellulose and other β-linked glucans (Hughs and McCully, 1975); the molecules also have a known affinity for chitin (Harrington and Raper, 1968). Calcofluor therefore has a role in elucidating the surface structural details of the many microalgae with cellulosic cell walls. For example the cellulose microfibrils that form the outer layer of the 20 - 40 μm appendages extending from the cell wall of the freshwater chlorococcalean alga *Acanthosphaera zachariasi* were highlighted by Calcofluor (Herth *et al.*, 1982). The thecal plate structure of members of the Dinophyceae, marine microalgae, has also been elucidated with this dye (Fritz and Triemer, 1985); several species of the toxic dinoflagellate genus *Alexandrium* are identified on the basis of the form of these cellulosic cell wall plates. The scales of *Chrysochromulina chiton* are composed of a β-1,4 glucan (Allen and Northcote, 1975) and it appears that the unmineralised surface scales of many *Chrysochromulina* species have similar fibrillar components.

The lectins (the name is derived from *legere* - to select) are non-enzymatic secretory proteins or glycoproteins that have the capability of acting as recognition molecules between cells and also have a suggested role in plant defence (Chrispeels and Raikhel, 1991). The lectins are derived from a variety of plant, animal and insect sources. The red macroalgal

genus *Ptilota* (Rhodophyceae) is a rich source, producing lectins which bind to glycoproteins as well as sugars (Rogers and Hori, 1993). Lectin from *Gracilaria verrucosa* (Rhodophyceae) suppresses the growth of the toxic microalga *Chattonella antiqua* (Raphidophyceae) (Tanabe *et al.*, 1993), which suggests a potential role in the control of toxic phytoplankton blooms.

Fluorescein isothiocyanate (FITC)-conjugated lectins are fluorochrome probes with the potential to aid in the differentiation of toxic from non-toxic strains of the same species and to discriminate between morphologically similar species without resorting to electron microscopy. This potential has already been tested in the differentiation of different genotypes of several microalgal species from the phyla Cyanophyceae, Dinophyceae and Conjugatophyceae (Costas *et al.*, 1993).

The lectins bind non-covalently (the binding is reversible) to sugars, but this binding ability is not induced via the immune system through antigenic stimulation (Brown and Hunt, 1978). They can be classified into groups on the basis of their specificity, as determined by the capacity of the various sugars to inhibit the haemagglutination of erythrocytes (Slifken and Doyle, 1990). The groups are glucose/ mannose, galactose/ N-acetyl-D-galactosamine, N-acetyl-glucosamine, fucose and sialic acids.

The analysis of the macromolecular topology of visible microalgal cell surfaces, which bear such potential receptors as glycoproteins, polysaccharides and chitin molecules can be carried out with lectins without chemically modifying those molecules, although factors such as hydrophobicity and accessibility will determine the nature of any lectin receptor. The localisation of various lectin receptor sites in secreted mucilage and on cell walls was determined for the freshwater desmid, *Cosmocladium saxonicus* (Surek and Sengbusch, 1981), and lectin binding patterns were shown to be species specific in a variety of freshwater microalgae (Sengbusch and Müller, 1983). This molecular diversity at the cell surface has been suggested as an added selection pressure to ensure specificity, for example through lectin-mediated

conjugation of microalgal gametes (Wiese and Shoemaker, 1970), and in evolutionary terms would have preceded diversity in morphological structure.

In this study the hypothesis that prymnesiophyte species which can not be differentiated by light microscopic observation can be identified with the aid of Calcofluor and by the differential binding of FITC-conjugated lectins is tested.

Method

Microalgae

The prymnesiophytes *Chrysochromulina apheles, C.camella, C.ericina, C.hirta, C.quadrikonta, Emiliania huxleyi* and *Gephyrocapsa oceanica,* the dinoflagellates *Gymnodinium sanguineum, Prorocentrum compressum* and *Scrippsiella* sp. and the diatoms *Pseudonitzschia pungens* var. *pungens* and *P.* cf. *pseudodelicatissima* were isolated from New Zealand coastal waters (Chapter two, Methods). *C.simplex, Prymnesium parvum, P.patellifera,* and *Pavlova* sp. were obtained from the University of Copenhagen, Denmark and *Isochrysis galbana* from MAF Fish, Mahanga Bay, Wellington.

The dinoflagellates *Alexandrium margalefii, A.ostenfeldii, A.minutum*, *Cachonina hallii* and *G.mikimotoi* were isolated by L.MacKenzie, Cawthron Institute, Nelson, *G.* cf. *breve* was isolated by A.Haywood, Cawthron Institute, and *Prorocentrum lima* (Spain) was obtained from the University of British Columbia, Canada.

Cultures were maintained in GP medium (Loeblich and Smith, 1968) at 18^0C, light intensity 100 μmol m^{-2} s^{-1} and light /dark cycle of 14:10 h.

Fluorescent probes

Calcofluor White M2R

A fresh solution of Calcofluor white M2R (1%; Sigma Chemical Co.) was made with distilled water. Microalgae were mounted on glass slides and the Calcofluor added to one edge of the cover slip (final concentration 0.05%). The algae were observed at the moving edge of the stain for several minutes to allow time for the dye to bind.

Lectins

Fresh solutions of FITC-conjugated lectins (100 μg .ml^{-1}; Sigma Chemical Co.) were made with filtered synthetic seawater (0.4 μm Nuclepore; Sigma Chemical Co), salinity 28$^{0}/_{00}$, pH8.0. Microalgal cell cultures were centrifuged (114 g; 12 minutes, 15^{0}C) and the lectin (Table 2.1) added to the microalgal pellet at 1 ml per 10 x10^{4} cells and incubated for 15 minutes at 20^{0}C. Unbound lectin was removed by washing in synthetic seawater (i.e. recentrifuging). The treated cells were mounted on siliconised glass slides and binding (cell fluorescence) and/or agglutination (cell clumping) observed under epifluorescence microscopy.
Lugol's iodine and gluteraldehyde were added to individual sub-samples of both *A.minutum* and *G.* cf. *breve* prior to treatment with UEA and ConA respectively to test whether fixation interfered with lectin binding. The cells treated and observed as above.

Specific carbohydrate inhibition of binding of FITC-labelled lectins

Carbohydrates specific for the lectins were added (50 mM final concentration) at the same time as the lectins were added in a separate set of experiments (Table 2.2). The microalgal cells were then treated in

Table 2.1 FITC-conjugated lectins used as probes

Name	Source	Specificity
ConA	*Canavalia ensiformis*	Methyl α-D-mannopyranoside; D-Mannose; D-Glucose
ECA	*Erythrina cristagalli* (coral tree)	α-Lactose; N-Acetyl-D-galactosamine; D-Galactose
HPA	*Helix pomatia* (snail)	N-Acetyl-D-glucosamine; N-Acetyl-D-galactosamine; D-Galactose
PEA	*Pisum sativum* (pea)	Methyl α-D-mannopyranoside; D-Mannose; D-Glucose
PHA	*Phaseolus limensis* (lima bean)	N-Acetyl-D-galactosamine
PNA	*Arachis hypogaea* (peanut)	α-Lactose; D-Galactose
PWM	*Phytolacca americana* (pokeweed)	N-Acetyl-D-glucosamine
SBA	*Glycine max* (soy bean)	N-Acetyl-D-galactosamine; D-Galactose; Methyl α-D-galactopyranoside
UEA	*Ulex europaeus* (gorse)	L-Fucose
WGA	*Triticum vulgaris* (wheat germ)	N,N′,N″-Triacetylchitotriose; N,N′-Diacetylchitobiose; Sialic acid

Table 2.2 Carbohydrates used to inhibit binding of FITC-labelled lectin.

Lectin	Carbohydrate (final conc. 50mM)
ConA, PEA	α-D-methyl mannopyranoside, glucose
PHA, SBA, ECA, HPA	N-Acetyl-D-galactosamine
PNA	α-Lactose
WGA	N,N′,N″-triacetylchitotriose
UEA	L-Fucose

the same way as the lectin-treated microalgae. Controls had lectin addition only. *A.minutum* and *G.mikimotoi* also had carbohydrate addition only (100 mM final concentration galactosamine and glucose respectively) as a further control.

Microscopy

Microalgae were examined following treatment with Calcofluor and FITC-conjugated lectin with an AHBT New Vanox Olympus photomicrographic epifluorescence microscope using UV (excitation, 375_{nm}; emission, $>420_{nm}$) and blue light (excitation, 490_{nm}; emission, 520_{nm}). Binding of the fluorescent probes was determined qualitatively at the time of observation and recorded as positive or negative. Kodak Ektachrome colour slide film was used, with the ASA at four times the advised setting, and photographs taken for confirmation of the results.

Results

Calcofluor white M2R

All the *Chrysochromulina* species tested fluoresced on the addition of Calcofluor, except *Chrysochromulina apheles* and *C.camella.* The spine scales of *C.ericina, C.quadrikonta* and *C.hirta* were highlighted and could be clearly observed with epifluorescence microscopy (Figure 2.1). The cell bodies of *Prymnesium parvum* and *P.patellifera* were clearly outlined with Calcofluor; *Isochrysis* sp. and *Pavlova* sp. were faintly outlined.

The two flagella of *Isochrysis* sp. and one of the two flagella of *C.ericina* and one of the four of *C.quadrikonta* autofluoresced in the absence of Calcofluor. The non-fluorescing flagella did not fluoresce with Calcofluor.

The coccolithophores did not fluoresce with the dye.

The thecal plates of the dinoflagellates *Alexandrium* and *Prorocentrum* (Peridiniales) were clearly outlined with the Calcofluor stain; the

Gymnodinium species tested (Gymnodiniales) did not fluoresce (Table 2.3).

FITC-conjugated lectins

All five *Chrysochromulina* species bound WGA; the fluorescence was clearly observed around the cell body in each case, but no fluorescence was noted on spine scales (Table 2.3).

None of the other prymnesiophytes tested fluoresced with any of the lectins.

Alexandrium margalefii fluoresced faintly with SBA and brightly with PNA; *A.ostenfeldii* fluoresced strongly with both PNA and HPA (Figure 2.2). The thecae of both species exhibited a faint autofluorescence without any lectin addition and this was taken into account in assessing lectin fluorescence.

A.minutum was the only species tested that bound (i.e. fluoresced with) UEA and *A.minutum* and *Scrippsiella* sp. were the only species tested to bind PWM. *Cachonina hallii* bound ConA (Table 2.3).

Gymnodinium sanguineum bound HPA and WGA, the latter forming a very fine fluorescent outline of the cell body, but did not bind ConA or PEA. *G.mikimotoi* and *G.* cf. *breve* both bound ConA and PEA. *G.* cf. *breve*, but not *G.mikimotoi*, also fluoresced strongly with PHA and a faint fluorescent cell outline was observed with SBA and WGA (Figure 2.2).

Prorocentrum lima (Figure 2.2) and *P.compressum* fluoresced with ConA; *P.compressum* also fluoresced weakly with ECA and WGA. Detritus in field samples also fluoresced and, although the reflectance was usually at one point only, in these instances no fluorescence was recorded.

The *Pseudonitzschia* species tested fluoresced with ConA and PHA (Table 2.3); the frustules of dead cells of both species, but not the healthy cells, fluoresced with WGA.

Table 2.3 FITC-conjugated lectins and Calcofluor used as fluorescent probes; their source and specificity.

	ConA	PHA	PEA	ECA	SBA	PWM	PNA	HPA	WGA	UEA	CALC
PRYMNESIOPHYCEAE											
Prymnesiales											
C.apheles	-	-	-	-	-	-	-	-	+	-	-
C.camella	NT	NT	NT	NT	NT	NT	NT	NT	NT	-	-
C.ericina	-	-	-	-	-	-	-	-	+	-	+
C.hirta	-	-	-	-	-	-	-	-	+	-	+
C.quadrikonta	-	-	-	-	-	-	-	-	+	-	+
C.simplex	-	-	-	-	-	-	-	-	+	-	+
Prymnesium parvum	-	-	-	-	-	-	-	-	-	-	+
P.patellifera	-	-	-	-	-	-	-	-	-	-	+
Pavlovales											
Pavlova sp	-	-	-	-	-	-	-	-	-	-	+
Isochrysidales											
Isochrysis sp	-	-	-	-	-	-	-	-	-	-	+
Coccolithophorales											
Gephyrocapsa oceanica	-	-	-	-	-	-	-	-	-	-	-
Emiliania huxleyi	-	-	-	-	-	-	-	-	-	-	-

Table 2.3 continued..

DINOPHYCEAE											
Gymnodiniales											
Gymnodinium* cf. *breve	+	+	+	-	+*p*	-	+	-	+*p*	-	-
G.mikimotoi	+	-	+	-	-	-	-	-	-	-	-
G.sanguineum	-	-	-	-	-	-	-	+	+*p*	-	-
Peridiniales											
Alexandrium margalefii	-	-	-	-	+	-	+	-	-	-	+
***A.ostenfeldii* (Timaru isolate)**	-	-	-	-	-	-	+	+	-	-	+
***A.ostenfeldii* (Kaitaia isolate)**	-	-	-	-	-	-	+	+	-	-	+
***A.ostenfeldii* (Wellington isolate)**	-	-	-	-	-	-	+	+	-	-	+
A.minutum	-	-	+*p*	+*p*	+*p*	+*p*	+	+	+*p*	+*p*	+
Cachonina hallii	+*p*	-	-	-	-	-	+*p*	+*p*	+*p*	-	+
***Scrippsiella* sp**	-	-	-	-	-	+*p*	-	-	-	-	+
Prorocentrales											
Prorocentrum compressum	+*p*	-	-	+*p*	-	-	-	-	+*p*	-	+
P.lima	+*p*	-	-	-	-	-	-	-	-	-	+
DIATOMOPHYCEAE											
Pennales											
Pseudonitzschia pungens* var.*pungens	+	+*p*	-	-	NT	-	NT	NT	-	-	NT
P.*cf.*pseudodelicatissima	+*p*	+*p*	-	-	NT	-	NT	NT	-	-	NT

NT: not tested; +*p*: pale fluorescence (weak binding); +: bright fluorescence (binding); -: no fluorescence (no binding).

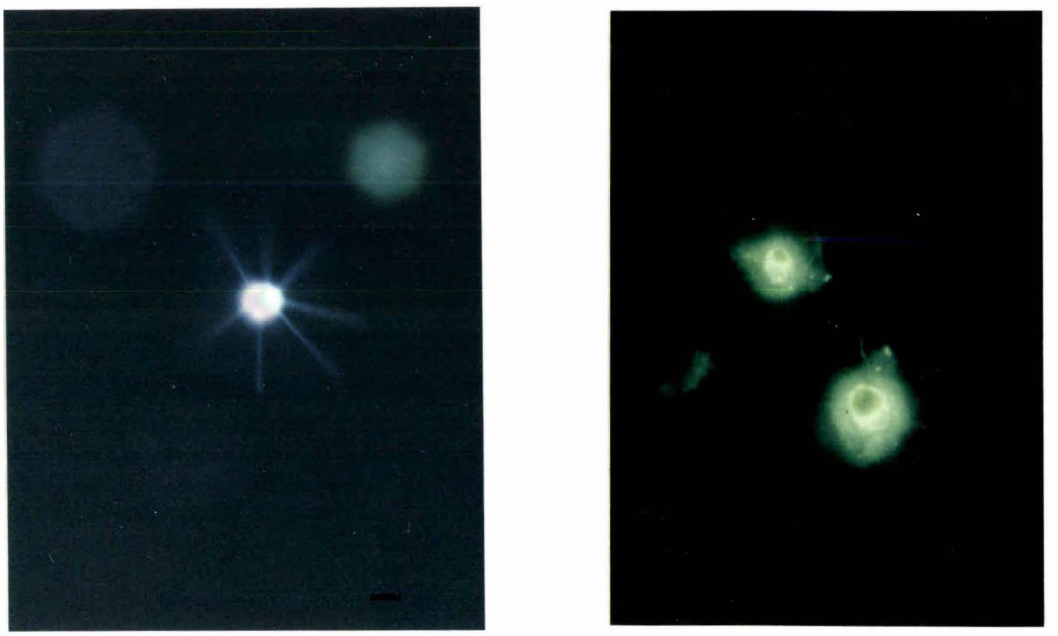
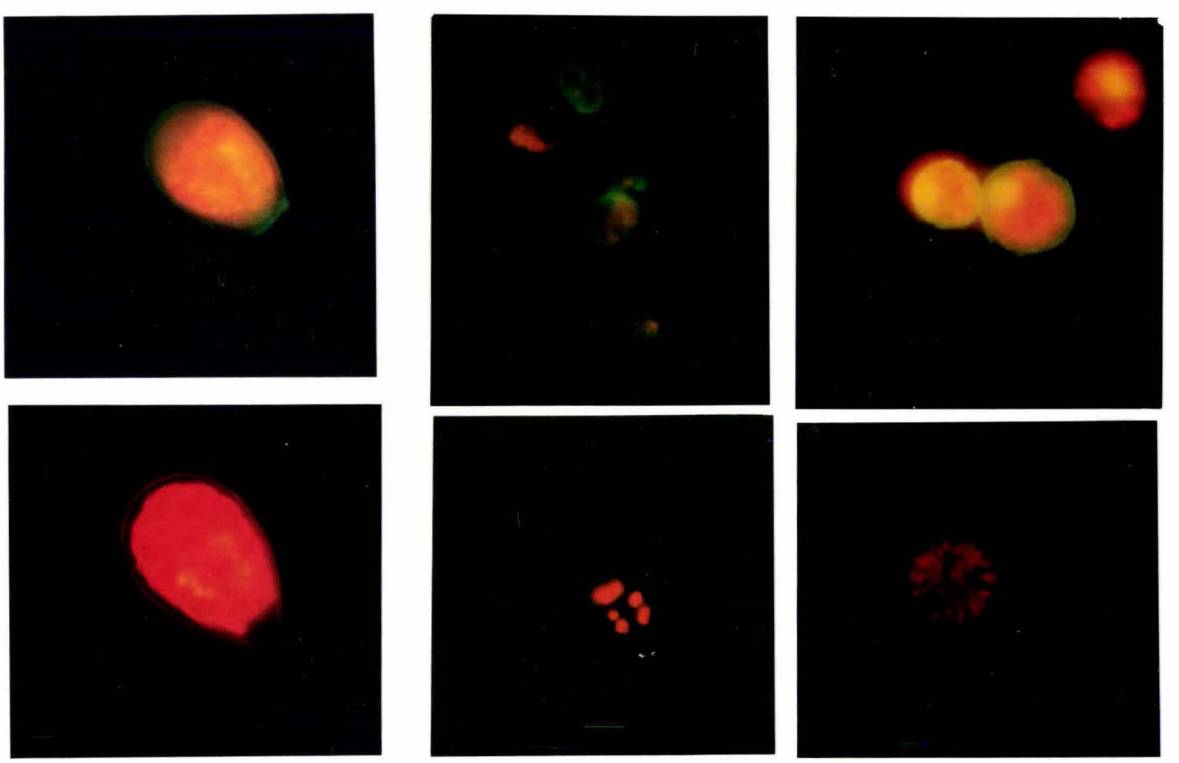


Fig. 2.1 The spine scales of ***Chrysochromulina ericina*** (L) and ***C.quadrikonta*** (R) fluorescing under UV light following the addition of the fluorescent dye, Calcofluor White. Bar = 5 μm.



(a) (b) (c)

Fig. 2.2 The thecae of dinoflagellates with (top) and without (lower) the addition of FITC-tagged lectins, photographed under blue light excitation: (a) *Prorocentrum lima* (ConA lectin), (b) *Gymnodinium* cf. *breve* (PHA lectin) and (c) *Alexandrium ostenfeldii* (HPA lectin).

Addition of Lugol's iodine or gluteraldehyde to *A.minutum* and *G.* cf. *breve* did not effect the binding of lectins, UEA and ConA respectively, to those microalgae.

Carbohydrate inhibition of binding of FITC-labelled lectins

Glucose failed to inhibit the binding of ConA to all species tested, including *G.* cf. *breve*; methyl α-D-mannopyranoside also failed to competitively inhibit the binding of ConA and PEA to all species tested except PEA to *G.* cf. *breve* (Table 2.4).

N-acetyl-D-galactosamine inhibited the binding (i.e. fluorescence decreased) of PHA, SBA, HPA and ECA to all species tested except for SBA to *A.margalefii* (Table 2.4).

Binding of PNA was inhibited by α-lactose in all species tested except *A.margalefii* (Table 2.4).

N-triacetylchitotriose (25 mM final concentration) caused some inhibition of binding of WGA to *A.minutum.*

The binding of UEA to *A.minutum* was not inhibited by L-fucose under the experimental conditions (Table 2.4).

A.minutum and *G.mikimotoi* remained healthy with the addition of galactosamine and glucose (100 mM) respectively.

Discussion

Fluorescent probes proved to be useful tools not only for the identification of morphologically-like species, but also for the confirmation of genus differentiation.

The fluorescence of stained spine scales of *C.quadrikonta* with Calcofluor White M2R allows identification of this species using light microscopy. However *C.ericina* and *C.hirta* still require electron microscopy for

Table 2.4 Carbohydrate inhibition of FITC-labelled lectins.

Treatment	*Gymnodinium* cf. *breve*	*G.mikimotoi*	*G.sanguineum*	*Alexandrium margalefii*	*A.ostenfeldii* (Timaru)	*A.minutum*	*Cachonina hallii*
ConA	+	+					+
ConA; glucose	+	+					+
ConA; mannopyranoside	+						+
PHA	+						
PHA; galactosamine	+*p*						
PEA	+					+	
PEA; mannopyranoside	-					+	
SBA	+*p*			+*p*		+	
SBA; galactosamine	-			+*p*		+*p*	
HPA			+		+*p*	+*p*	+*p*
HPA; galactosamine			+*p*		-	-	-
PNA				+*p*	+	+*p*	+*p*
PNA; lactose				+*p*	+*p*	-	-
ECA						+*p*	
ECA; galactosamine						-	
UEA						+*p*	
UEA; fucose						+*p*	
WGA						+*p*	
WGA; chitotriose						-/+*p*	

+p: pale fluorescence (weak binding); +: bright fluorescence (binding); -: no fluorescence (no binding).

species confirmation as their spine scales are of a similar length and their differences in form are not clearly defined at the lower magnifications of light microscopy, despite fluorescence.

The FITC-conjugated lectins enabled the differentiation of those species of *Chrysochromulina* tested from *Prymnesium parvum* and *P.patellifera.* The genus *Chrysochromulina* was initially separated from the genus *Prymnesium* on the basis of the longer haptonemal length and haptonemal coiling in *Chrysochromulina* (Manton and Leadbeater, 1974) and on the existence of only one scale type in *Prymnesium.* However it is now recognised that species with long haptonema do not always exhibit coiling, for example *C.parkeae* (Green and Leadbeater, 1972) and *C.spinifera* (Pienaar and Norris, 1979), and that there are species with short non-coiling haptonema that do have dimorphic scales (Pienaar and Kleizen, 1976). The discrimination of the genera on these bases is therefore untenable and the possibility of combining them has been raised (Pienaar and Norris, 1979). There is more recent evidence, however, that the genera do differ at the ultrastructural level in the internal structure of their flagellar apparatus. *Chrysochromulina* species possess broad flagellar roots with relatively few microtubules, whereas members of the genus *Prymnesium* have multimembered flagellar roots (Moestrup and Thomsen, 1986).

The binding of wheat germ lectin (WGA) to all of the *Chrysochromulina* species tested, but to neither of the *Prymnesium* species, supports the decision to retain the two separate genera. WGA appears to bind to the cell membrane rather than to the unmineralised scales, as no spine scales have been observed fluorescing. It is possible that lectin receptor sites are present on the scales, but inaccessible to the lectins; non-binding is not necessarily indicative of the lack of receptor molecules.

The differential binding of lectins to the dinoflagellates suggests that this qualitative technique has great potential in the identification of toxic bloom species. The saxitoxin-producing *Alexandrium* species can be extremely difficult to differentiate without painstaking microscopic

analysis of their thecal plate structures; *A.minutum* is difficult to differentiate from local isolates of *Cachonina hallii* and *Scrippsiella* sp., non-toxic species that commonly occur with *A.minutum* in New Zealand waters. Currently research effort is being concentrated on the development of genetic probes and immuno-chemical assays for the identification of species which, it would seem, could be more simply determined by the FITC-conjugated lectin technique. As fixatives did not alter the binding of the lectins to those microalgae tested, it is possible that field samples containing mixed phytoplankton populations could be treated with the differentiating lectin and the species identification confirmed by the presence or absence of fluorescence.

Prorocentrum compressum was differentiated from *P.lima* by WGA, both species binding to ConA. However *P.lima* did not bind to PHA, ECA or SBA, which is in contrast to the results of Costas *et al.*, (1993), although they were working with different clones. A New Zealand strain of *P.lima* was isolated in August, 1994, from Rangaunu Harbour, Northland (L.Rhodes) and it will be valuable to determine whether this isolate has a similar toxin profile and, if not, whether there are binding differences between these geographically separate strains.

Many species of the genus *Gymnodinium* are notoriously difficult to identify to species level and *G.mikimotoi* and *G.* cf. *breve* are particularly difficult. The former produces ichthyotoxins, which are harmless to humans if present in shellfish, whereas the latter produces the neurotoxic brevetoxin which has been responsible for the closure of shellfisheries in New Zealand since 1993. As both toxins give a positive reaction in mouse bioassays, currently used to detect brevetoxin in shellfish, it is important to differentiate between the species with confidence. The FITC-lectin technique warrants further testing as a simple tool for their differentiation.

The *Pseudonitzschia* species (Diatomophyceae) were not differentiated by the lectins tested and in this instance immuno-chemical assays might be a

more effective method of rapid identification, particularly of the toxic, domoic acid producing, forms.

The results of the carbohydrate inhibition assays were variable; glucose, fucose and methyl mannopyranoside were the least effective at the concentrations added and neither galactosamine nor lactose inhibited the binding of SBA or PNA to *A.margalefii.* It is possible that other more competitive sugars were present on *A.margalefii's* cell surface or that the sugars were present in greater concentrations on the microalgal cell surface than were added for the competitive binding assay. Galactosamine, lactose and triacetylchitotriose competed successfully with the other microalgae tested for those lectins with which they had an affinity. It appears that ConA was tightly bound at the molecular surface of *G.mikimotoi, G.* cf. *breve* and *C.hallii,* PEA to that of *A.minutum* and SBA and PNA to that of *A.margalefii.*

The application of this technique to the prymnesiophytes, and the dinoflagellates, warrants further research; the genus *Gymnodinium* in particular warrants more intensive study.

Chapter three: GROWTH CHARACTERISTICS OF PRYMNESIOPHYTES

Introduction

The taxonomy of many of the prymnesiophytes in the order Prymnesiales have been described (Green *et al.*, 1989), but prior to 1988, when the toxic species *Chrysochromulina polylepis* killed massive numbers of caged salmon in Scandinavian waters (Skjoldal and Dundas, 1989), there was little emphasis on the investigation of the cultural requirements and growth characteristics of these nanoflagellates. As toxic prymnesiophyte blooms have continued to occur in the Scandinavian fiords (Aune *et al.*, 1992), with associated caged fish deaths, there has been a recognition of the urgency to understand the environmental triggers that lead to the rapid growth of these organisms and to the development or enhancement of toxicity.

Studies to date include the determination of the effect on growth rate of light intensity and temperature for the toxic species *Prymnesium patelliferum* (Larsen and Paasche, 1993), *P.parvum* (Shilo, 1971) and *C.polylepis* (Edvardsen and Paasche, 1992), but little is known about the growth characteristics of non-toxic species. The ability of some species of *Chrysochromulina* to assimilate carbon heterotrophically has been demonstrated (Pintner and Provasoli, 1968) and as many as one tenth of the described species of *Chrysochromulina* are known to be mixotrophic, combining heterotrophic and/or phagotrophic feeding behaviour with photosynthesis (Parke *et al.,* 1955,1956; Jones *et al.,* 1993). A requirement for vitamins, in particular thiamine and vitamin B_{12}, (Pintner and Provasoli, 1968) and selenium (Harrison *et al.,* 1988; Dahl *et al.*, 1989) has been demonstrated for some prymnesiophyte species.

In New Zealand there has been a rapid development of the aquaculture industry over the last decade with a consequent need for understanding of the growth

habits of locally occurring and potentially toxic nanophytoplankton. In this study the *in vitro* growth characteristics of six New Zealand *Chrysochromulina* isolates are determined and compared with those of the toxic northern hemisphere isolate *C.polylepis.* Their individual preferences for oceanic, coastal or estuarine waters and for sub-tropical, temperate or sub-antarctic water temperatures are inferred from the *in vitro* studies. The competitive advantages of some species due to, for example, their ability to grow at low light intensities, to tolerate a wide range of salinities or to phagocytose, are discussed.

The hypothesis that prymnesiophytes produce enzymes which hydrolyse substrates such as cellulose (found in dinoflagellate thecae), chitin or protein, making them available for heterotrophic assimilation, is tested. In particular, *Prymnesium parvum* causes the death in dual culture of other species of microalgae, and also the brine shrimp *Artemia salina.* *P.parvum* characteristically clusters around the dead organisms (personal observation) and the possibility that death is due to hydrolase production, rather than the secretion of the toxin prymnesin is explored.

The ubiquitous and most abundant of microalgae, are the coccolithophores (Coccolithophorales), with *Emiliania huxleyi* and *Gephyrocapsa oceanica* dominating in Australasian waters (Hallegraeff, 1984).

The taxonomy of the coccolithophores has been intensively studied (Young, 1993), but only northern hemisphere isolates of *E.huxleyi* and *Cricosphaera* sp. have undergone detailed physiological examination. For example the effect of changing light intensity on growth and photosynthesis has been elucidated (Jeffrey and Allen, 1964; Paasche, 1968) and it is known that *E.huxleyi* survives within a temperature range of $<0^{0}$C to 29^{0}C, whilst *G.oceanica* survives within a temperature range of 13^{0}C to 26^{0}C (McIntyre and Bé, 1967). Both species occur at depths down to 50 m in oceanic waters, but tend to be nearer the surface at the northern and southern temperature limits (Honjo, 1977).

The present study determines the *in vitro* growth characteristics of New Zealand isolates of *G.oceanica, E·huxleyi* and *Pleurochrysis* sp. and relates these to the current species distribution around coastal New Zealand.

Methods

Microalgae: isolation and identification

Chrysochromulina acantha, C.apheles, C.camella, C.ericina, C.hirta, C.quadrikonta, Gephyrocapsa oceanica, Emiliania huxleyi and *Pleurochrysis* sp. were isolated from samples collected from New Zealand coastal waters with either a 10 μm mesh net or a Van Dorn bottle sampler (refer Chapter one, Methods). Cells were isolated by picking out with fine bore Pasteur pipettes and transferring into CHRY (Andersen *et al.,* 1991) and GP (Loeblich and Smith, 1968) media in Multiwell^{TM} tissue culture plates (Becton Dickinson). Germanium dioxide (0.5 mg l^{-1} final concentration) was added to inhibit diatom growth.

Chrysochromulina polylepis (C285) was obtained from the Provasoli-Guillard Centre Culture Collection at Bigelow Laboratory for Ocean Sciences, West Boothbay Harbour, Maine, USA. It was originally isolated from Scandinavian waters during the May-June bloom of 1988. Cultures were established in CHRY and GP media, and grew consistently well at 15^0C.

Prymnesium parvum was provided by F.H.Chang, NIWA, Wellington and was maintained in GP medium.

The initial identification and the continued purity of the New Zealand *Chrysochromulina* isolates and *C.polylepis* (Larsen and Moestrup, 1989) were confirmed by comparison of transmission electron micrographs with those in the literature. Whole cells and individual scales, directionally shadow cast with platinum, were examined under a Philips 201c transmission electron microscope and photographed; coccolithophores were identified by scanning electron microscopy (refer Chapter one, Methods and Results).

Culture and maintenance of isolates

Cultures were established in GP and CHRY media (pH7.9), made up with filtered oceanic seawater, salinity $34^0/_{00}$. Maintenance cultures, held in 50 ml culture bottles, were transferred from 100 μmol m^{-2} s^{-1} light intensity to 30 μmol m^{-2} s^{-1} at late exponential phase. Temperatures were maintained at 18^0C ($\pm$ 1^0C).
Experimental cultures of *Chrysochromulina* species were grown in a modified GP medium in which synthetic sea water (Gross, 1967) replaced oceanic sea water, vitamin addition was doubled, sodium selenite was included (as for CHRY medium), soil extract was reduced to one tenth of the normal GP level, TES (N-tris[hydroxymethyl]-methyl-2-aminoethane-sulfonic acid; final concentration 0.01 M) was used as a buffer, as it did not affect growth of the algae, and pH was adjusted to 7.9 with 1 M NaOH prior to sterilisation (121^0C, 15 min). Experimental cultures (20 ml begun with a 10% inoculum derived from a culture in exponential phase) were grown in 50 ml acid-washed glass tubes (Kimax^{TM}) with teflon caps or, in the instances when microalgae would not grow in tubes, in sterile plastic bottles (50 ml). Incubation was at a light intensity of 100 μmol m^{-2} s^{-1} with a 14:10 h light:dark cycle and a temperature of 18^0C ($\pm$ 1^0C) unless otherwise stated.
Coccolithophores were cultured as above, but with pH adjusted to 8.4.

Growth Measurements

Exponential growth of the microalgae was measured by chlorophyll *a* increases using a Turner Designs Fluorometer (Model 10-005R), with the filter combination for *in vivo* fluorescence (light source: 10-045 blue lamp; excitation filter: 10-050 colour specification 5-60; emission filter: 10-051 colour specification 2-64). Cultures of *Prymnesium* species (20 ml) grew successfully in the glass fluorometer tubes and microalgal growth could be measured directly by placing the tubes in the fluorometer. Cultures of *Chrysochromulina* species (20 ml) were grown in 50 ml plastic bottles and transferred to the sterile

fluorometer tubes for growth measurements, as growth in the tubes was variable.

Fluorometer readings correlated well with cell counts during the exponential phase of growth, but not during the stationary phase. At stationary phase, cell counts were determined using an inverted IMT-2 Olympus microscope. Cultures were sampled, preserved with Lugol's iodine (20 μl per 100 ml; greater amounts caused cell lysis) and allowed to settle in Utermöhl chambers for 4 hours prior to counting.

Growth rates (number of doublings per day) were derived from the means of a minimum of four replicates. ANOVA was used to determine the statistical significance of the results, with the Tukey-Kramer method (Sokal and Rohlf, 1981) applied to give significant differences.

Heterotrophy

Axenic microalgal cultures were obtained by adding 0.002% Penicillin G and 0.0003% streptomycin sulphate to the microalgal culture medium. Purity of cultures was checked by streaking on to marine bacteriological agar plates (Difco, USA) and incubating at 25^0C. Axenic in this context means that bacteria did not grow under the described conditions.

Axenic cultures were grown in quadruplicate in either f2 (Guillard, 1975) or modified GP medium with additions of 0.1% of either casein hydrolysate, acetate (sterilised at 121^0C, 15 min), glucose or glycerol (filter sterilised; 0.2 μm nuclepore) before addition to sterile medium. Cultures were incubated at 18^0C at light intensities of 100, 30 or 0 μmol m^{-2} s^{-1}.

Enzyme studies

(a) **Cellulases.** Cellulase production was determined for *P.parvum, C.ericina, C.polylepis* and *C.quadrikonta* by assaying cell free culture medium and whole cells for cellulase activity.

Heterocapsa triquetra is a dinoflagellate bearing a cellulose theca. *Prymnesium parvum* was observed clustering around cells of *H.triquetra* during toxin bioassays (refer Chapter 5, Results) and it was hypothesised that the prymnesiophyte was utilising the cells as a nutrient source by hydrolysing the cellulosic thecae with extracellular hydrolases. To test this hypothesis *H.triquetra* (25 ml in 150 ml Erlenmeyer flasks grown under standard conditions in GP medium; 14 day old culture) was inoculated with *C.ericina, C.polylepis, C.quadrikonta* or *P.parvum* (25 ml of 7 day old culture; controls GP medium only). Controls had GP medium only added. These dual cultures were incubated at 20^0C, 100 μmol m^{-2} s^{-1} light intensity and 14:10 h light/dark regime. Samples (5 ml) of the cultures were harvested at 0 h and after 5 days growth (for both cell counts and for assaying for cellulase) and were monitored for morphological changes during growth under a light microscope (magnification x600).

Harvested cultures were centrifuged (9,900 g) and the supernatant frozen. The pellet of microalgal cells was resuspended in 1 ml acetate buffer (sodium acetate, 70 ml 0.2 M: acetic acid, 30 ml 0.2 M; pH 5; final concentration 0.05 M), sonicated (20 seconds x 3, with 20 second intervals) and frozen.

The assay for cellulase involved measuring the reducing groups produced when cellulose was incubated with supernatant or resuspended cells.

Carboxymethylcellulose (Sigma) was dissolved or microcrystalline cellulose (AVICEL) was suspended in 0.2 M sodium acetate - 0.2 M acetic acid (acetate) buffer (5 mg 1 ml^{-1}; pH 5), the former to measure endo-glucanase activity and the latter to measure exo-glucanase activity. Culture supernatant (0.1 ml) was incubated with the cellulose substrate at 30^0C for 0 or 30 min. The assay was also run for *P.parvum* culture supernatant at 17^0C for 30 min. The reaction was stopped by the addition of para hydroxy-benzoic acid hydrazide (PAHBAH) reagent. Samples were also run (i) without cellulose substrate (buffer only) and (ii) with cellulose and commercial cellulase (5 mg 1 ml^{-1} acetate buffer). The cellulase was from *Trichoderma viride* (Sigma; activity 1-5 units mg^{-1} solid).

Reducing substances were measured using the PAHBAH procedure (Lever, 1973). Following the addition of the PAHBAH reagent, samples and standards were held at 100^0C in a water bath for 10 min., then cooled in an ice bath.

Distilled water (4 ml) was added and the tubes read at 420_{nm} on a spectrophotometer (against a control tube (i) containing acetate buffer).

(b) **Chitinases.** Assays for chitinase production in *P.parvum* were carried out following the growth of cultures as for cellulase production above, but with (i) nauplii of the arthropod *Artemia salina* (refer Chapter 5, Methods) or (ii) colloidal chitin (0.2 mg ml^{-1}) (Shimahara and Takiguchi, 1988) instead of cellulose. *A.salina* was chosen as it contains chitin in its procuticle (Villee *et al.,* 1973).

Pellets were obtained by centrifugation (as above) from 6 day old *P.parvum* cultures and were resuspended in imidazole buffer (5 ml; 0.1 M) containing ethylenediamine tetraacetic acid (EDTA; 0.1 mM) and dithiothreitol (DTT; 0.1 mM). The buffer, pH 6.2, was made up in $35^{0}/_{00}$ sodium chloride.

Sample (1 ml of supernatant or resuspended pellets) was added to 1 ml chitin (15 mg ml^{-1} in 0.1 M imidazole buffer containing sodium chloride ($17.5^{0}/_{00}$)) and held on ice.

A positive control with chitinase added (from the marine fungus *Corollospora maritima* provided by D.Grant, 81 Mount St., Nelson, New Zealand) and a negative control with filtered seawater only were included in the assay.

The tubes were held at 37^{0}C in a water bath and samples (800 μl) were taken at 0 h and after 3 h incubation. Samples were held at 100^{0}C for 5 minutes then cooled and centrifuged (9,900 g; 5 min.). Reducing substances in the supernatants were measured by the PAHBAH procedure described above, but with glucosamine rather than glucose standards.

(c) **Proteases.** Protease activity in *P.parvum* and *C.ericina* was investigated as follows: axenic cultures were grown in GP medium under standard conditions with the addition of 0, 0.5 or 0.05% bovine serum albumin (BSA). Controls contained no BSA. Cultures were sampled and the supernatant and pellet obtained by centrifugation (as for cellulase assay). *C.ericina* cultures containing BSA were also grown with the addition of a loopful of cream agarlytic rod-shaped bacterium (*Pseudomonas* sp.), isolated from the same seawater sample as *C.ericina*. Controls included *C.ericina* with bacteria but no BSA, and GP medium with bacteria only. Hide powder azure (5 mg; Sigma) (Rinderknecht *et al.*, 1968) was added to 0.5 ml TES buffer (10 mM) (made up in filtered

seawater, pH 8.0) and 0.5 ml of supernatant or pellet in acid washed capped tubes. Tests were in duplicate. Tubes were incubated for 2 hours at 37^0C and inverted to mix every 5 minutes. The assay mixtures were then passed through glass wool (in short Pasteur pipettes held over spectrophotometer cuvettes) and the absorption of supernatants read at 595_{nm}.

Phagotrophy

Microalgae were cultured in tissue culture plates. Fluorescently-labelled beads (0.5 μm diameter, soaked in bovine serum albumin), graphite, *Porphyridium cruentum, P.purpureum* (Rhodophyceae) and a fluorescing bacterium, *Pseudomonas* sp. (from the Cawthron culture collection), were added at early or late exponential phase and at stationary phase. Non-axenic and axenic *Chrysochromulina* cultures were tested. In non-axenic cultures DAPI (4'6-diamidino-2-phenylindole) was used to detect ingested bacteria (Porter and Feig, 1980).

Acrylic acid analyses

High-performance liquid chromatographic determinations of acrylic acid followed the method of Brown (1979).

Results

Growth characteristics of Prymnesiales

Selenium/cobalt addition

C.quadrikonta, previously grown in selenium-free GP medium, exhibited an increase in growth rate (GR; doublings d^{-1}) with the addition of 0.02 μM selenium (in the form of sodium selenite); GR increased significantly from 0.59 to 0.82 ($P<0.01$). Final yields of cultures (total cell counts at stationary phase)

were all double that of the control with selenium additions of from 0.02 to 0.1 μM.

No significant difference in growth rate was noted when selenium was added to previously selenium deprived cultures of *C.acantha, C.apheles, C.camella, C.ericina* or *C.hirta* (Table 3.1).

Table 3.1 Selenium as a limiting growth factor in *Chrysochromulina* species.

Microalga	*Se supplement requirement*
C.acantha	-
C.apheles	-
C.camella	-
C.ericina	-
C.ericina[a] (North Pacific)	+
C.hirta	-
C.polylepis[b] (Scandinavia)	+
C.quadrikonta	+
C.strobilus[c]	+

References: [a] Harrison *et al.*, 1988; [b] Dahl *et al.*, 1989; [c] Pintner and Provasoli, 1968.

Cobalt was normally added to GP medium at 0.11 μM. At 0.23 μM *C.quadrikonta* ceased growth, but survived for five days; at 0.57 μM cells died immediately.

Nitrogen source

There was no significant difference in the growth rates (GR, doublings per day), of *C.apheles, C.camella, C.ericina, C.polylepis* or *C.quadrikonta* when grown in GP medium with potassium nitrate, urea or ammonium chloride as nitrogen source. *C.acantha* had faster GRs with potassium nitrate ($P<0.01$) and ammonium chloride ($P<0.05$) than with urea and *C.hirta* with urea and ammonium chloride than with potassium nitrate ($P<0.05$) (Table 3.2).

Table 3.2 Growth rates (doublings per day) of *Chrysochromulina* species cultured with different nitrogen sources. Bars indicate a significant difference in growth rate ($P<0.05$; ANOVA, Tukey Kramer).

Microalga	*Potassium nitrate*	*Ammonium chloride*	*Urea*
C.acantha	0.9	0.8	0.7
C.apheles	0.4	0.4	0.3
C.camella	0.8	0.9	0.9
C.ericina	1.1	1.1	0.9
C.hirta	0.8	1.2	1.2
C.polylepis	1.5	1.5	1.8
C.quadrikonta	0.7	0.9	0.7

Temperature

Growth rates (doublings d^{-1}) were faster for *C.acantha* ($P<0.05$), *C.apheles*, *C.camella* and *C.quadrikonta* (all $P<0.01$) at 20^0C than at 15^0C. There was no significant difference between between 20^0C and 25^0C for *C.acantha* or *C.apheles*, whereas *C.quadrikonta* and *C.camella* both had a faster GR at 25^0C than at 20^0C ($P<0.05$). None of these species grew, although some cells survived for a week, at 10^0C. Freshly inoculated cultures of all species died when transferred to 30^0C, although when stationary cultures of *C.apheles* were transferred to 30^0C they survived for several days.

C.polylepis, C. ericina and *C. hirta* all grew at a faster rate at 15^0C than at 10^0C ($P<0.01$). There was no significant difference in GR between 15 and 20^0C. All

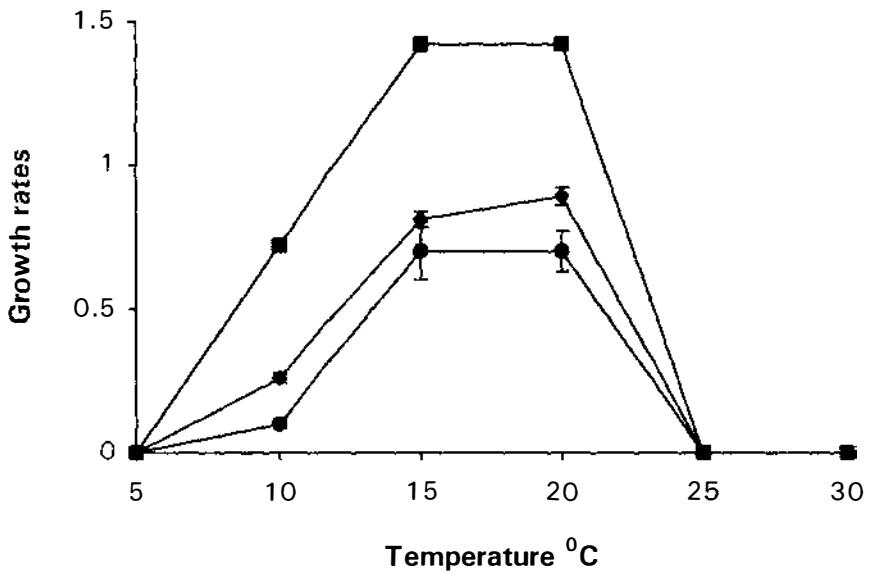
three species died at 25^{0}C; none grew (although some cells remained viable) at 5^{0}C (Figure 3.1).

Salinity

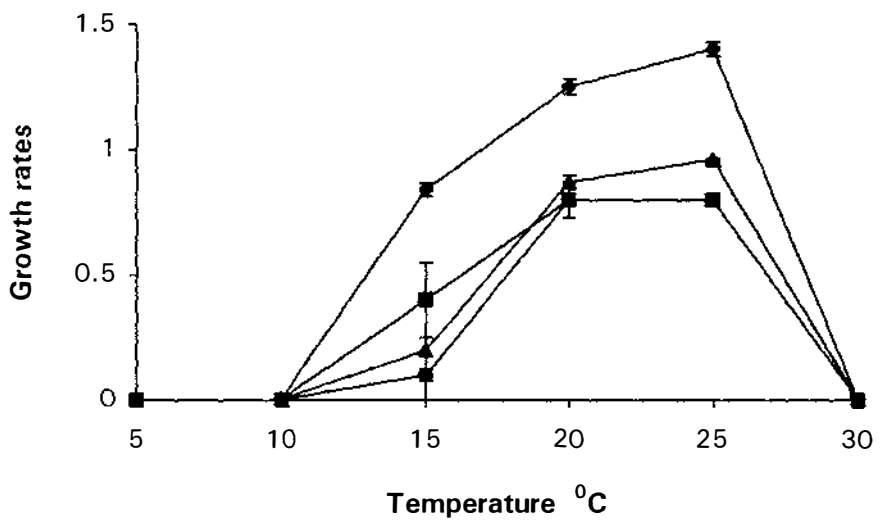
C.quadrikonta and *C.polylepis* had faster GRs at salinities of 17 - 23^0/$_{00}$ than at 29^0/$_{00}$ ($P<0.01$) under the experimental conditions. Neither grew at 35^0/$_{00}$.
C.hirta grew optimally at 23^0/$_{00}$ ($P<0.01$), although it grew between 10 and 35^0/$_{00}$.
C.ericina grew optimally between 17 - 35^0/$_{00}$, although growth lagged at 35^0/$_{00}$. Growth was slower, as it was for *C.apheles*, at 42^0/$_{00}$ ($P<0.01$). *C.apheles* and *C.camella* grew optimally between 23 - 35^0/$_{00}$ ($P<0.01$). *C.camella* failed to grow at 17^0/$_{00}$ and growth occurred at 42^0/$_{00}$, but only at 25^{0}C and with urea as the nitrogen source (Figure 3.2).
C.acantha grew optimally between 29 - 35^0/$_{00}$, but still grew well at 42^0/$_{00}$.

pH

Under the experimental conditions there was no significant difference in GR for *C.quadrikonta* between pH7.0 and pH8.7.
C.ericina, *C.hirta* and *C.acantha* grew optimally between pH7.5 and 8.7; *C.acantha* did not grow, whereas *C.hirta* and *C.ericina* had slow GRs, at pH7.0 ($P<0.01$). *C.apheles* grew optimally between pH7.5 and 8.4 ($P<0.01$).
C.camella grew optimally at pH8.0. Growth was significantly slower at pH7.5 and pH8.4 ($P<0.01$).
Growth of *C.polylepis* was optimal between pH8.4 and 8.7 ($P<0.01$; Figure 3.3).

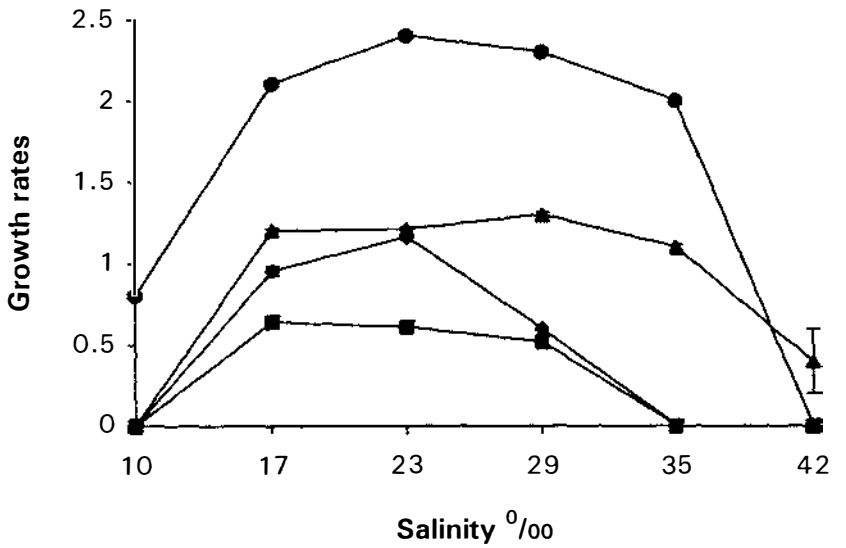


(a)

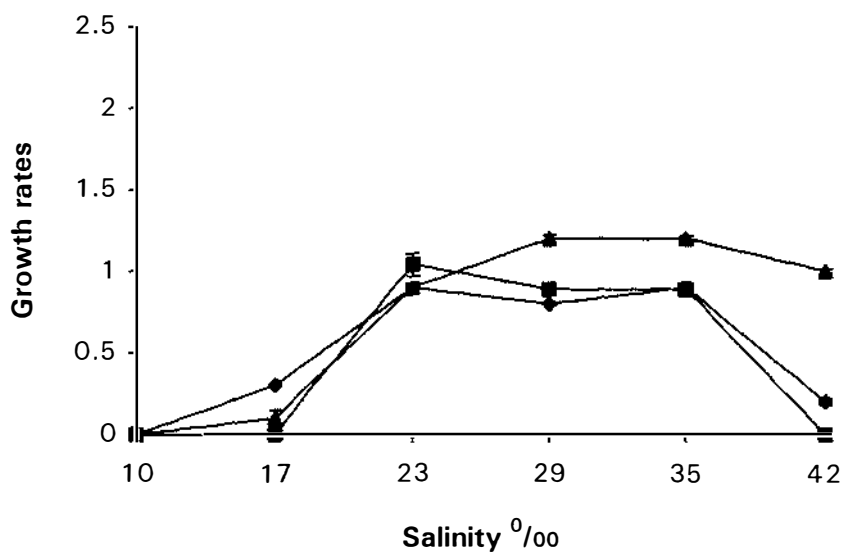


(b)

Fig. 3.1 Growth rates (doublings per day) of several *Chrysochromulina* species at different temperatures: (a) *C.polylepis* (■), *C.ericina* (◆) and *C.hirta* (●); (b) *C.acantha* (■), *C.apheles* (●), *C.quadrikonta* (◆) and *C.camella* (▲). Bars indicate standard error ($n=4$), but are obscured by symbols in some instances.

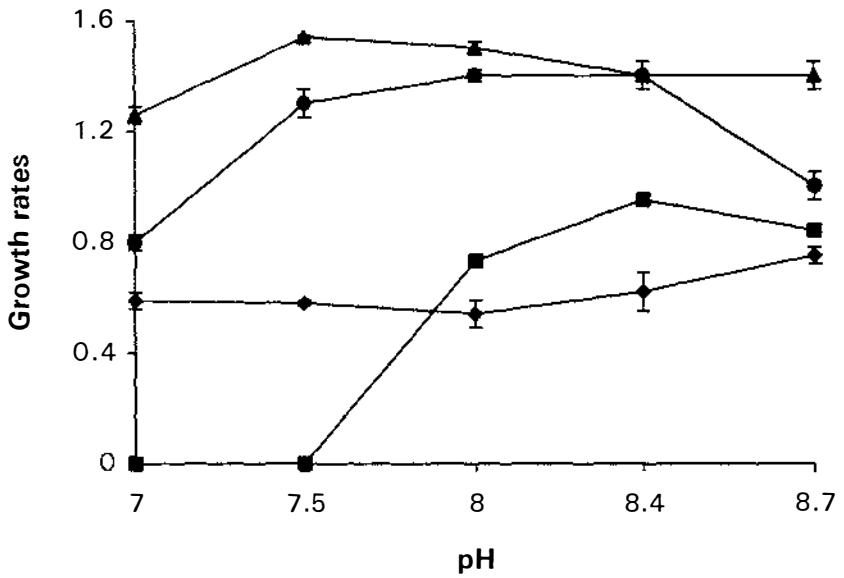


(a)

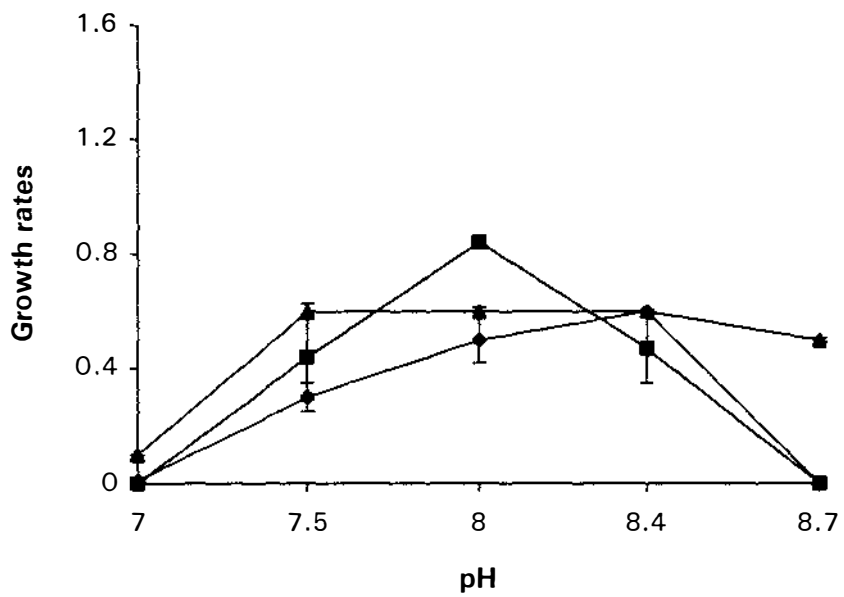


(b)

Fig 3.2 Growth rates (doublings per day) of several *Chrysochromulina* species at different salinities: (a) *C.ericina* (▲), *C.polylepis* (♦), *C.hirta* (●) and *C.quadrikonta* (■); (b) *C.acantha* (▲), *C.apheles* (♦) and *C.camella* (■). Bars indicate standard error ($n=4$), but are obscured by symbols in some instances.



(a)



(b)

Fig. 3.3 Growth rates (doublings per day) of *Chrysochromulina* species at different pH: (a) *C.polylepis* (■), *C.ericina* (▲), *C.hirta* (●) and *C.quadrikonta* (◆); (b) *C.camella* (■), *C.acantha* (▲) and *C.apheles* (◆). Bars indicate standard error ($n=4$), but are obscured by symbols in some instances.

Aeration

Aeration of batch cultures of *C.apheles, C.camella, C.ericina, C.hirta* and *C.quadrikonta* caused some inhibition of growth, but this was not statistically significant.

Light

No significant difference in GR was recorded for *C.camella, C.ericina, C.polylepis* or *C.quadrikonta* between 25 and 175 μmol m^{-2} s^{-1}. *C.acantha* and *C.hirta* grew slower at 25 than at 50 μmol m^{-2} s^{-1} and *C.acantha* also grew slower at 50 than 75 μmol m^{-2} s^{-1}. *C.apheles* grew faster at ≥100 μmol m^{-2} s^{-1} (Table 3.3). Under constant light the growth of *C.quadrikonta* was inhibited.

Table 3.3 Growth rates (doublings per day) of *Chrysochromulina* species cultured at different light intensities. Bars indicate a significance difference in growth rates ($P<0.05$; ANOVA; Tukey Kramer).

	Light intensity (µmol m^{-2} s^{-1})						
Microalga	**25**	**50**	**75**	**100**	**125**	**150**	**175**
C.acantha	0.7 <--->	1.0 <--->	1.2	1.1	1.2	1.3	1.2
C.apheles	0.6	0.9	1.1	1.0 <--->	1.5	1.4	1.2
C.camella	1.0	1.0	1.0	1.0	1.0	1.0	1.0
C.ericina	0.7	0.7	0.9	1.0	1.0	0.9	NT
C.hirta	0.8 <--->	1.2	1.3	1.4	1.2	1.2	NT
C.polylepis	0.9	1.1	1.1	1.1	1.1	1.1	1.0
C.quadrikonta	NT	0.6	0.7	0.7	0.9	0.7	0.8

NT: not tested

Heterotrophy

There was no indication that carbon was obtained heterotrophically by *C.quadrikonta* grown in f2 medium, nor by *C.ericina* and *C.hirta* grown in modified GP medium. No growth occurred in the dark, and no effect on growth was noted at 100 μmol m^{-2} s^{-1} when either glucose, glycerol or acetate were added to the medium. At 40 μmol m^{-2} s^{-1}, neither glucose nor acetate had an effect on the growth of *C.quadrikonta*, but glycerol inhibited growth. Addition of casein hydrolysate caused death of the microalgal cells at all light intensities. No other species were tested.

Hydrolase activity:

No reducing groups were detected in any of the culture supernatants or microalgal pellets of *C.polylepis, C.quadrikonta, C.ericina* or *P.parvum* on assaying for cellulase activity (lower limit of detection: 0.3 μmoles h^{-1} ml^{-1}). No reducing groups were detected in culture supernatants or microalgal pellets of *P.parvum* when assayed for chitinase activity (lower limit of detection: 0.05 μmoles h^{-1} ml^{-1}).

Protease activity was not detected by the hide powder azure assay method in either the supernatants or pellets of axenic cultures of *P.parvum and C.ericina* grown in GP medium with or without bovine serum albumin (BSA) addition, nor with bacterial addition but no BSA. Only in cultures of *C.ericina* to which both BSA and a marine bacterium (*Pseudomonas* sp.) were added was protease activity indicated by the hide powder azure assay. Fluorometer readings of cultures of *C.ericina*/BSA/bacteria were 5000 fluorometer units after 4 days growth, compared with 420 fluorometer units for the axenic control, which represented a ≈10-fold increase in chlorophyll *a* in the test culture.

Phagotrophy

Cells of *C. ericina* could be observed, under UV excitation, taking up, and after

10 to 20 seconds ejecting, fluorescently-labelled beads (0.8 μm). None of the other particles tested were phagocytosed.

Fluorescent beads were observed within cells of *C.acantha*, although such observations were rare.

Phagotrophy was not observed in *C.quadrikonta, C.camella, C.polylepis, C.apheles* nor *C.hirta.* Graphite formed a coating around the algal cells, outlining the spine scales, but was not observed within cells. DAPI stained bacteria were not observed internally.

Acrylic acid

C.quadrikonta had a distinctive "rotten cabbage" smell in culture, typical of dimethylsulphide (DMS), which diminished over time. No acrylic acid was detected in cultures of *C.quadrikonta*. None of the other species were tested.

Growth characteristics of Coccolithophorales

Nitrogen source

There was no significant difference in growth rate (GR) for *E. huxleyi*, *G. oceanica* or *Pleurochrysis* sp. given the different nitrogen sources (Table 3.4).

Table 3.4 Growth rates of coccolithophores cultured with different nitrogen sources. No significant differences in growth rate (doublings d^{-1}) were recorded (ANOVA; Tukey Kramer).

Microalga	*Potassium nitrate*	*Ammonium chloride*	*Urea*
Emiliania huxleyi	1.4	1.9	1.4
Gephyrocapsa oceanica	1.2	1.8	1.4
Pleurochrysis sp.	0.7	1.0	0.8

Temperature

No significant difference in GR of cultures of *E.huxleyi* was recorded between 15 and 25^{0}C. GRs were slower at 10^{0}C ($P<0.01$). *G.oceanica* entered the exponential growth phase and reached maximum cell densities earlier at 25^{0}C than at 20^{0}C, although GRs were not significantly different. However GRs of cultures incubated at 15^{0}C were slower ($P<0.01$). Cells of both species died at 30^{0}C.

Pleurochrysis sp. grew optimally at 20^{0}C ($P<0.05$). Cultures barely survived at 25^{0}C and died at temperatures of 10^{0}C and at 30^{0}C (Figure 3.4).

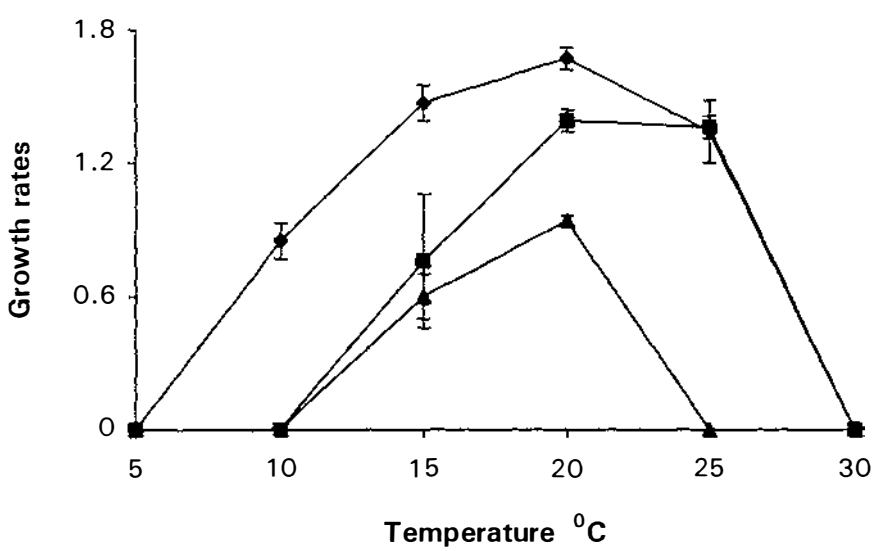


Fig. 3.4 Growth rates (doublings d^{-1}) of *Emiliania huxleyi* (♦), *Gephyrocapsa oceanica* (■) and *Pleurochrysis* sp. (▲) at different temperatures. Bars indicate standard error ($n=4$).

Salinity

The coccolithophores were grown at salinities of 10, 17, 23, 29, 35 and 42$^0/_{00}$. The fastest GRs for *E.huxleyi* were in media with salinities of 29$^0/_{00}$ ($P<0.01$).

GR was slower at $35^{0}/_{00}$ ($P<0.01$) and cells survived, but grew extremely slowly at $42^{0}/_{00}$.

Optimal GRs for *G.oceanica* were between 17 and $29^{0}/_{00}$; GRs were significantly slower at $35^{0}/_{00}$ ($P<0.05$).

Pleurochrysis sp. did not survive long enough in culture for a salinity optimum to be obtained.

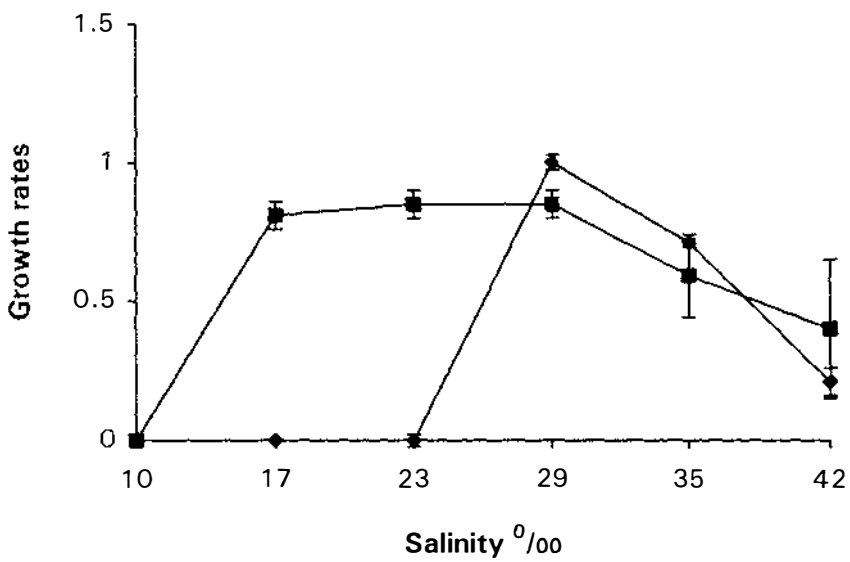


Fig. 3.5 Growth rates (doublings d^{-1}) of *Emiliania huxleyi* (♦) and *Gephyrocapsa oceanica* (■) at different salinities. Bars indicate standard error ($n=4$).

pH

E.huxleyi had a faster GR at pH7.5 than at 7.0 ($P<0.01$), but no significant differences in GRs were noted between pH7.5 and 8.9. Faster GRs were obtained for *G.oceanica* at pH8.4 than at 7.9 ($P<0.01$), but there was no significant difference in growth between cultures grown at pH8.4 and 8.9 (Figure 3.6).

Pleurochrysis sp. exhibited a slow growth rate under the experimental culture conditions. There was no significant difference in GR between pH7.9 and 8.6. Cultures did not grow at pH7.5 or 8.9.

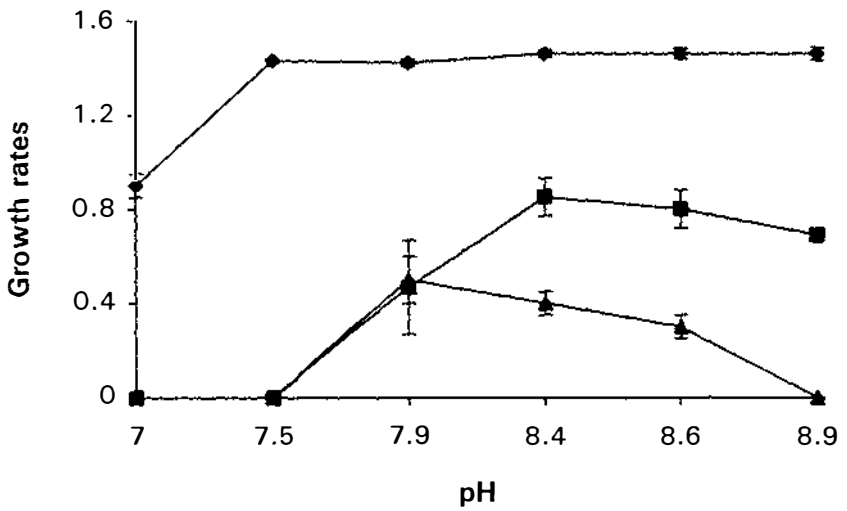


Fig. 3.6 Growth rates (doublings d^{-1}) of *Emiliania huxleyi* (♦), *Gephyrocapsa oceanica* (■) and *Pleurochrysis* sp. (▲) at different pH. Bars indicate standard error ($n=4$), but are obscured by symbols in some instances.

Light

There was no variation in GR for either *E.huxleyi* or *G.oceanica* at 50 - 150 μmol m^{-2} s^{-1} light intensity under otherwise optimal conditions. GR was slower at 20 μmol m^{-2} s^{-1} ($P<0.01$).

Pleurochrysis sp. did not survive long enough in culture for light intensity optima to be obtained.

The fastest GR recorded in this study for *E.huxleyi* was 1.89 doublings per day and for *G.oceanica* 1.39 doublings per day.

Discussion

Prymnesiales

Six morphologically dissimilar *Chrysochromulina* species, *C.acantha, C.camella, C.ericina, C.hirta, C.quadrikonta* and. *C.apheles*, were isolated from New Zealand coastal waters.

C.quadrikonta, a previously unrecorded quadriflagellate species, produced a "rotten cabbage" smell in early cultures, a trait which has also been associated with the fresh-water *C.breviturrita* (Wehr *et al.*, 1985). The related prymnesiophyte, *Phaeocystis pouchetii*, is known to produce dimethylsulfide (DMS) and acrylic acid from dimethylsulfoniopropionate, an osmoregulator of algal cells (Gibson *et al.*, 1990). The odour might have been associated with DMS production in *C.quadrikonta,* but acrylic acid production was not detected in the *in vitro* cultures analysed.

The local isolates fell into two distinct groups as regards temperature preferences. *C.quadrikonta, C.camella* and *C.apheles* favoured warmer temperatures and failed to grow at 10^0C. These species grew optimally at 25^0C, but died at 30^0C. *C.quadrikonta* was recorded at Waimangu Point, Firth of Thames in May 1994 (MAF Regulatory Authority operational research phytoplankton programme) where it survived winter sea surface temperatures of as low as 12^0C and a summer high of 22^0C in 1993. The *in vitro* temperature range within which *C.quadrikonta* survived and grew closely matched the *in vivo* temperature range recorded at Waimangu Point. The greatest numbers there occurred between May 4 - 11, when sea surface temperatures ranged from 17 - 18^0C.

C.ericina and *C.hirta* grew over the same temperature range as the northern hemisphere isolate, *C.polylepis.* They survived at 5^0C, growing slowly at 10^0C and optimally from 15 - 20^0C. Both species died at 25^0C. During the 1988 Scandinavian bloom *C.polylepis* appeared to predominate at temperatures between 6.7 and 8.8^0C (Kaas *et al.,* 1991), although the *in vitro* optimum in this study was 15 - 20^0C.

Despite the different maxima and minima for the two groups, all would have been able to survive, if not grow, throughout the year in the north-eastern waters of the North Island and the northern waters of the South Island (Figure 1.1). The range of average monthly temperatures in the Marlborough Sounds, from which *C.ericina, C.camella* and *C.hirta* were isolated, was from 18^{0}C in summer to 10^{0}C in winter for 1987 - 1993 (Regal Salmon Limited, unpublished data). It appears that at least some of New Zealand's coastal prymnesiophytes are existing at the lower extreme of their temperature range and this might explain why they are usually recorded in extremely low numbers. Most species in this study were isolated between March and May, before temperatures had dropped below 16^{0}C (refer Chapter one, Results). The consistently lower temperature range recorded in Big Glory Bay, Stewart Island, of 10 - 16^{0}C (MacKenzie, 1991), where *C.pringsheimii* has been recorded in high numbers (refer Chapter four, Results), suggest that the warmer temperature tolerant group of *Chrysochromulina* isolates would be excluded from the far south.

C.polylepis occurred throughout the water column during the 1988 Scandinavian bloom, but chlorophyll *a* readings were highest where salinities were ≈28^0/$_{00}$ (Skjoldal and Dundas, 1989). The New Zealand toxic-bloom former, *Prymnesium calathiferum* (Chang, 1985), occurred in the brackish upper layers of coastal waters at salinities of from 3 to 25^0/$_{00}$. This is consistent with the present study in which all the New Zealand isolates tested exhibited fastest growth rates in brackish water.

All the New Zealand isolates grew at a relatively wide range of pH (from pH7.5 - 8.6), with only *C.camella* and *C.apheles* not growing at pH8.6. *C.polylepis* did not grow at the low of pH7.5, but did grow at pH8.6.

A selenium requirement has been reported for a northern hemisphere isolate of *C.ericina* (Harrison *et al.*, 1988) and for the Scandinavian isolate of *C.polylepis* (Dahl *et al.*, 1989), but not for North-Pacific isolates of the latter species. A selenium requirement was exhibited by *C.quadrikonta* in this study. *C.strobilus*, which has been closely aligned with *C.camella,* has also been reported to have a selenium requirement (Pintner and Provasoli, 1968), but the New Zealand isolate of *C.camella* did not. None of the other New Zealand isolates were limited by

selenium. As the concentrations of selenium added to the GP medium (0.04 μM) at no stage caused growth inhibition of any of the species, this element is now routinely included in all prymnesiophyte media.

Acid rain, and consequent release of cobalt from soils, was hypothesised as being responsible for the toxic *C.polylepis* bloom in Scandinavia in 1988 (Sangfors, 1988). In the case of *C.quadrikonta* small additions of cobalt to the standard GP medium resulted in cessation of growth, and greater additions in death of cells. These results do not support the hypothesis, at least for this species.

Phagotrophic uptake of particles occurred in *C.ericina* and rarely in *C.acantha*. *C.ericina* develops non-motile amoeboid forms in culture (refer Chapter 1; Results) and it is feasible that if these forms settle out of the water column on to sediments that their phagotrophic capability would allow survival for indefinite periods. No phagotophic uptake of beads was noted for *C.hirta,* although phagotrophy has been fully described for a Japanese strain of this species (Kawachi *et al.,* 1991). Phagotrophy has also been described for *C.polylepis* (Nygaard and Tobiesen, 1993).

Heterotrophy at low light intensities (although not in the dark) has been reported for some prymnesiophytes, and survival in the dark has been enhanced with glycerol addition (Pintner and Provasoli, 1968), but uptake of soluble carbon did not occur in the species tested and growth of *C.quadrikonta* was inhibited by glycerol in dim light. However, the addition of glycerol to CHRY medium proved critical in bringing live cultures of *C.polylepis* to New Zealand from the USA.

The hypothesis that hydrolases, such as cellulase, chitinase and protease, are produced by prymnesiophytes and assist in the degradation of complex substrates prior to heterotrophic uptake by the microalgae was not supported by the results of this study. *Prymnesium parvum* commonly adheres to dead organisms and organic detritus (personal observation), but neither it nor the phagotrophic *C.ericina* secreted proteases to degrade the available protein. However, the rapid growth rate of *C.ericina* cells in the presence of bacteria which did secrete proteases suggests that either the algae were limited for amino acids or that the bacteria were releasing other vital growth factors. Such

interactions between marine prokaryotes and microalgae warrant further investigation, not only to ascertain the role of bacteria in microalgal bloom formation, but whether bacteria have a role in bloom collapse.

The causes of the toxic Scandinavian *Chrysochromulina* blooms in recent years are still speculative, although the exclusion of predators due to prymnesin toxin production by the microalga was one likely cause of the 1988 *Chrysochromulina polylepis* bloom (Dahl *et al.*, 1989). The temperatures that favoured the onset of the *Chrysochromulina polylepis* bloom would inhibit growth of the New Zealand isolates. However the species investigated (excepting *C.camella,* but including *C.polylepis*) appear to flourish in slightly brackish coastal waters and to tolerate a wide range of light intensities. This might give some competitive advantage over other phytoplankton species, by enabling the prymnesiophytes to survive periods of low light intensity and varying salinity throughout the water column.

Coccolithophorales

The growth studies of the New Zealand isolates of *E.huxleyi*, *G.oceanica* and *Pleurochrysis* sp. highlighted the broader tolerance ranges of temperature and pH of *Emiliania. E.huxleyi* and *G.oceanica* grew, albeit slowly, in dim light (20 μmol m^{-2} s^{-1}) and grew equally well from 50 - 150 μmol m^{-2} s^{-1}. This was not surprising, as *E.huxleyi's* tolerance of low light intensity *in vitro* has been observed previously (Jeffrey and Allen, 1964) and *G.oceanica* has been recorded growing at the limits of the photic zone (Honjo, 1977). The light-scattering ability of the coccolithophores at high light intensities has also been noted (Berge, 1962) and might well be a factor in the wide tolerance range.

In this study the optimum salinity for *E.huxleyi* was $29^0/_{00}$ and for *G.oceanica* 17 - $29^0/_{00}$, with both species growing at salinities of as high as $42^0/_{00}$. The wide range of salinities and temperatures under which *G.oceanica* can survive and grow is not surprising for such a ubiquitous species. In contrast, *E.huxleyi* is surprisingly stenohaline, with no growth recorded at $23^0/_{00}$. Coccoliths might allow the maintenance of a favourable ecosystem between the cell membrane

and the outer calcareous scales (Manton, 1986), which could provide a buffering effect against changes in the chemical composition of the surrounding water. It has been suggested that iron availability might be a factor limiting phytoplankton growth (Gribbin, 1991) but this was not supported by the growth studies of the New Zealand isolates.

The fastest GR recorded for *E.huxleyi*, of 1.89 doublings per day, was comparable with the GR of 1.85, which was recorded for a northern hemisphere strain of *E.huxleyi* (Paasche, 1968). This exceeds the maximum recorded in this study, of 1.39, for *G.oceanica*.

The growth characteristics of the prymnesiophytes warrant further studies, particularly in the light of developing models for bloom forecasting. The prymnesiophytes are a ubiquitous group, with *E.huxleyi* being the most abundant of all microalgae. The toxic species are, in some cases, a recent phenomenon and an understanding of their growth requirements and characteristics prior to the appearance of toxicity, would in hindsight have been invaluable. As the culture requirements of more species become established, further studies of this group will be able to be undertaken.

Chapter four: PRYMNESIOPHYTE BLOOMS IN NEW ZEALAND

Introduction

Toxic and noxious prymnesiophyte blooms

Prymnesiophyte blooms are now a major concern world-wide. Ichthyotoxic species have caused economic losses through the deaths of fin-fish due to the production of a proteinacious haemolysin, prymnesin.

The genus *Prymnesium* Massart ex Conrad has a history of ichthyotoxicity. *Prymnesium parvum* was responsible for fish kills in Palestinian fish ponds in the 1930s (Otterstrøm and Steeman-Nielsen, 1939) and caused the deaths of farmed salmon and rainbow trout in the Norwegian fiords in the northern summers of 1989 (Kaartvedt *et al.*, 1991) and 1990 (Aure and Rey, 1992). Blooms of this species appear to be favoured by low salinity waters and increased toxicity has been correlated with high nitrogen:phosphate ratios (Shilo, 1967).

Blooms of the genus *Chrysochromulina* (Lackey), in particular *C.polylepis* and *C.leadbeateri,* have occurred almost yearly since 1988, in some instances killing millions of dollars worth of caged fish in the north of Norway (Tangen, 1989; Tangen, 1991; Aune *et al.*, 1992). In April - May 1992 fish kills due to blooms comprising a suite of *Chrysochromulina* species were reported in Danish waters. The causative species of that incident have yet to be identified (Knipschilt, 1992). Many species of *Chrysochromulina* had been recorded in the Scandinavian region prior to these blooms, but none had been described as ichthyotoxic (Leadbeater, 1972a,b).

Until recently no *Chrysochromulina* species isolated from New Zealand waters had been shown to be ichthyotoxic. A bloom of *C.pringsheimii*

occurred in Big Glory Bay, Stewart Island, in November 1990, but did not harm the caged salmon in the vicinity (L.MacKenzie, unpublished data). However *Prymnesium calathiferum* was implicated in wild fish kills in the north-east of New Zealand in the summer of 1983 (Chang, 1985; Chang and Ryan, 1985). Fish and shellfish died at Bream Bay (Figure 4.1) during a bloom dominated by the non-toxic diatom *Cerataulina pelagica*, but with the newly discovered *P. calathiferum* being a sub-dominant species. The fish deaths were attributed to anoxia (Taylor *et al.,* 1985), but as cell-free extracts of cultures of *P.calathiferum* were toxic to the fish *Gambusia* sp., an ichthyotoxic cause of death was possible (Chang, 1985).

Phaeocystis pouchetii (Prymnesiales; Phaeocystaceae) is not known as an ichthyotoxin producer, but a bloom of this species caused a severe reduction in fish catches in Tasman Bay during the spring of 1981 (Figure 4.1), mainly due to clogging of nets due to the copious amounts of mucilage produced by that species. Deaths of biota in the bay were attributed to asphyxiation caused by the mucilage rather than by a toxin (L.MacKenzie, unpublished data). However acrylic acid and dimethylsulphide, produced by *P.pouchetii* (Sieburth, 1960), could conceivably have an effect on the health of captive fish.

The presence of ichthyotoxic prymnesiophytes in coastal waters and the possibility of development of toxicity in non-toxic species are now recognised as potential threats to the aquaculture industry in New Zealand. Monitoring of the water in the vicinity of fish-farms is currently carried out weekly to gain prior warning of potential blooms.

Coccolithophore blooms

Non-toxic coccolithophore blooms (Coccolithophorales) typically colour the water a milky-green and cells can number up to 1.2 $x10^{8}$ l^{-1} (Berge, 1962). *Emiliania huxleyi* is the most abundant of the coccolithophores, at

times constituting up to 50% of the total microflora of tropical seas and up to 100% in subarctic and subantarctic waters (Balch *et al.*, 1991).

The fossil record indicates that *E.huxleyi* was a rare species in the New Zealand region until 85 000 years ago, when its increase coincided with a decrease in the abundance of *Gephyrocapsa* coccoliths (Thierstein *et al.,* 1977; Rhodes *et al.*, 1994a). Both species were first recorded by light microscopy in New Zealand waters in 1961 (Norris, 1961) and later confirmed by electron microscopy (Moestrup, 1979; Burns, 1977). Coccolithophore populations of 0.1 - 2.3 $x10^{5}$ cells l^{-1} were recorded in oceanic surface waters to the east of New Zealand during August 1974 (Nishida, 1979), with *E.huxleyi* dominating the samples and *G.oceanica* present in low numbers. *E.huxleyi* was also abundant during the "Tasman Bay slime" of 1981 in New Zealand, in which *Phaeocystis pouchetii* was responsible for copious mucilage production (Chang, 1983).

In the last few years there has been a greater public awareness of phytoplankton blooms in New Zealand, due mainly to the media publicity given to farmed fish deaths and shellfish toxins. This has led to an increased reporting of sea water discolouration, which in turn has led to more frequent sampling and identification of bloom species. Blooms of *E.huxleyi* have been recorded in the Marlborough Sounds, Palliser Bay and Big Glory Bay since 1990. Fish cages were towed from Big Glory Bay in November 1992 during such a bloom due to increased mortalities of caged salmon, although no microalgal toxicity was demonstrated (Rhodes *et al.*, 1994a).

G.oceanica was a co-dominant species in a spring-1992 bloom which extended more than 200 km along the north-east coast of the North Island, from north of Ti Point down to the Hauraki Gulf (Haywood, 1993; Rhodes *et al.*, 1993; Rhodes and Haywood, 1993). This bloom immediately preceded a major toxic event in summer 1993 during which the whole of New Zealand's multi-million dollar shellfish industry was closed. The event was attributed to toxic dinoflagellates (MacKenzie

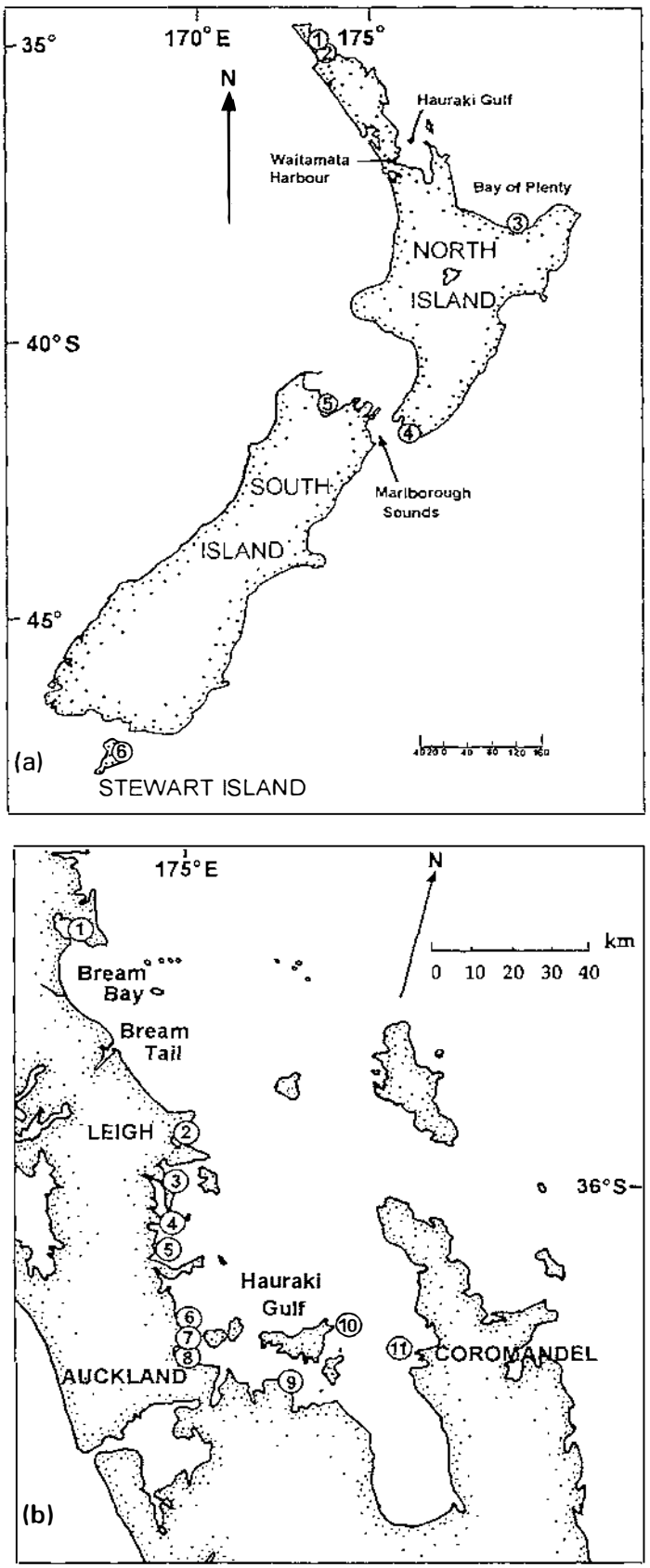
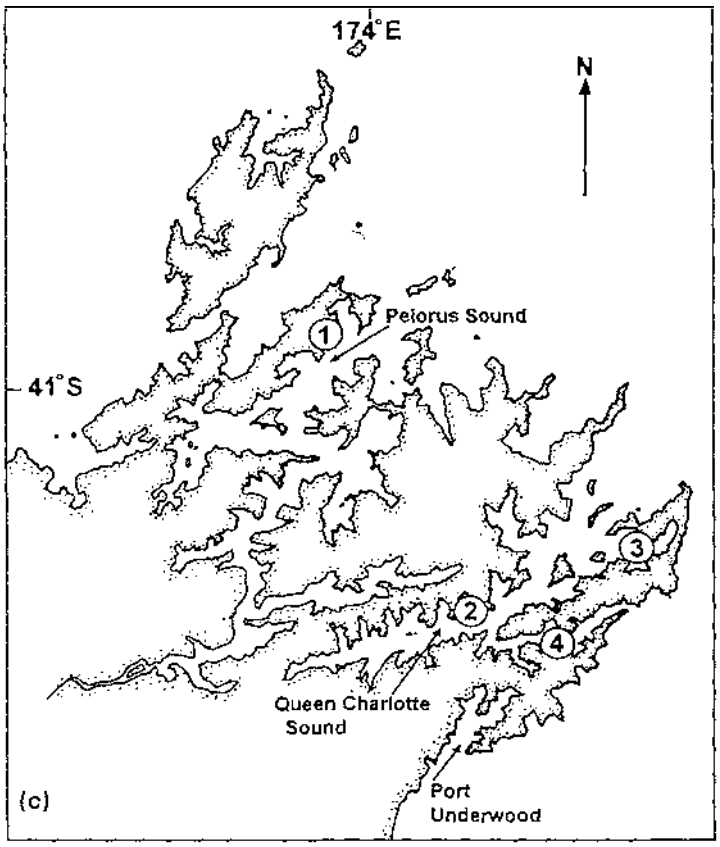
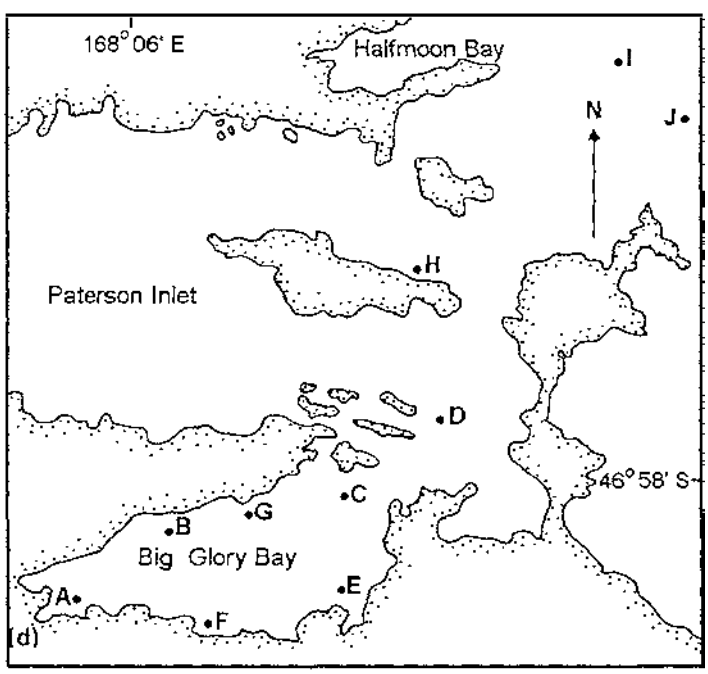


Fig. 4.1 Maps showing sites of sampling and bloom events referred to in this study. (a) New Zealand: 1, Houhora Heads in Rangaunu Bay; 2, Whangaroa Heads; 3, Ohiwa Harbour; 4, Palliser Bay; 5, Tasman Bay; 6, Big Glory Bay. **(b) the Hauraki Gulf:** 1, Marsden Point; 2, Ti Point; 3, Snells Beach; 4, Mahurangi Harbour; 5, Orewa Beach; 6, Browns Bay; 7, Takapuna Beach; 8, Duders Beach; 9, Waimangu Point; 10, Waiheke Island; 11, Coromandel. Continued over page.





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(c) Marlborough Sounds: 1, Waihinau Bay; 2, Ruakaka Bay; 3, East Bay; 4, Te Pangu Bay.

(d) Big Glory Bay, Stewart Island. (A - J indicate sampling sites).

and Rhodes, 1993; Smith *et al.*, 1993; Bradford-Grieve *et al.*, 1993).

This study presents the details, from inception to demise, of the prymnesiophyte blooms of 1992 - 94 in New Zealand waters, in particular the blooms of the coccolithophores *G.oceanica* and *E·huxleyi.* The possible triggers for bloom development are discussed and comparisons drawn between the 1992 *E.huxleyi* bloom and the 1990 *C.pringsheimii* (L.MacKenzie, unpublished data) and 1989 raphidophyte (*Heterosigma akashiwo*) blooms (MacKenzie, 1991; Chang *et al.*, 1990) in Big Glory Bay, Stewart Island.

Methods

Phytoplankton monitoring of microalgal species

Sea water samples were investigated during phytoplankton monitoring of Big Glory Bay in Stewart Island and Waihinau, Ruakaka, East and Te Pangu Bays in the Marlborough Sounds on behalf of aquaculture clients. Integrated samples (one sampling, 0 - 10 m) were collected weekly at the various sites with a hose sampler and split into one Lugols' iodine treated and one untreated sample (50 ml each) couriered to Cawthron for identification and counting of microalgae. Samples were also sent from Leigh by W.Ballantine (Leigh Marine Laboratory), the Hauraki Gulf by B.Hickey (Auckland Regional Council) and Big Glory Bay by S.Marwick (Big Glory Seafoods). These were collected as discrete samples with a water bottle sampler (in the case of Auckland Regional Council sampling was carried out from a helicopter). Samples were also received through the MAF Regulatory Authority operational research phytoplankton programme (refer Chapter one, Methods). Further samples were sent *ad hoc* by members of the public. Fixed (Lugol's iodine) and unfixed samples were collected as Lugol's tended to destroy the calcareous scales of coccolithophores making species identification difficult.

Biomass estimation

Samples (10 ml) were settled in Utermöhl chambers and microalgae were counted and measured under an inverted microscope (x100 magnification for species of >15 μm; x400 - 600 for species <15 μm). Cell volumes were estimated using standard geometric formulae (coccolithophores were treated as spheres and raphidophytes as ellipsoids of revolution). Sea water samples collected at Leigh Marine Reserve between August and October 1992 were filtered (0.4 μm nitrate/acetate filters) and dried (Haywood, 1993). Filters were treated with microscopic immersion oil, which made them translucent, and the ratios of *G.oceanica* to *E.huxleyi* estimated microscopically.

Identification, culture and toxicity testing of microalgal species

Flagellates, dinoflagellates and diatoms were identified by light microscopy and coccolithophores by scanning electron microscopy (refer Chapter one, Methods). Microalgal isolates from sea water samples were cultured in sea water based nutrient media (refer Chapter three, Methods) and tested for toxicity using *Artemia salina* bioassays (refer Chapter five, Methods).

Climate data and water quality analyses

Marine and atmospheric climate records (Evans 1992) and chlorophyll measurements (Haywood 1993; Rhodes *et al.* 1993) for Leigh Marine Reserve were obtained from Leigh Marine Laboratory. Big Glory Bay data were provided by the Chemistry Department, University of Otago (Rhodes *et al.*, 1994a) and S.Marwick (Big Glory Bay Seafoods Limited, unpublished data). Salinity (Orion conductivity/salinity meter, Watson Victor) and temperature profiles were recorded whenever possible for discrete samples collected by water bottle from the water column. Sea

water temperatures were also obtained from Regal Salmon Limited (unpublished data).

Results

Northland Bloom

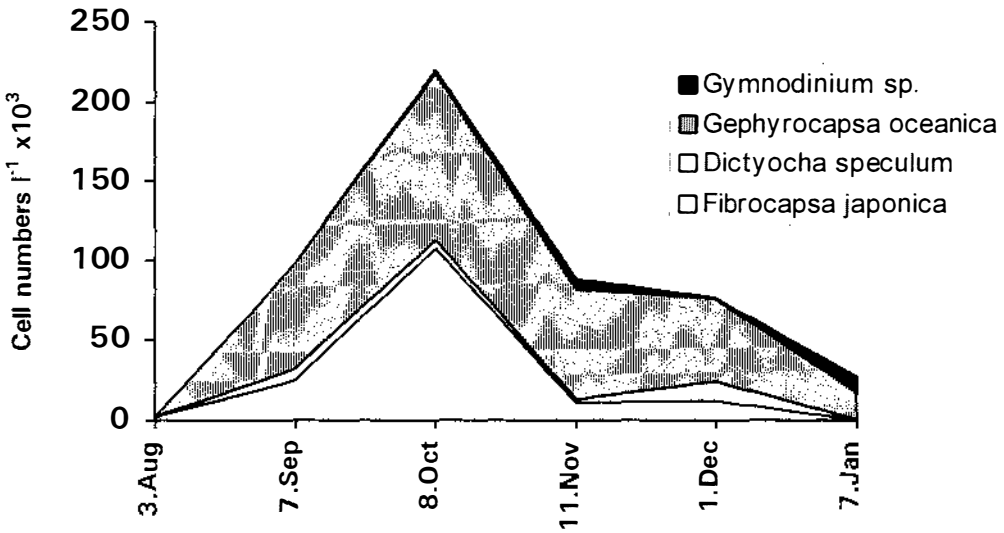
August 1992 - January 1993:

A phytoplankton bloom extended for 200 km down the north-eastern coastline of New Zealand, from Bream Tail in the north to Coromandel in the south, from late August through to December, 1992 (Rhodes *et al.*, 1993). At Leigh Marine Reserve *G.oceanica* (maximum 1.1 $x10^5$ cells l^{-1}) was co-dominant with the raphidophyte *Fibrocapsa japonica,* (maximum 1.1 $x10^5$ cells l^{-1}; Figures 4.2a and 4.3). The cell volumes of *F.japonica* and of *G.oceanica* were estimated as 1.5 $x10^3.\mu m^{-3}$ and 0.5 $x10^3.\mu m^{-3}$ respectively. The total estimated cell volume for *F.japonica* (1.7 $x10^8$ $.\mu m^{-3}$) was more than three times that of the coccolithophore (5.5 $x10^7$ $.\mu m^{-3}$; Figure 4.2b).

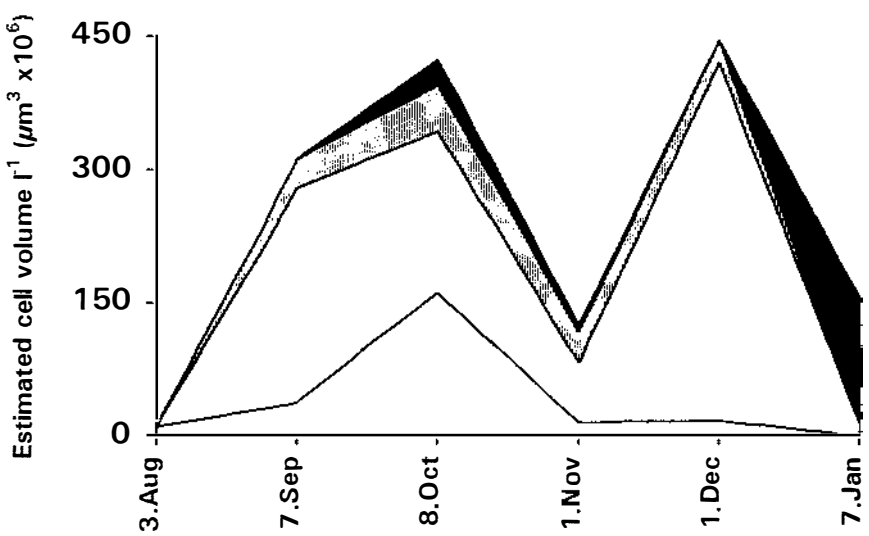
The silicoflagellate *Dictyocha speculum* and the dinoflagellates *Gymnodinium* spp. (the latter later identified as *G.* cf. *breve* and *G.mikimotoi*; Smith *et al.,* 1993) were also identified as important components of the bloom at Leigh (Figures 4.2 and 4.3). Other dinoflagellates and diatoms did occur, but in low numbers (Table 4.1).

The ratio of cells of *E.huxleyi* to *G.oceanica* was 1:20 in the August samples. In September, at the onset of the bloom, the ratio was approximately 1:40. By mid-October, at the height of the bloom, this ratio had dropped back to ≈1:14.

Further south, at Coromandel, the raphidophyte *Heterosigma akashiwo* replaced *F.japonica* as the dominant raphidophyte (Table 4.2).



(a)



(b)

Fig. 4.2 Cell numbers (a) of individual bloom species at Leigh, August 1992 to January 1993 and estimated cell volumes (b) of those species, obtained with standard geometric formulae.

Table 4.1 Phytoplankton species present, and their abundance, at the height of the raphidophyte dominated bloom at Leigh, 8 October 1992. Abundance scale (cells l^{-1}): 1, ≤500; 2, 501-5000; 3, 5001-10 000; 4, >100 000. (N.B. No cell counts between 10 000 and 100 000.)

Dinophyceae	Abundance	**Bacillariophyceae**	Abundance
Ceratium furca	1	*Leptocylindricus danicus*	2
Dinophysis acuminata	1	*Navicula* sp.	2
Gymnodinium sp (50 µm)	1	*Nitzschia* sp.	1
Gymnodinium sp (10 µm)	2	*Pleurosigma* sp.	1
Gyrodinium/Gymnodinium sp.	2		
Gyrodinium glaucum	1	**Dictyophyceae**	
Oxytoxum sp.	2		
Prorocentrum triestinum	2	*Dictyocha speculum*	3
Protoperidinium sp.	1		
Scrippsiella sp.	2	**Cryptophyceae**	
Prymnesiophyceae		*Cryptomonas* sp.	3
Emiliania huxleyi	1	**Raphidophyceae**	
Gephyrocapsa oceanica	4	*Fibrocapsa japonica*	4

The spring/summer bloom was succeeded in January by a dinoflagellate bloom, which included the toxic species *Gymnodinium* cf. *breve* and *G.mikimotoi* (=*Gyrodinium aureolum*) (Smith *et al.*, 1993), although counts of *G.oceanica* at Leigh, Takapuna Beach, Duders Beach and Waiheke Island, persisted at a constant 3.0 - 4.0 x10^{4} cells l^{-1}.

During the raphidophyte-coccolithophore bloom, only sea water samples from Coromandel, and then only the isolate *Heterosigma akashiwo* from that sample, gave a positive response to the *Artemia salina* bioassay. *G.oceanica* was not toxic (Table 4.3).

Table 4.2 Comparison of cell numbers of dominant species at sites throughout the north-eastern coastal region of New Zealand, 13 November 1992. A, Leigh Marine Reserve; B, Ti Point; C, Snells Beach; D, Mahurangi Harbour; E, Orewa Beach; F, Browns Bay; G, Waitemata Harbour; H, Coromandel (refer Fig. 4.1). Cell numbers are thousands per litre.

Site	A	B	C	D	E	F	G	H
Gephyrocapsa oceanica	104.9	65.4	45.2	35.8	82.7	10.0	<0.1	<0.1
Fibrocapsa japonica	10.6	19.0	5.8	1.4	<0.1	2.8	<0.1	1.2
Heterosigma akashiwo	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	10.2
Dictyocha speculum	5.4	2.0	1.6	1.2	0.3	0.4	<0.1	1.4
Gymnodinium sp.	6.4	14.6	19.8	14.0	<0.1	0.2	<0.1	0.8

Table 4.3 Species isolated, cultured and tested for ichthyotoxicity by the *Artemia salina* bioassay during the raphidophyte-coccolithophore bloom of 1992.

Microalga	***Toxicity***
Dictyocha speculum	-
Emiliania huxleyi	-
Fibrocapsa japonica	-
Gephyrocapsa oceanica	-
Gymnodinium sanguineum	-
Gymnodinium sp.	-
Heterosigma akashiwo	+

November 1993:

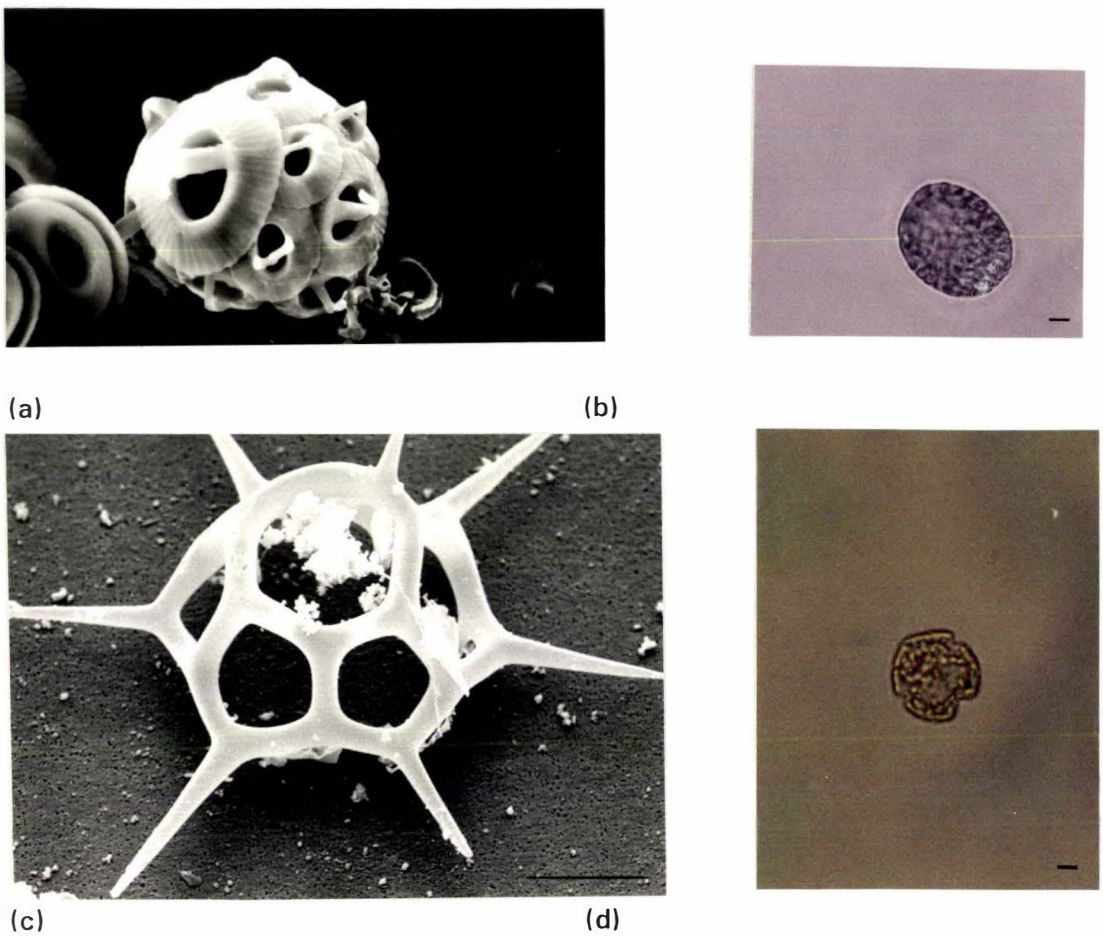
In November, at Rangaunu Bay and Whangaroa Heads in the far north, *Pseudonitzschia australis*, identified by scanning electron microscopy was recorded at a maximum 6.0 $x10^5$ cells l^{-1}. Both *P.australis* and vast numbers of large cells of *G.oceanica* (12 μm diameter) were observed in the digestive glands of scallops sent to the Cawthron for analysis at that time (Figure 4.4). The diatom and the coccolithophore were isolated from sea water samples, cultured in sea water based nutrient medium (refer Chapter two, Methods) and analysed by HPLC for domoic acid (analysis carried out by D.White) (Rhodes and White, 1993). No domoic acid was detected in the cultured microalgae at that time, but more recent isolates of *P.australis* have proved toxic (unpublished data).

May 1994:

A prymnesiophyte bloom was recorded at Waimangu Point in the Firth of Thames, 10 May 1994. Cell numbers reached 60.5 $x10^3$ l^{-1} at that time, but had disappeared by the following week. The species was identified by both light microscopy and transmission electron microscopy as *Chrysochromulina quadrikonta.*

Big Glory Bay bloom

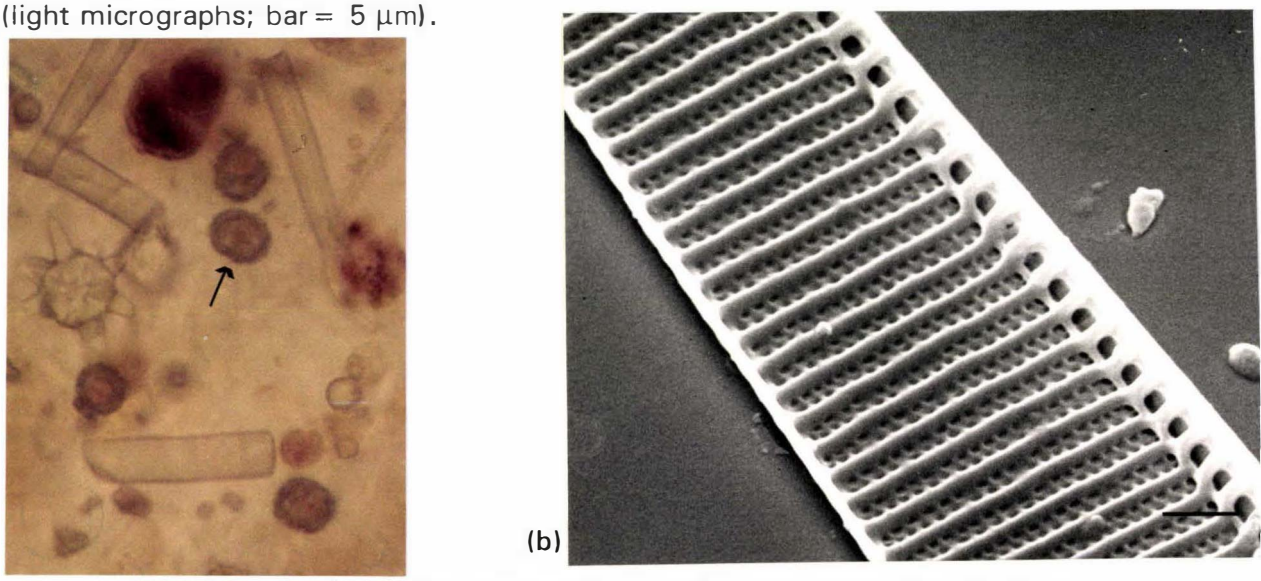
A bloom coloured the water a milky green at Big Glory Bay (Figure 4.1) throughout November, 1992. Coccolithophores were already abundant in water samples analysed on 22 October (L.MacKenzie, personal communication), but early in November *E.huxleyi*, identified by scanning electron microscopy (Tappan, 1980), developed into an almost unialgal bloom. By 16 November a small gymnodinoid (10 μm) had replaced *E.huxleyi* as the bloom dominant (6.0 $x10^5$ cells l^{-1}). On 22 November the coccolithophores were concentrated in the north-west of the bay, with numbers (close to 9.0 $x10^6$ cells l^{-1}) being four times those recorded in the south-east of the bay. Some mortalities of salmon occurred at that time and most of the sea cages were towed to refuge sites outside the



(a) (b)

(c) (d)

Fig. 4.3. Micrographs of the dominant species involved in the Northland bloom, spring-summer 1992: (a) *Gephyrocapsa oceanica* (scanning electron micrograph; bar = 10 μm); (b) *Fibrocapsa japonica*; (c) *Dictyocha speculum*; (d) *Gymnodinium* sp. (light micrographs; bar = 5 μm).



(a) (b)

Fig. 4.4. Light micrograph of *Gephyrocapsa oceanica* and scanning electron micrograph of *Pseudonitzschia australis*: (a) *G.oceanica* in the digestive glands of scallops containing domoic acid from Rangaunu Bay, Northland; (b) *P.australis* from sea water samples. Bar = 1 μm.

bay in Paterson Inlet as a precautionary measure. Coccolithophore numbers had declined by 26 November, but were still distributed unevenly throughout the bay (Figure 4.5; 4.6). The bloom species continued to diversify to include *Leptocylindricus danicus* (4.5 $x10^5$ cells l^{-1}; Diatomophyceae) and lesser numbers of *Diplopsalis* sp. (1.4 $x10^5$ cells l^{-1}; Dinophyceae). Silicoflagellates and raphidophytes were also present. Coccolithophores were always concentrated well within the bay, with extremely low numbers at sites just within the entrance of the bay and beyond the entrance in Paterson Inlet.

On 27 November strong westerly winds led to the dissipation and collapse of the bloom and by 28 November cell numbers were down to 88 $x10^3$ cells l^{-1}. *Chaetoceros* spp. (2.0 $x10^5$ cells l^{-1}) succeeded the coccolithophore bloom throughout December.

A.salina bioassays for toxicity of water samples from the bay, and of cultured isolates of *Emiliania huxleyi*, were negative.

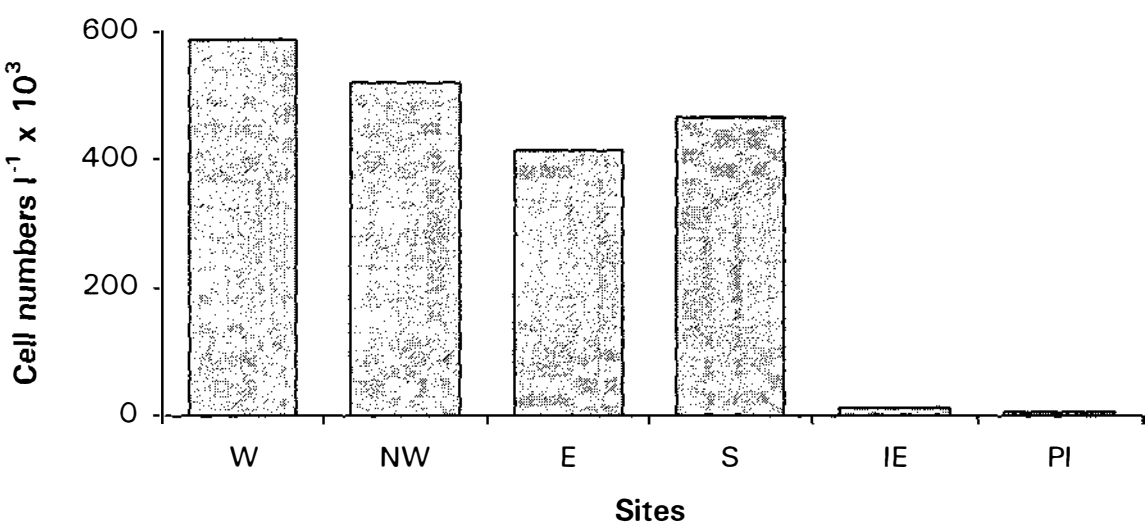


Fig. 4.5. Distribution of *Emiliania huxleyi* within Big Glory Bay and Paterson Inlet, 26 November 1992: W, west of bay; NW, north-west; E, east; S, south-east; IE, inside entrance; PI: outside bay in Paterson Inlet (refer Fig. 4.1).

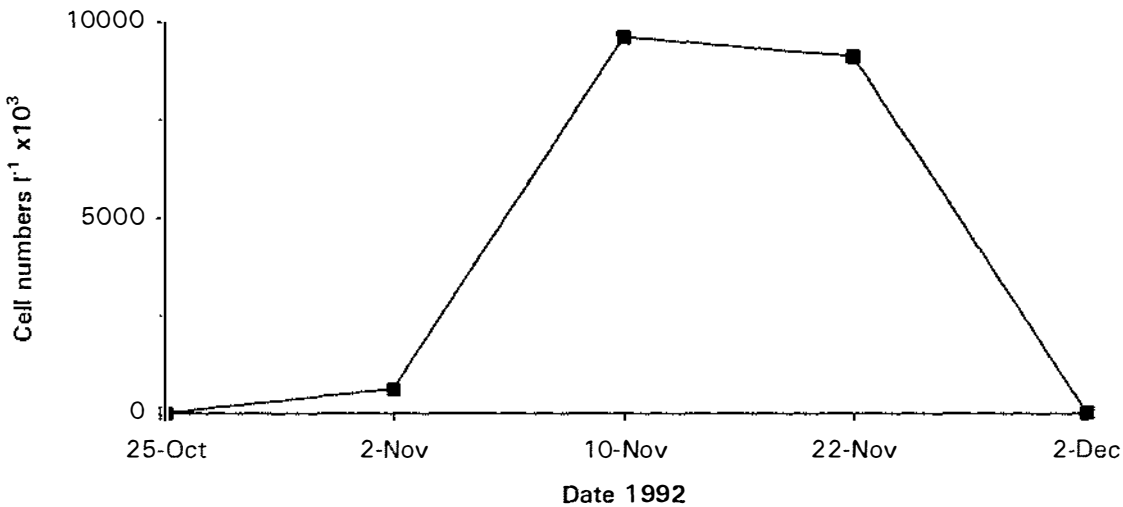


Fig. 4.6. Maximum cell numbers of *Emiliania huxleyi* from onset to demise of a bloom at Big Glory Bay (NW), Stewart Island, October-December 1992.

Tasman Bay/Marlborough Sounds

G.oceanica was present in the Marlborough Sounds in high numbers in March 1992 (1.1 $x10^5$ cells l^{-1}), when temperatures averaged 16°C, but was replaced by *E.huxleyi,* both in the Sounds and Tasman Bay during December 1992 through to December 1993. From December 1992 to February 1993 *E.huxleyi* was a dominant species in the water column not only in Tasman Bay (Figure 4.1) (4.0 $x10^5$ cells l^{-1}), but in the inner Pelorus Sound (2.0 $x10^5$ cells l^{-1}), Port Underwood (2.0 $x10^5$ cells l^{-1}) (MacKenzie *et al.*, 1993) and Queen Charlotte Sound (6.0 $x10^5$ cells l^{-1}). Diatoms dominated in the outer sounds. On two occasions blooms were pin-pointed by aerial survey prior to sampling (Rhodes *et al.*, 1993; MacKenzie, 1993).

The coccolithophores, at times indicated by a milky-green colouration of the water, were often accompanied by mixed populations of dinoflagellates at those sites.

Discussion

A phytoplankton bloom, which occurred during spring/summer 1992, stretched for 200 km down the north-east coastline of the North Island of New Zealand and comprised a suite of species. The co-dominants in the bloom were the coccolithophore *Gephyrocapsa oceanica,* the potentially ichthyotoxic flagellates *Fibrocapsa japonica* and *Dictyocha speculum* and the toxic dinoflagellates, *Gymnodinium* cf. *breve* and *G.mikimotoi* (Rhodes and Haywood, 1993). *Emiliania huxleyi* was also present at a tenth the number of *G.oceanica* cells in this northern New Zealand bloom.

Unusually cold sea surface temperatures (SST) were related to the El-Niño phase of the Southern Oscillation and the consequent climatic patterns occurring at that time. The SSTs were the lowest on record, being $<13^{0}$C at the onset of the bloom and reaching 18^{0}C, a seasonal low, in December.

The germination of cysts of *F.japonica* has been linked to periods of low temperature, with high rates of germination *in vitro* if cysts are held at 12^{0}C (Yoshimatsu 1987). It is probable that the low SSTs were the forcing factor for the 1992 bloom (Rhodes *et al.*, 1993).

The dominant microalga present in samples taken from Coromandel in November 1992 was *Heterosigma akashiwo*, numbers of which exceeded those of *F.japonica* at this site by a factor of ten. By then SSTs in the Hauraki Gulf had increased from the previous month's low to between 17.4 and 18.1^{0}C. The reason for the domination of *H.akashiwo* at Coromandel in 1992 is unclear, as there is no data on nutrient conditions at that time, but growth stimulation due to warmer sea temperatures or to run-off following heavy rainfall are both possibilities.

The nearest equivalent climatic event, in summer 1983, also featured low SSTs and a major widespread bloom. On that occasion the dominant species was a diatom with an associated toxic prymnesiophyte (Chang, 1985; Taylor *et al.*, 1985).

It is significant that in January, 1993, following the collapse of the 1992 bloom, a further toxic dinoflagellate dominated bloom developed, which included the neurotoxin producer *Gymnodinium* cf. *breve.* In this later episode, shellfish toxins caused the total disruption of the bivalve industry in New Zealand. There is substantial evidence that the chemical conditioning of the water column during blooms is an important factor in species succession (Gauthier and Aubert, 1981), and it is conceivable that the earlier bloom played a role in the composition and toxicity of the subsequent bloom.

It is very rare that a bloom is monitored from inception to completion; most blooms are investigated reactively. The substantial data available in this instance should prove valuable in developing predictive models for the future.

The MAF Regulatory Authority operational research phytoplankton programme (MAF ORPP) was fully established by September 1993, in response to the toxic dinoflagellate bloom, and data collected at 17 sites, from Houhora Heads in the far north down to Ohiwa Harbour in the Bay of Plenty, was available for analysis.

A raphidophyte bloom, similar in timing, numbers and extent to the previous year's bloom, was recorded in spring 1993. Vast stretches of discoloured water were reported by fishers down the length of the north-east coastline and up to 20 km from the shore. *H.akashiwo* replaced *F.japonica* at the end of November and at Marsden Point (Figure 4.7) the coccolithophore *Syracosphaera* sp. (Figure 1.13) peaked ($>37.4 \times 10^3$ cells l^{-1}) during November, along with the co-dominant diatom *Leptocylindricus danicus. D.speculum* occurred in low numbers (a maximum of 0.4×10^3 cells l^{-1} during October), but *G.oceanica* had, as in 1992, a continued presence (maximum 60.3×10^3 cells l^{-1}, 30 November; the decrease in numbers at the November 24 sampling was most likely due to patchiness). *G.* cf. *breve* caused levels of the breve-like toxin to escalate during November and December (New Zealand marine biotoxin management board reports, November and December 1993).

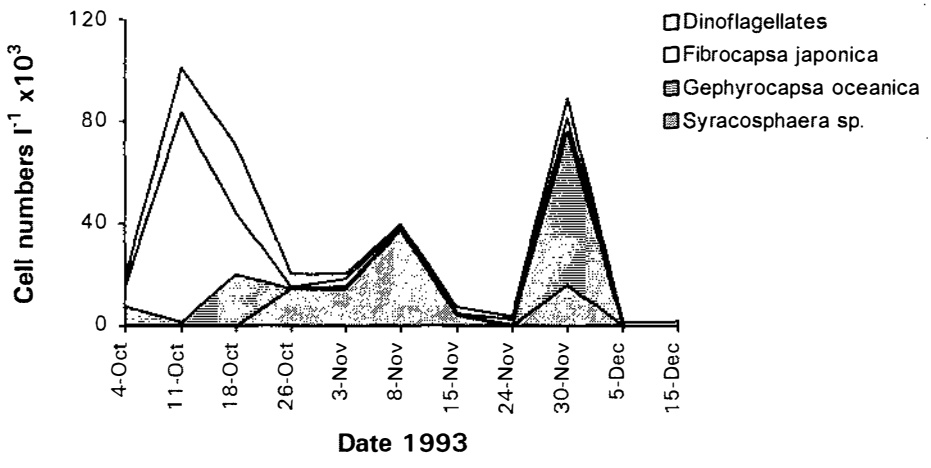


Fig. 4.7. Cell numbers of bloom species at Marsden Point, October to December 1993.

The *F.japonica* bloom was again recorded throughout the spring and early summer of 1994. The golden-brown blooms, typically described as "stringy", were preceded by extensive *Myrionectra rubrum* blooms (also recorded in the Marlborough Sounds) and followed by equally extensive orange-red *Noctiluca scintillans* blooms. The El Niño SSTs continued throughout 1993 - 94 (MAF ORPP data; W.Ballantine, personal communication), supporting the hypothesis that they were the forcing factor behind the unusually extensive and long-lasting bloom events.

G.oceanica also bloomed in Jervis Bay, north-east Australia, from mid-December 1992 to late January 1993 (Blackburn and Cresswell, 1993), but it was suggested that the unique mono-specific bloom there was driven by the intrusion of continental-slope water entering the bay, rather than by temperature anomalies (SSTs were >18ºC). Whether this event was linked to the El Niño weather patterns occurring in the South Pacific region can only remain speculative.

G.oceanica was also present in the Marlborough Sounds in high numbers in March 1992, when SSTs averaged 17.3ºC - 17.8ºC, below the

seasonal average of between 19°C - 20°C for the inner sounds (Regal Salmon Limited, unpublished data). Salinities were within the range of 33.6°/oo - 34.7°/oo. SSTs and daylength were similar to those prevailing at the same time in Jervis Bay, Australia, where *G.oceanica* was blooming. As in Australia, when other species were present in the water column with the coccolithophore they were predominantly dinoflagellates. *G.oceanica* was replaced by *E.huxleyi* during the winter months.

Coccolithophores were dominant around much of New Zealand's coastline during the summer of 1993. The north-east of the North Island was a major exception, with dinoflagellates being dominant and responsible for both neurotoxic and paralytic shellfish toxicity in that area.

Domoic acid (the causative agent of amnesic shellfish poisoning) was one more toxin to appear in northern shellfish in November 1993. It was detected in scallops collected at Rangaunu Bay and at Whangaroa Heads (Figure 4.1) by mouse bioassays (MBMB reports, November-December 1993) and the presence of domoic acid was confirmed by HPLC analysis (10 and 270 mg .kg^{-1} digestive gland contents respectively) (Rhodes and White, 1993). *G.oceanica* and *Pseudonitzschia* sp. were the co-dominant species present in the water column and in scallop digestive glands at that time, but cultures of both species tested negative for domoic acid. Howver, more recent isolates of *P.australis* have proved toxic and it is probable that it was the diatom that was responsible for the toxicity.

G.oceanica was not noted in the far south at any time; the colder waters during winter months would have prevented its establishment as *in vitro* cultures of *Gephyrocapsa* isolated from the Sounds were killed at 10°C (refer Chapter two, Results). SSTs at Stewart Island ranged from 10 - 16°C (MacKenzie, 1991) and it is not surprising that *E. huxleyi*, which tolerates lower temperatures than *G.oceanica* (McIntyre and Bé, 1967), replaced *Gephyrocapsa* in those waters.

The November 1992 bloom of *E.huxleyi* in Big Glory Bay, Stewart Island was at times unialgal and at other times accompanied by a mixed

population of dinoflagellates, in particular a small (10 μm) gymnodinoid. SSTs at that time ranged from 13°C outside the entrance to the bay to 15°C in the shallower reaches of the bay. Despite the slow growth rate of *E.huxleyi* at lower temperatures, it would still have had an advantage over the many microalgal species which have slower growth rates even under optimal conditions. Many dinoflagellates, for example, have optimum growth rates of ≈1.0 division per day (unpublished data); under optimal conditions *E.huxleyi* has a growth rate of 1.9 divisions per day.

In Big Glory Bay there is a potential for increased phytoplankton growth due to high inputs of nitrogen from feed wastage and salmon excreta combined with low rates of flushing (Hare and Brash, 1993). It is feasible that the ammonia levels in the bay, enhanced by the thousands of tonnes of farmed fish, favour microalgal species with an ammonium preference. The growth rate of *E.huxleyi* is optimised by growing cells in media with ammonium salt as the nitrogen source (Rhodes *et al.,* 1994a).

When N:P ratios are calculated from nitrate and phosphate concentrations both prior to and during bloom incidents at Big Glory Bay, it is clear that nitrate was depleted during the 1990 *Chrysochromulina pringsheimii/E.huxleyi* bloom and that the concentration was low in parts of the bay during the 1992 *E.huxleyi* bloom, presumably due to uptake by the microalgae (Table 4.4). However in 1989, when *H.akashiwo* bloomed, molar ratios of N:P were higher than the pre-bloom data (Rutherford *et al.*, 1988). It would appear that the coccolithophore rapidly depletes the water of nitrogen.

At the time of the bloom some salmon mortalities occurred and it is reasonable to conclude that the cause of these salmon deaths was not (at least directly) due to oxygen depletion. Toxicity was not indicated by the *Artemia salina* bioassay.

E.huxleyi is a known dimethyl sulphide (DMS) producer (Keller *et al.*, 1989). No analyses for DMS nor hydrogen sulphide (toxic to fish at low

levels) were carried out during the November bloom. It is possible that such emissions could have been responsible for the fish mortalities, but

Table 4.4 Nitrogen: phosphate (N:P) molar ratios calculated from water column analyses of samples from within Big Glory Bay, Stewart Island, 1988 - 1992. These results are compared with N:P ratios during the Scandinavian *Chrysochromulina* bloom, 1988.

Month/Year	*Bloom status*	*N:P molar ratio*
2/1988[1]	No bloom	4.0 - 8.5
1/1989[2]	*Heterosigma akashiwo* bloom	12.8 - 16.6
11/1990[3]	*Chrysochromulina pringsheimii/ Emiliania huxleyi* bloom	0.7
11/1992[4]	*Emiliania huxleyi* bloom	0.8 - 14.0
5-6/1988[5]	Scandinavian *C.polylepis* bloom	10.0 - 30.0 (av. 24.0)

References: 1, Rutherford *et al.*, 1988; 2, Chang *et al.*, 1993; 3, L.MacKenzie, (unpublished data); 4, Rhodes *et al.*, 1993; 5, Lindahl and Dahl, 1990.

this suggestion can only remain speculative. Testing for sulphide gas production by coccolithophores is worth considering in any future coccolithophore bloom event.

It is evident that coccolithophores are an important component of New Zealand's coastal waters as they are elsewhere in the world's oceans. The predominance of *G.oceanica* cells in toxic scallop guts in November 1993 indicates that the coccolithophores were present in Northland waters and ingested in substantial numbers. Widely distributed milky-green patches, typical of waters in which the reflective calcareous scales of coccolithophores predominate, were observed up to 20 km off-shore during aerial surveys of the north of the South Island and the south of the North Island coastlines in January 1993; the pale green colour of the water correlated with the presence of *E.huxleyi* in the sea water samples

analysed from some of those sites (MacKenzie, 1993). Coccolithophores were again dominant around the New Zealand coastline in summer 1993-94 and in the Marlborough Sounds in spring 1994 (personal observation). Other prymnesiophytes have been implicated in isolated bloom events, in particular *Phaeocystis pouchetii, C.pringsheimii* and *C.quadrikonta.* The latter has developed into nuisance blooms in Japanese waters, where it coloured oysters yellow, and cultures of a New Zealand isolate produced low levels of haemolytic activity against salmon erythrocytes (refer Chapter five, Section 2, Results). However, it is the coccolithophores which have not only bloomed on many occasions and over large geographic areas, but which have persisted as an important component of the microflora at sites right around New Zealand for the full term of this study.

Chapter five: MICROALGAL INTERACTIONS

Section one: ALLELOPATHY

Introduction

Allelopathy has been defined as the release of secondary metabolites by plants (including microalgae) into the environment and the consequent impact of these chemical excretions on other plant species (Rice, 1984).
The development of allelopathic interactions between plants has been ascribed to the pressures of limited resources, with competition for these resources being exhibited through interference (a "chemical warfare" hypothesis) rather than exploitation (Tilman, 1982).
An antithetical view is that microalgae can and do co-exist through the partitioning of resources (Titman, 1976). Further, that a complex of feedback processes drives communities towards homeostasis with excreted chemical substances enriching the environment, but not necessarily having an energetic alimentary value (Gauthier and Aubert, 1981; Whitfield, 1988). According to this hypothesis the survival of populations genera, rather than of individual species within those genera, is believed to be the driving force (Whitfield, 1988).
The suspicion that organisms could affect one another in other than predator-prey interactions is not new. In 1924 it was suggested (Johnstone *et al.*, 1924) that "... there are what we may call group symbioses on the great scale so that the kind of plankton which we may expect to be present in a certain sea area must depend, to some extent, on the kind of plankton which was previously present."

The role of phytochemicals in the succession and blooming of marine microalgal species is a little-explored field. In laboratory experiments excreted substances inhibitory to diatoms have been detected in filtrates of the dinoflagellate *Prorocentrum micans* (Uchida, 1977) and the diatoms *Phaeodactylum tricornutum* (Sharp *et al.*, 1979), *Ditylum brightwellii* and *Skeletonema costatum* (Rijstenbil, 1989). Sometimes called ectocrines, such secretions have been postulated for the raphidophyte *Olisthodiscus luteus* (=*Heterosigma akashiwo;* Pratt, 1966) and a green microalga, *Nannochloris* sp. (Moon and Martin, 1985). The ectocrines from the latter, called "aponin" (apparent oceanic natural cytolin), inhibited the growth of the neurotoxic dinoflagellate *Gymnodinium breve*. Exotoxins produced by the closely related *G.mikimotoi* (=*Gyrodinium aureolum*) had an allelopathic effect on a range of other microalgae (Gentien and Arzul, 1990). The ichthyotoxic prymnesiophyte, *Chrysochromulina polylepis,* isolated from a Scandinavian bloom in 1988, inhibited growth of the diatom *Skeletonema costatum* in laboratory experiments (Myklestad *et al.,* 1993).

Most experiments to date have been carried out *in vitro* and it is debatable as to whether chemical mediators have an impact on the natural succession of algae in the oceans and whether they can, in fact, override such factors as nutrient availability, physical conditions, water column stability or grazing pressure. However, the Scandinavian *Chrysochromulina* bloom was characterised by the exclusion of nearly all competitors and predators, and *in vitro* were weakly inhibitory to the growth of diatoms, blue mussels and cod (Skjoldal and Dundas, 1989), which supports the hypothesis that the bioactive compounds produced by *C.polylepis* gave that species a competitive advantage *in vivo*.

Microbial antagonisms can be mediated by a range of substances including toxins, antibiotics and growth regulators. Several prymnesiophyte species produce the toxin prymnesin, which is a complex of hydrophilic and hydrophobic compounds (Yariv and Hestrin, 1961; Kozakai *et al.*, 1982; Yasumoto *et al.*, 1990) exhibiting ichthyotoxic,

haemolytic, cytotoxic and neurotoxic activities (Shilo and Rosenberger, 1960; Meldahl *et al.*, 1993). Two pure toxins have recently been purified, prymnesin 1 and 2, which have polyhydroxy-polyene-polyether features (Igarishi *et al.*, 1994); the allelopathic effects of the toxic species *Prymensium parvum* and *P.patellifera* on other microalgae are recorded in this study.

The antibiotic acrylic acid is produced, with dimethylsulfide, from dimethylpropiothetin by the bloom-forming prymnesiophyte *Phaeocystis pouchetii* (Sieburth, 1960; Ackman *et al.*, 1966; Guillard and Hellebust, 1971; Eberlein *et al.*, 1985) and might have a role in the competitive success of this alga. *P.pouchetii* forms mucilage enveloped colonies when grown in a dilute marine medium, whereas free-swimming flagellated forms dominate in high nutrient level media. Colonial forms can release up to 30 times the amount of extracellular substances that free cells do, about 2% of the total material released being acrylic acid (Guillard and Hellebust, 1971). It would appear that nutrient limitation stimulates allelochemical production, at least in this organism.

The coccolithophore, *Cricosphaera* sp. (Prymnesiophyceae), produces gibberellin-like substances in both its motile and non-motile phases, and cytokinin-like substances in the motile phase. This raises the possibility of an interplay occurring between growth promoters and inhibitors (Hussain and Boney, 1971). Gibberellins and cytokinins have small but significant effects on the growth of phytoplankton, the effect being dependent on the state of the inoculum (Bentley-Mowat and Reid, 1969).

Inoculum levels can have a marked effect on growth interactions. In comparisons of mixed batch and continuous cultures of the dinoflagellates *Gymnodinium splendens*, *Prorocentrum micans* and *Scrippsiella faeroense*, it appeared that growth was regulated mainly by nutrient competition during the exponential phase and that the species with the greater inoculum would dominate. Inhibition by metabolic products was effective only at maximum cell densities (Kayser, 1979).

Factors that affect the amounts of allelochemicals produced by plants include light quality, intensity and duration, temperature, other allelopathic agents, the age of the organisms involved, any pathogens or predators present and the genetic base of the plants (Rice, 1984). The activity of chemicals can also fluctuate over time. This can be due to changes in the concentrations of allelochemicals being produced, perhaps due to diurnal effects, or to the complexities of multiple substance excretions.

The role of allelopathy in microalgal species succession and bloom development is still not fully understood, and might have been underestimated. With blooms impacting on the aquaculture industry there is a need to determine the extent of involvement of allelopathy, not only to assist in the development of reliable bloom prediction models, but to enable assessment of allelochemicals as possible management tools.

In the previous chapter (refer Chapter four, Discussion), it was suggested that the raphidophyte/coccolithophore dominated bloom that occurred in the Hauraki Gulf, New Zealand, in spring/summer, 1992, played a role in determining the composition and toxicity of the phytoplankton that succeeded it in January, 1993. The major focus of this study was on the allelopathic interactions between phytoplankton species *in vitro*. Preliminary attempts were also made to identify the inhibitory agents. The intention was to highlight interactions of ecological significance within the New Zealand microalgal flora, but the time taken to isolate and optimise growth conditions of local strains led to the acquisition and testing of microalgae from a variety of sources (Table 5.1). From this screening programme two species, the known prymnesin producer, *Prymnesium patellifera* (Prymnesiophyceae), and *Chlamydomonas coccoides* (Chlorophyceae), both of which inhibited the growth of other microalgae, were selected for further investigation. However the inhibitory activity decreased in these cultures over time. The results of this study, the implications of allelopathic interactions and the problems inherent in such studies *in vitro* are discussed.

Methods

Microalgae

The microalgae used in the broad screening programme for allelopathic interactions are listed, with their source, in Table 5.1. Many were chosen because of known toxicity, others were tested because of their availability.

Screening for chemical interactions

The screening for allelopathic interactions was carried out using two methods: (a) Dual cultures were set up in 20 ml GP medium (Loeblich and Smith, 1968) in 50 ml sterile plastic containers (Salmon Smith Biolab, NZ Ltd.). Two selected species were inoculated together (5% final volume of inoculum of each of the exponentially growing cultures, unless stated otherwise in results). Cell counts and qualitative observations were carried out daily; subsamples of I ml were transferred to Utermöhl chambers (fixed with Lugol's iodine (0.2 μl ml^{-1}). Interactions were considered inhibitory when cell numbers in the control culture were significantly higher than in the test culture, or when microscopic examination indicated lysis or deformation of at least 50% of cells. Controls with same species inoculum were also tested.

Chlamydomonas coccoides (14 $x10^7$ cells l^{-1} initial inoculum) and *Chattonella antiqua* (15 $x10^4$ cells l^{-1} initial inoculum) were also grown as dual cultures in GP medium (20 ml). The inocula for the 2 species were either added together, with *C.antiqua* added 2 days prior to *Chlamydomonas coccoides* or with *Chattonella antiqua* added 2 days after *Chlamydomonas coccoides.* Subsamples were taken over a 10 day

Table 5.1. Microalgal species investigated for allelopathic interactions.

PRYMNESIOPHYCEAE Hibberd 1976	
Isochrysis galbana	MAFFisheries, Mahanga Bay, NZ
Phaeocystis pouchetii	L.Rhodes, Cawthron, Nelson, NZ
Prymnesium parvum and *P. patellifera*	F.H.Chang, Oceanographic Inst., Wellington, NZ; Univ. Copenhagen, Denmark.
CHLOROPHYCEAE Wille sensu Silva 1982	
Chlamydomonas coccoides	MAFFisheries, Mahanga Bay, NZ
Dunaliella salina and *D. tertiolecta*	" "
Nannochloris sp.	" "
CRYPTOPHYCEAE GS West et Fritsch 1927	
Chroomonas salina	MAFFisheries, Mahanga Bay, NZ
Cryptomonas maculata	CSIRO, Tasmania (Cs-85)
DIATOMOPHYCEAE Rabenhorst 1864	
Chaetoceros sp.	L.Rhodes, Cawthron, Nelson, NZ
Ditylum brightwellii	L.Rhodes, Cawthron, Nelson, NZ
Melosira sp.	" "
Navicula sp.	" "
Nitzschia closterium	" "
N. seriata	L.Rhodes, Cawthron, Nelson, NZ
Pseudonitzschia. pseudodelicatissima	I.M.B.*, Halifax, NS (F-269-A)
Pseudonitzschia pungens f. multiseries	I.M.B.* (NP-23;NPM;MD-1A;TKA-2-34)
Pseudonitzschia pungens f. pungens	I.M.B.* (NPBRWD-A)
Skeletonema costatum	" "
Thalassiosira oceanica	CSIRO, Tasmania (Cs-67)
T. pseudonana	MAFFisheries, Mahanga Bay, NZ
DINOPHYCEAE GS West et Fritsch 1927	
Amphidinium carterae	CSIRO, Tasmania (Cs-212)
A. carterae	L.Rhodes, Cawthron, Nelson, NZ
Gymnodinium sanguineum	CSIRO, Tasmania (Cs-35)
G. sp.	L.Rhodes,Cawthron, Nelson, NZ
Peridinium balticum	CSIRO, Tasmania (Cs-38)
Prorocentrum gracile and *P. micans*	" " (Cs-80, Cs-27)
P. lima	Univ. British Columbia, Canada (514)
P. lima	" " (Spain)
Scrippsiella sp.	CSIRO, Tasmania (Cs-168)
PRASINOPHYCEAE Christensen sensu Moestrup et Throndsen 1988	
Pyramimonas grossii and *P.* sp.	MAFFisheries, Mahanga Bay, NZ
Tetraselmis chuii and *T. suecica*	" "
RAPHIDOPHYCEAE Chadefaud ex Silva 1980	
Chattonella antiqua	CSIRO, Tasmania (Cs-171)
Fibrocapsa japonica	" " (Cs-235)
Heterosigma akashiwo	F.H.Chang, Wellington, NZ
H. akashiwo	CSIRO, Tasmania (Cs-96)
RHODOPHYCEAE Rabenhorst 1863	
Porphyridium purpureum	" " (Cs-25)

***IMB: Institute of Marine Biosciences**

growth period for cell counts and microscopic observations of cell condition (refer Chapter three, Methods).

(b) Dual cultures were also grown in glass U-tubes, with the species separated by nuclepore filters (0.2 μm; Figure 5.1). Cultures were counted daily (as above).

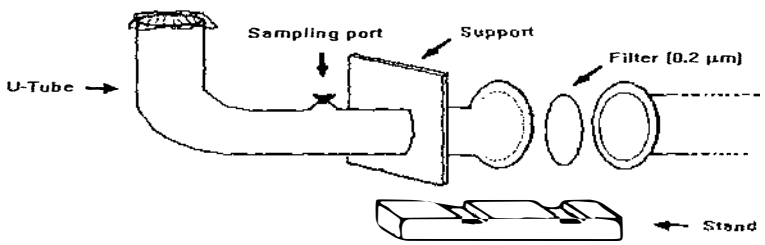


Fig. 5.1. U-tube apparatus used in the screening for allelochemicals in marine microalgae.

Acrylic acid

Acrylic acid (1 to 50 mM final concentration) was added to *Chattonella antiqua* (≈800 cells per ml GP medium in tissue culture plate wells; Becton Dickonson) to assess its effect.

Cell-free filtrates

Cell-free filtrates (0.2 μm nuclepore) were obtained from exponential and stationary phase cultures grown under standard conditions (refer Chapter 3, Methods). These were tested against selected microalgae as above, but with the filtrates replacing one of the microalgal species (refer screening for chemical interactions).

Partial characterisation of the filtrates was carried out as follows: filtrates were heat treated by immersing the culture vessel in a water bath (100^0C) for 10 min.

Dialysis of filtrates was in dialysis tubing (Spectrum Medical Industries, Inc., USA) previously boiled in 0.2% Na_2 EDTA solution and rinsed with distilled water. Filtrates (2 ml; controls were GP medium) were suspended in the dialysis tubing in a 1 and a 2 day old culture (20 ml) of *C.antiqua* and microscopic observations and cell counts of *C.antiqua* were made daily.

A 5-fold concentration of filtrates was obtained by addition of Sephadex G-15 (11.2 g in 30 ml filtrate; Pharmacia Fine Chemicals, Sweden).

Fatty acid analyses: analysis of filtrates for fatty acid methyl esters was by gas chromatography (GC): the sample (200 ml) was extracted with chloroform, the chloroform removed and the extract esterified with boron trichloride prior to analysis by GC using a thermal gradient between 180^{0}C and 225^{0}C. Separation was carried out on a wide-bore capillary column with flame ionisation detection (AOAC, 1990).

Results

Screening results

In bialgal and U-tube experiments three prymnesiophytes, *Prymnesium parvum, P.patellifera* and *Phaeocystis pouchetii* (Prymnesiophyceae) had an inhibitory effect in both exponential and stationary phases of growth on other microalgae (Table 5.2).

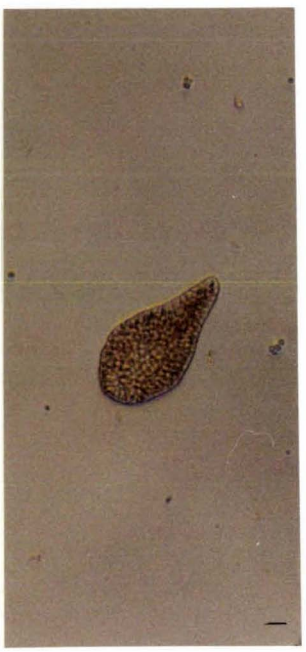
Chattonella antiqua was the most profoundly affected alga (Figure 5.2), although in the case of *P.pouchetii* the cells only appeared deformed when trapped within that prymnesiophyte's mucilage. *Amphidinium carterae, Peridinium balticum* and *Prorocentrum gracile* also exhibited deformities when grown in dual culture with the *Phaeocystis pouchetii*.

Prorocentrum lima (stationary phase cultures only), *Gymnodinium* sp., *Scrippsiella* sp. (Figure 5.2), *Nitzschia closterium* and *Chlamydomonas coccoides* also caused deformation of cells of *Chattonella antiqua.* The results of each experiment were of duplicates only (due to the number of

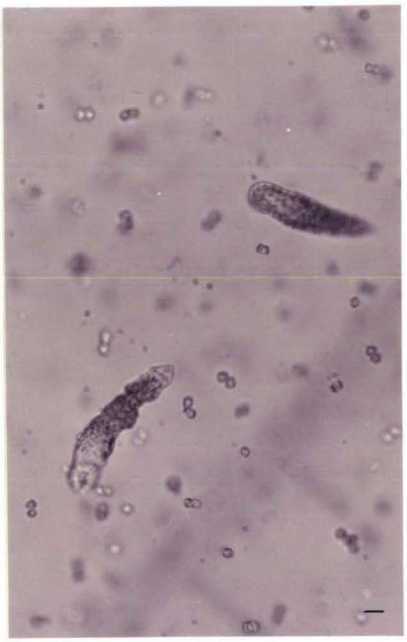
Table 5.2 Microalgal species tested in dual cultures for allelopathic interactions.

Microalga 1	***Microalga 2***	***Allelopathic effect:***	
		Microalga 1	***2***
Prymnesium parvum	*Chattonella antiqua*	-	**D**
"	*Chrysochromulina quadrikonta*	-	**ND***
"	*Heterosigma akashiwo*	-	-
"	*P.patellifera*	-	-
P.patellifera	*Chattonella antiqua*	-	**D**
"	*Chrysochromulina quadrikonta*	-	**ND***
"	*Fibrocapsa japonica*	-	**ND;DF***
C.quadrikonta	*Chattonella antiqua*	-	-
"	*Gymnodinium sanguineum; G.* sp.	-	-
"	*H.akashiwo*	-	-
"	*Phaeocystis pouchetii*	-	-
"	*Porphyridium cruentum*	-	**ND;DF**
"	*Prorocentrum micans*	-	-
"	*Pseudonitzschia* spp.#	-	-
"	*Scrippsiella* sp.	-	-
"	*Skeletonema costatum*	-	-
Phaeocystis pouchetii	*Amphidinium carterae*	-	**ND;DF**
"	*C.antiqua*	-	**ND;DF**
"	*G.* sp.	**ND**	-
"	*Navicula* sp.	-	-
"	*Peridinium balticum*	-	**ND;DF**
"	*Prorocentrum gracile*	-	**ND**
H.akashiwo	*Cryptomonas maculata*	-	-
"	*Chattonella antiqua*	-	**DF**
"	*Nitzschia closterium*	-	-
"	*N.seriata*	-	-
Chattonella antiqua	*Chaetoceros* sp.	-	-
"	*Chlamydomonas coccoides*	**ND;DF**	-
"	*Chroomonas salina*	-	-
"	*Dunaliella salina; D.tertiolecta*	-	-
"	*G.sanguineum;*	**DF**	-
"	*Isochrysis galbana*	-	-
"	*Melosira* sp.	-	-
"	*Nannochloris* sp.	-	-
"	*Nitzschia closterium*	**DF**	-
"	*P.lima*	**DF**	-
"	*Pyramimonas grossii; P.*sp.	-	-
"	*Scrippsiella* sp.	**DF**	-
"	*Tetraselmis chuii; T.suecica*	-	-
"	*Thalassiosira oceanica; T.pseudonana*	-	-
***Scippsiella* sp.**	*Ditylum brightwellii*	-	**ND**

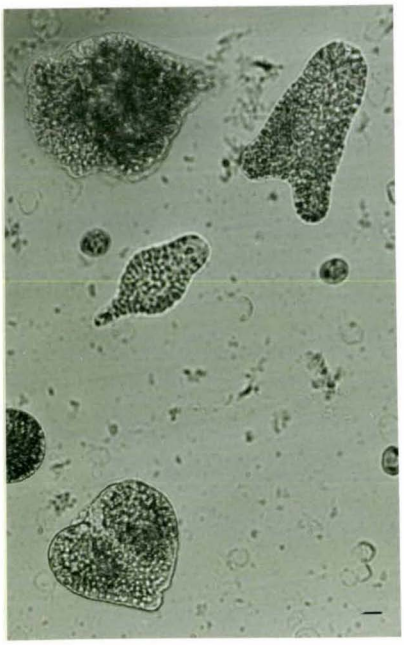
Refer Table 5.1. ND, cell numbers decreased; D, cells died; DF, cells were deformed; - , no effect; *, effect only when *P.patellifera* and *P.parvum* nitrogen limited.



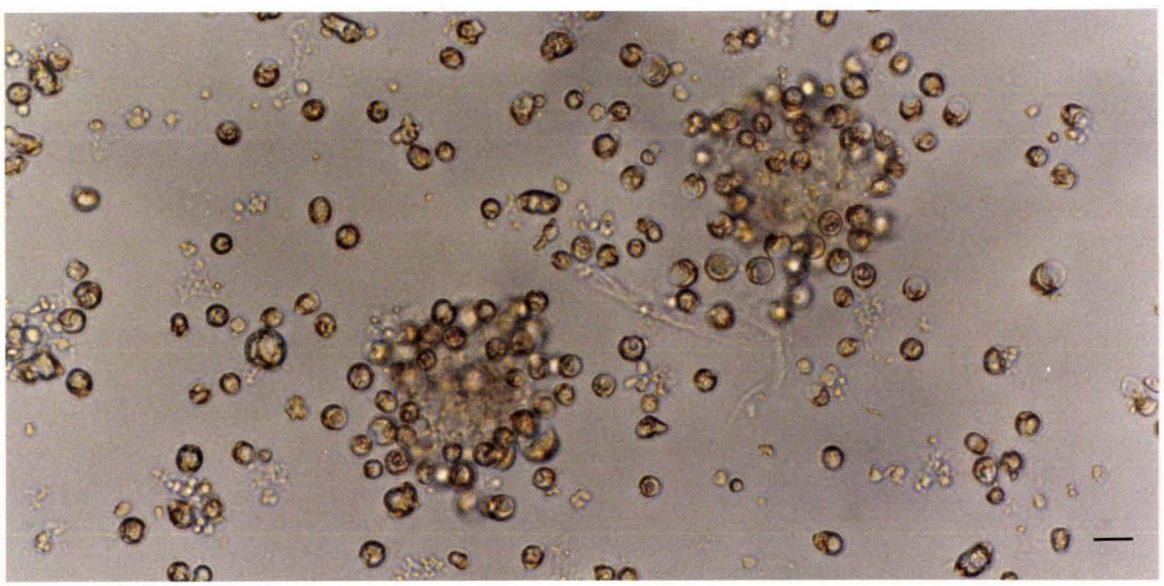
(a)



(b)



(c)



(d)

Fig. 5.2 Results of dual culture experiments: *Chattonella antiqua* cells in monoculture (a) and in dual culture with (b) *Phaeocystis pouchetii* (5 day old co-culture), (c) *Scrippsiella* sp (7 day old co-culture) and (d) *Prymnesium parvum* (7 day old co-culture; *C.antiqua* cells are lysed and surrounded by *P.parvum* cells). Bar = 10 μm.

combinations of species tested), but were replicated at least twice when activity was indicated, before being considered worthy of further investigation.

Chlamydomonas coccoides caused inhibition of growth of *Chattonella antiqua* (i.e. a decrease in cell numbers in comparison with the control). The inhibitory effect took 3 days to take effect when *C.antiqua* had been growing for 2 days prior to addition of *C.coccoides*, but was immediate when the inocula were added together or when *C.coccoides* was added 2 days earlier than *Chattonella antiqua* (Figure 5.3).

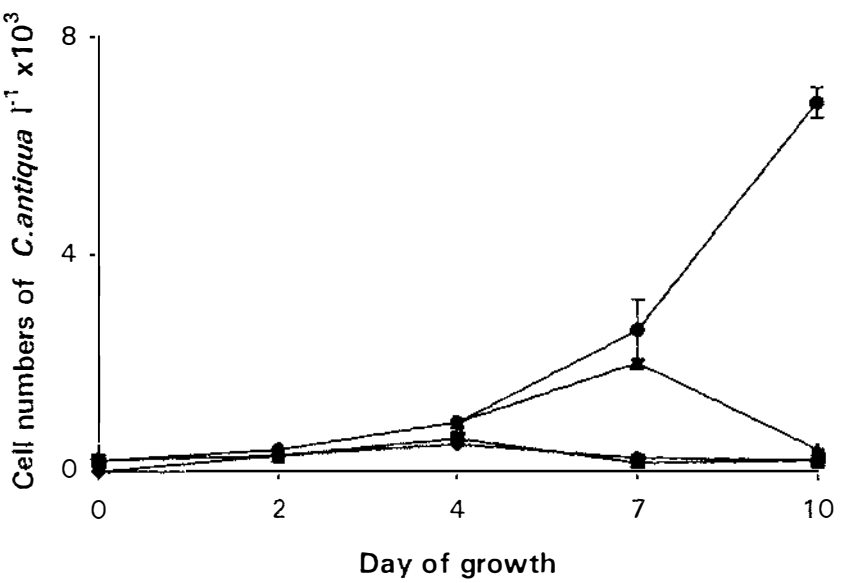


Fig. 5.3 The effect of addition of *Chlamydomonas coccoides* to exponential phase cultures of *Chattonella antiqua*. Results are means of duplicates only (bars indicate std.dev.). Control of *C.antiqua* only (●); both cultures added together (■); *C.coccoides* (14.0 x10^6 cells l^{-1}) added 2 days after *C.antiqua* (▲) and *C.coccoides* added 2 days before *C.antiqua* (♦).

C.antiqua was selected as a target organism for further studies due to its apparent sensitivity to other inhibitory species and its consistent growth pattern *in vitro*. The possibility that the deformations were due to osmotic effects on addition of culture filtrates was tested by adding 0.01% through to 2.5% mannitol to otherwise standard cultures of *C.antiqua*. Addition of 2% had no effect on the microalgal cells. Only the

2.5% mannitol addition elicited an effect: cells rounded up 30 min after addition, but with a full recovery at 24 h.

A marked decrease in inhibitory activity was noted over time in both cell cultures and culture filtrates of *Scrippsiella* sp., *Gymnodinium* sp. and *Nitzschia* sp. (i.e. *C.antiqua* was no longer affected); *Phaeocystis pouchetii* failed to elicit an inhibitory response with cell free filtrates. *Prorocentrum lima*, a known producer of okadaic acid, a diarrhetic shellfish toxin (Murakami *et al.*, 1982), was not selected for further study as it only caused inhibition in stationary phase (24 day old culture filtrates); cultures having an effect during exponential phase as well as stationary phase were considered more likely to be producing extracellular substances rather than degradation products due to senescence. *Prymnesium patellifera* and *Chlamydomonas coccoides* were selected for further comparative investigations as their inhibitory effects on *Chattonella antiqua* were able to be replicated at that time.

Inhibitory effects of cell free filtrates of Phaeocystis pouchetii, Prymnesium patellifera *and* Chlamydomonas coccoides *on* Chattonella antiqua *in laboratory experiments*

Filtrates of *Phaeocystis pouchetii* cultures had no effect on *Chattonella antiqua* when added to cultures of that microalga. The effect of acrylic acid (produced by *P.pouchetii*) on *C.antiqua* was also tested. Cells lysed immediately with the addition of ≥10 mM acrylic acid and after 2 h with the addition of 5 mM, but were unaffected after 24 h with 1 mM.

As a result of the screening experiments cultures of *Prymnesium patellifera* and *Chlamydomonas coccoides* were grown under standard culture conditions and with nitrogen and phosphate limitation, and incubated at temperatures of 10^0C to 25^0C. Cell free filtrates of these cultures were then tested against *Chattonella antiqua* at different stages of growth. Filtrates from exponential through to stationary phase cultures of *P.patellifera,* grown under standard conditions, inhibited the growth of

C.antiqua, the greatest activity being observed in the stationary phase cultures (≥10 d growth).

Filtrates of *Chlamydomonas coccoides,* sampled on days 8 and 12 of growth, caused inhibition of *Chattonella antiqua* cells (both a decrease in cell numbers and deformation of living cells) and the activity was greatest in cultures grown at 14^0C under nitrogen and/or phosphate limitation. Filtrates from cultures of less than 8 days growth or from ten days growth had no inhibitory effect. These promising results, which demonstrate a bimodal activity, were replicated, but activity then diminished, despite growth under a variety of nutrient regimes and environmental conditions. Concentration with Sephadex G-15 was unsuccessful in regaining the original activity, as were efforts to induce the effect by adding culture filtrates of both heat and UV killed whole cells of *C.antiqua* to cultures of *Chlamydomonas coccoides*.

The *C.coccoides* filtrate activity was not impeded by dialysis tubing; *Chattonella antiqua* outside the tubing became deformed.

Heat treatment of 8 d old culture filtrates caused a loss of activity, but not of 12 d old cultures (i.e. *C.antiqua* cells became deformed).

Fatty acid analyses: analysis by gas chromatography indicated that most of the fatty acids in the cultures were less than C16. As no more active filtrate was available no further analyses were carried out.

Discussion

The allelochemical interactions determined for *Chlamydomonas coccoides* (Chlorophyceae) in this study were of particular interest as bimodal peaks of activity, dependent on the stage of growth, were exhibited. Cell free culture fluids were similarly active and the different effects of heat treatment on these two activities suggests that either two different compounds were being secreted or that a conversion of one to another different but still active compound was occurring. The low molecular weight of the active fractions, as indicated by dialysis, points to a non-

proteinaceous agent. Unfortunately the loss of inhibitory activity in the microalgal cultures over time, despite intensive efforts to re-induce it, ended this line of investigation.

Prymnesium parvum and *P.patellifera* (Prymnesiophyceae) caused inhibition of the growth of species from several microalgal classes during this study, but *P.patellifera* rapidly lost its inhibitory activity *in vitro*. However, *P.parvum* retained its activity for several years in culture and because of the ease of replication of its toxic effect it was selected as a positive control for the bioassays for ichthyotoxicity that were developed and assessed, and which are described in Section two.

Decreases and/or losses in activity *in vitro* have been noted by other researchers (Taylor, 1993), e.g. *Gymnodinium mikimotoi* (syn. *Gyrodinium aureolum*) progressively lost its anti-algal activity over a period of 9 months in culture (Gentien and Arzul, 1990).

Dual cultures of *Phaeocystis pouchetii* (Prymnesiophyceae) and *Chattonella antiqua* (Raphidophyceae) resulted in deformation of those *C.antiqua* cells that contacted the prymnesiophyte's mucilage. Cells immediately outside the mucilage were unaffected and there was no effect of cell free filtrates of *P.pouchetii* on *C.antiqua* cells. The effect could have been due to the physical properties of the mucilage or to the activity of acrylic acid. The effect of acrylic acid could have been direct (i.e. algicidal) or indirect, for example by lowering bacterial levels and thus the concentrations of vitamins secreted by those microbes. Acrylic acid (at 5 mM final concentration) was shown to lyse *C.antiqua* cells, but the hypothesis was not supported in this instance as acrylic acid was not detected in the laboratory cultures.

The ecological role of marine allelochemicals is poorly understood, but it is clear that many microalgae do secrete a variety of compounds into their immediate environment, conditioning the water and thus optimising the chances for a particular alga or suite of algae to dominate. For example, *Chlamydomonas reinhardtii* (Chlorophyceae), a freshwater relative of

C.coccoides, produces a mixture of long chain fatty acids which inhibit the growth of other algae (Proctor, 1957).

The linking of toxin production to associated bacteria (possibly endosymbionts) needs to be considered in claims of allelochemical production by microalgae; saxitoxin is not only produced by *Alexandrium* species, but is produced by the bacterium, *Moraxella* sp., isolated from within cells of *Protogonyaulax tamarensis* (syn. *Alexandrium tamarense*) (Ogata et al., 1990; Kodama et al., 1988).

Section two: MICROALGAL TOXICITY BIOASSAYS

Introduction

The recurrent toxic microalgal events in New Zealand over the last decade have highlighted the need for rapid detection systems for both shellfish toxins and ichthyotoxins. Caged fish are particularly vulnerable to toxic blooms and in this study bioassays have been developed and refined for the rapid and inexpensive detection of ichthyotoxic microalgae. In particular, New Zealand prymnesiophyte isolates have been assessed for toxicity using the known prymnesin producer *Prymnesium parvum* as a positive control. *P.calathiferum,* discovered in New Zealand waters, has already been shown to be ichthyotoxic to *Gambusia* sp. (Chang, 1985).

Prymnesin has been a difficult toxin to purify and many of the toxin classifications in the literature have arisen from tests run on impure samples. Recently, however, two structurally-related toxins, prymnesin-1 and prymnesin-2, have been purified from *P.parvum.* Both toxins are haemolytic, ichthyotoxic and induce calcium ion influx into rat glioma cells (Igarishi *et al.*, 1994).

The raphidophyte *Chattonella antiqua* and the dinoflagellate *Heterocapsa triquetra* were evaluated as bioassay organisms for prymnesin against brine shrimps (*Artemia salina*) (Persoone and Wells, 1987) and veliger paua larvae (*Haliotis iris*). *C.antiqua* was chosen as it had proved to be a sensitive bioassay organism during the screening for allelopathic interactions (Section one) and *H.triquetra* was selected due to its reputed sensitivity to prymnesin (O.Moestrup, personal communication).

In another more quantitative assay, culture extracts from New Zealand prymnesiophyte isolates and known ichthyotoxin producers from culture collections overseas were tested for haemolytic activity against freshly isolated salmon erythrocytes (Edvardsen *et al.,* 1990). The advantages and disadvantages of this system are discussed.

Method

Microalgae

Microalgae tested for ichthyotoxicity against bioassay organisms included New Zealand prymnesiophytes isolated during this study (refer Chapter one, Results), the raphidophytes *Heterosigma akashiwo* and *Fibrocapsa japonica* isolated during the 1992 Northland bloom, toxic dinoflagellates isolated during other bloom events in New Zealand (Rhodes *et al.*, 1993) and species investigated for allelopathic interactions (refer Table 5.1). *Prymnesium parvum* (provided by F.H.Chang, NIWA, Wellington) and *Chrysochromulina polylepis* (Provasoli-Guillard Centre, Bigelow Laboratories, Maine, USA) were used as positive (toxic) controls.

The dinoflagellate *Heterocapsa triquetra* and the raphidophyte *Chattonella antiqua* were selected as bioassay organisms. *H.triquetra* was isolated from Nydia Bay, Marlborough Sounds, July 1993, during a bloom of that microalga. *C.antiqua* was obtained from CSIRO, Tasmania. Both species were rendered axenic and maintained in GP medium under standard culture conditions (refer Chapter three, Methods).

Cultures were rendered axenic with 0.002% Penicillin G and 0.0005% streptomycin sulphate and purity was checked by streaking on standard bacteriological marine agar (Difco, USA).

Artemia *and paua larval bioassays*

The dehydrated eggs of the brine shrimp *Artemia salina* (Brooklands Aquarium, San Fransisco) were purchased from a local pet store. Eggs were hydrated in filtered sea water (25^{0}C), to hatch the nauplii, for 48 h prior to carrying out the bioassays. (Nauplii younger than 24 h are less sensitive to the microalgal toxins). Hatched nauplii were checked under the microscope and only those in good condition and of similar size were

transferred by Pasteur pipette to tissue culture plate wells (Becton Dickinson) for bioassays.

Two day old veliger larvae of paua (*Haliotis iris*) (maintained in filtered sea water at 20^{0}C) were transferred to tissue culture wells and used for bioassays.

Microalgal species were tested for toxicity against *A.salina* and *H.iris* using modifications of the standard *A.salina* bioassay method (Persoone and Wells, 1987). The bioassays were carried out in quadruplicate in tissue culture plates, with 10 organisms added per well. Microalgal cultures were grown under phosphate and nitrogen limitation, and under a range of temperatures (10, 15, 20 and 25^{O}C), salinities (18, 25 and $34^{0}/_{00}$) and light regimes (50, 100 and 150 μmol m^{-2} s^{-1}), before testing for toxicity at both exponential and stationary phases of growth. Samples of cultures were then added (2 ml) to the bioassay wells and the bioassay organisms were observed at 0, 1, 2, 4, 8 and 24 h. Bioassays were held under standard microalgal growth conditions between observations. The time for 50% of the *Artemia* to die (tD_{50}) or to exhibit morbidity (tM_{50}) was then recorded for the particular number of microalgal cells added. Morbidity was arbitrarily scaled as +, slight distress; ++, marked twitching, but swimming; +++, nauplii on bottom of well, twitching.

Microalgae as bioassay organisms

The microalgal bioassays were carried out in tissue culture wells. The test microalgae (50 cells of *Chattonella antiqua* or 200 cells of *Heterocapsa triquetra*) were added to the assay microalga (2 ml of stationary phase culture, unless stated otherwise) in the wells. Subsamples for counting were taken at 0 h and 24 h. The condition of the cells was observed microscopically at regular intervals throughout the bioassay.

Transwell™ tissue culture plates with porous cell culture inserts (0.4 μm nuclepore; Costar, Maine) were also used with both microalgae and

A.salina; the bioassay organism was held in the outer well and the test microalgal cells in the insert. TD_{50} or tM_{50} was recorded.
All bioassays were incubated under standard microalgal culture conditions and were carried out in quadruplicate. Both negative (media addition only) and axenic positive controls were run for each assay.

Assays for haemolysis using salmon erythrocytes

Erythrocytes were extracted from salmon (*Oncorhynchus tshawytscha*) anaesthetised, in a bucket of sea water, with phenoxyethanol (0.3 ml l^{-1}). The salmon were then swabbed at the extraction site with methanol and blood was collected with a sterile syringe pre-rinsed with diluent* from a heparin lined tube to prevent clotting. The needle was inserted beside the anal fin and into the artery running along the backbone of the anaesthetised fish (Figure 5.4). The blood obtained was immediately injected into heparin lined "venoject" tubes (1 ml per tube) containing 9 ml of diluent*. Tubes were maintained at $\approx 10^{0}$C with ice packs until refrigerated and the erythrocytes were used within 24 h of extraction.
Toxin extraction: Toxin was extracted from the microalgal cultures using a modification of the method of Edvardsen *et al.* (1990) as follows: cultures (600 ml in 1 l Erlenmeyer flasks) were grown under various nutrient and culture regimes, harvested during exponential and stationary phase of growth and frozen. Methanol (300 ml) was added to each frozen culture and thawing was hastened by holding in a 34^{0}C water bath. Cultures were filtered (Whatman GF/C), the filters rinsed with methanol (2 x50 ml) and the filtrate transferred to separating funnels. Dichloromethane (100 ml) was added and the funnel contents were mixed by gently tipping 180^{o} x12. Mixing was repeated after approximately 15 minutes. The bottom dichloromethane fraction was collected in round bottom evaporating flasks and the separation repeated twice. The final total dichloromethane fraction of 300 ml was dried by rotary evaporation

at 33^{0}C, the resultant extract redissolved in ethanol (100 μl) and then tris (pH7; 0.01 M final conc.) added.

Assay procedure: The assay (again a modification of the method of Edvardsen *et al.*, 1990), was carried out in 1.5 ml centrifuge tubes. Extract (700 μl) was added to erythrocytes in diluent (500 μl). A positive control of saponin (2 g l^{-1} diluent; 700 μl), a commercial haemolysin, and a negative control of diluent were also run. Following incubation at 10^{0}C for 2 h, the tubes were centrifuged (9,900 *g*) for 5 minutes, then read on a spectrophotometer (540_{nm}) zeroed against a diluent blank. The results of the positive control were taken as 100% haemolysis and the test results related to this figure.

Erythrocytes were also assayed with saponin only, using both Fish rbc and Alsever's diluents* as above to assess the suitability of the diluents.

*Diluents:

Fish rbc diluent (adjusted to pH7.4).	g l^{-1} distilled water
sodium chloride	8.77
potassium chloride	0.24
magnesium sulphate	0.31
tris[hydroxymethyl]aminomethane (tris)	1.48
Alsever's diluent	
glucose	18.66
sodium chloride	4.18
sodium citrate	8.0
citric acid	0.55

Results

Artemia salina *bioassays*

None of the New Zealand prymnesiophyte isolates tested showed any effect on the brine shrimps, even when aged cultures (2 to 6 month old) were used (see Table 5.3). The positive controls achieved consistently shorter tD_{50} results when phosphate was limiting: *Prymnesium parvum*, tD_{50} 4 h; *Chrysochromulina polylepis*, tD_{50} 8 h, tM_{50} (+ + +) 6 h.

P.parvum gave the same tD_{50} result whether the test inoculum was from a 2, 4, 6, 8, 10 or 50 day old culture, and whether grown at $17.8^0/_{00}$ through to $34.9^0/_{00}$. Death was sudden and not preceded by a morbid or stressed stage.

C.polylepis elicited a tD_{50} of 18 h with 2 day old cultures, 10 h with 4 day old cultures, and 8 h in older cultures. The tM_{50} was also reached earlier when the culture was in stationary phase, decreasing from 8 h for 2 day old cultures to 4 h with 10 day old cultures. Results were consistent with both axenic and non-axenic cultures. The activity of *P.parvum* diminished after 3 years in culture, and the culture of *C.polylepis* has since been lost.

All the *Heterosigma akashiwo* (Raphidophyceae) strains tested caused morbidity, but the *A.salina* nauplii recovered if they were removed from the test wells and placed in filtered sea water. A tM_{50} of 8 h was recorded for the Stewart Island strain under standard growth conditions during exponential phase. When the bioassay was incubated at 15^0C the tM_{50} was 1 h. All the other *H.akashiwo* isolates tested caused morbidity (tM_{50} 1 h) whatever the growth phase, bioassay incubation temperature or salinity (within the range tested of $17.8^0/_{00}$ through to $34.2^0/_{00}$). *Fibrocapsa japonica* had no effect on *A.salina.*

The dinoflagellate *Gymnodinium sanguineum* formed a unialgal bloom in Okiwi Bay, Marlborough Sounds, 10 March 1993, and seawater samples from the bloom caused a distress reaction in *A.salina.* This species is not

known to be toxic and will be investigated in another study. *Prorocentrum lima*, an okadaic acid producer, consistently caused the death of *A.salina* in bioassays. Death of the brine shrimp was sudden and not preceded by a morbid response, i.e. no twitching was observed. Cultures of *Amphidinium carterae* (CSIRO) did not cause the death of *Artemia* when grown in medium with a salinity of $17^{0}/_{00}$, but did in media of 25 - $34^{0}/_{00}$. *Amphidinium carterae* (CSIRO) gradually lost the ability to cause death or distress after two years in culture (Table 5.3). *A.carterae* (Wellington) had no effect on *Artemia.*

Paua larva bioassays

Many of the microalgae tested against *Artemia salina* were also tested against two day old paua larvae (*Haliotis iris*). *Prymnesium parvum* (3600 $x10^3$ cells l^{-1}) caused the death of all larvae within 1 h (Table 5.4; Figure 5.5). Culture filtrate also caused immediate signs of morbidity in the larvae, with all dying within 3 h.

When *Heterosigma akashiwo* (various strains) (≈5000 $x10^3$ cells l^{-1}), *Prorocentrum lima* (2300 $x10^3$ cells l^{-1}), *Amphidinium carterae* (6000 $x10^3$ cells l^{-1}) and *Chrysochromulina polylepis* (2300 $x10^3$ cells l^{-1}) were tested, the larvae stopped swimming, withdrew into their shells and closed their operculum. Death was therefore difficult to determine. Other prymnesiophyte species tested had no effect on the larvae, which continued to swim for 24 h.

Because of an unforseen difficulty in obtaining paua larvae this bioassay was discontinued.

Microalgal bioassays

Microalgae causing death or morbidity in *Artemia*, and *Alexandrium ostenfeldii,* were tested against *Chattonella antiqua* and *Heterocapsa triquetra* (Table 5.3). *H.triquetra* was killed by *Prymnesium parvum*

Table 5.3 Results of *Artemia salina* bioassays for detection of microalgal toxicity. The time taken (h) for 50% of *A.salina* to die (tD_{50}) or become morbid (tM_{50}) is related to cell numbers of microalgae. *Prymnesium parvum* (F.H.Chang) and *Chrysochromulina polylepis* (Bigelow Laboratory, USA) provided positive controls.

Microalgal species	tM_{50}	tD_{50}	Algal cells ml^{-1} $x10^3$
Prymnesiophytes			
Chrysochromulina polylepis (positive control)	6	8	1.4
C.apheles (Northland, 1994)	NE	NE	2.0
C.camella (Marlborough Sounds, 1992)	NE	NE	2.0
C.ericina (Marlborough Sounds, 1993)	NE	NE	2.0
C.latilepis (Northland, 1994)	NE	NE	2.0
C.quadrikonta (Coromandel, 1993)	NE	NE	2.0
Emiliania huxleyi (Stewart Island, 1992)	NE	NE	4.0
Gephyrocapsa oceanica (Leigh, 1992)	NE	NE	4.0
Prymnesium parvum (positive control)		4	2.0
Raphidophytes			
Fibrocapsa japonica (Northland, 1992)	NE	NE	3.0
Heterosigma akashiwo (Stewart Is, 1989)	8	A	3.6
H.akashiwo (Marlborough Sounds, 1992)	1	A	6.8
H.akashiwo (Leigh, 1992)	1	A	4.0
H.akashiwo (Coromandel, 1992)	1	A	4.0
H.akashiwo (Omaha, 1993)	4	A	1.8
Dinoflagellates			
Alexandrium minutum (Sounds, 1993)	NE	NE	0.5
Alexandrium ostenfeldii (Kaitaia, 1992)	NE	NE	0.5
Alexandrium ostenfeldii (Timaru, 1993)	NE	NE	0.5
Amphidinium carterae (Sounds, 1992)	2	8	0.4
A.carterae (Wellington, 1993)	NE	NE	0.4
Gymnodinium mikimotoi (Southland, 1994)	8	30	0.5
G.sanguineum (Sounds, 1993)*	2	A	0.8
Prorocentrum lima (514)	4	12	0.4
Prorocentrum lima (Spain)		7	0.4

NE: No effect; A: *A.salina* morbid but alive after 24 h. Morbidity refers to *Artemia* that are on bottom of test vessel, but twitching. Quadruplicate bioassays, each with 10 *A.salina*, were carried out with stationary phase cultures (GP medium ; 18°C; 100 μmol m^{-2} s^{-1}; 10:14 h dark/light), except for *G.sanguineum*, which was a seawater sample. Bioassays incubated at 18°C; 100 μmol m^{-2} s^{-1}.

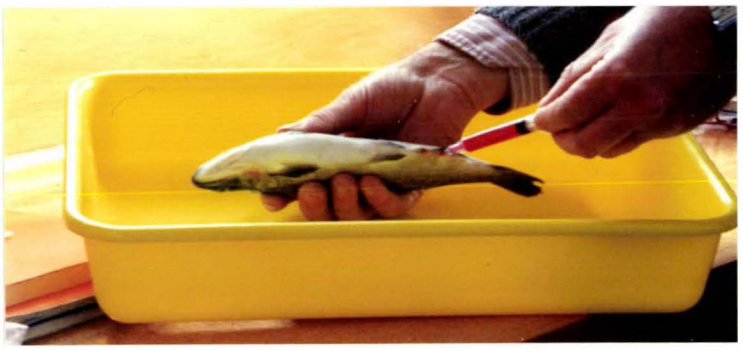
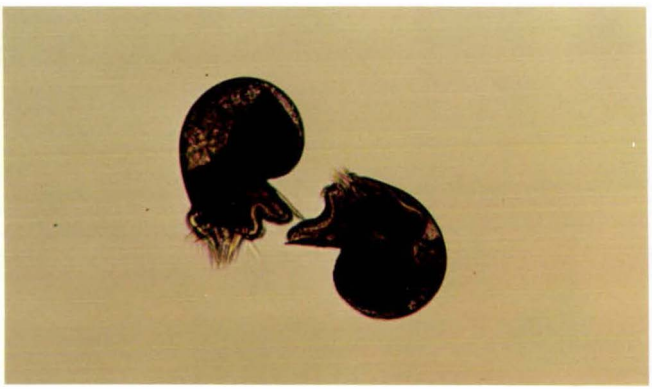
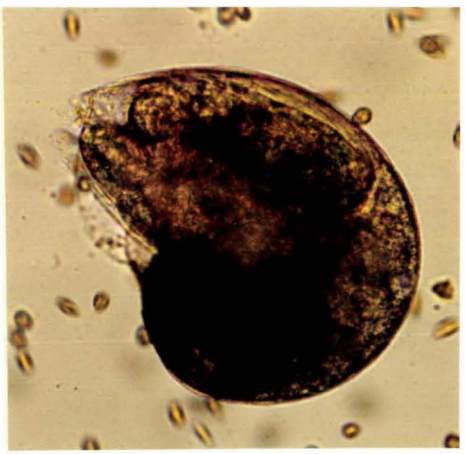


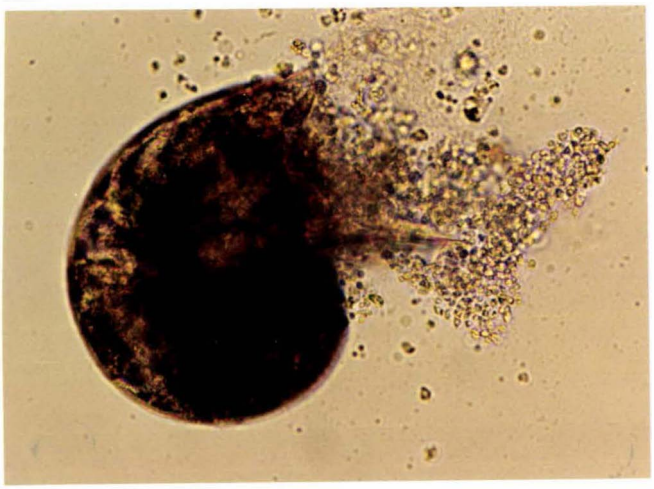
Fig. 5.4 Extraction of blood from anaesthetised salmon (*Oncorhynchus tshawytscha*).



(a)



(b)



(c)

Fig. 5.5 Paua larval bioassays: (a) Healthy paua veliger larva, (b) operculum closing in presence of *Prymnesium parvum* (F.H.Chang) and (c) death of paua larva caused by incubation with *P.parvum.*

Table 5.4 Results of *Haliotis iris* bioassays for detection of microalgal toxicity. The time taken (h) for 50% of *H.iris* to die (tD_{50}) or to close their operculum (tM_{50}) is related to cell numbers of microalgae. *Prymnesium parvum* (F.H.Chang) and *Chrysochromulina polylepis* (Bigelow Laboratory, USA) provided positive controls.

Microalgal species	tM_{50}	tD_{50}	Algal cells ml^{-1} x10^3
Prymnesiophytes			
Chrysochromulina polylepis (positive control)	1	A	2.3
C.camella (Marlborough Sounds, 1992)	NE	NE	2.0
C.quadrikonta (Coromandel, 1993)	NE	NE	2.0
Prymnesium parvum (positive control)		1	3.6
Raphidophytes			
Heterosigma akashiwo (Stewart Is, 1989)	1	A	5.0
H.akashiwo (Marlborough Sounds, 1992)	1	A	5.0
H.akashiwo (Leigh, 1992)	1	A	5.0
H.akashiwo (Coromandel, 1992)	1	A	5.0
Dinoflagellates			
Amphidinium carterae (Sounds, 1992)	8	A	6.0
Prorocentrum lima 514	NE	NE	2.3
Prorocentrum lima Spain	4	A	2.3

NE: No effect; A: *H.iris*: operculum closed but larvae alive at 24 h. Quadruplicate bioassays, each with 10 larvae, were carried out with stationary phase cultures (GP medium; 18°C; 100 μmol $m^{-2}s^{-1}$; 10:14 h dark/light), except for *G.sanguineum*, which was a seawater sample. Bioassays incubated as above.

(strains from F.H.Chang and Denmark; Figure 5.6a,b), *Amphidinium carterae, Prorocentrum lima* and *Heterosigma akashiwo. C.antiqua* was killed (the cells rounded up and then disintegrated) by *P.parvum* (F.H.Chang) and became morbid with the addition of *Alexandrium ostenfeldii* (Table 5.5; Figure 5.6c,d).

Cell free filtrates of the test organisms were also bioassayed for toxicity against both *C.antiqua* and *Heterocapsa triquetra,* but with negative results.

Table 5.5 Evaluation of *Chattonella antiqua* and *Heterocapsa triquetra* as bioassay organisms by testing with known toxic microalgal species. The time taken (h) for 50% of *C.antiqua* or *H.triquetra* to die (tD_{50}) or to become morbid (tM_{50}) is related to microalgal cell numbers.

	C.antiqua		*H.triquetra*		
Toxic microalgae tested	**tM_{50}**	**tD_{50}**	**tM_{50}**	**tD_{50}**	**Microalgal cells ml^{-1} $x10^3$**
Alexandrium ostenfeldii (Timaru)	0.1	A	NE	NE	0.4
Amphidinium carterae (Sounds)	NE	NE		8	1.0
Heterosigma akashiwo (Nelson)	NE	NE		24	0.9
Prorocentrum lima (Spain)	NE	NE	5	10	0.2
Prymnesium parvum (CSIRO)	1	3	5	24	580.0
P. parvum (Denmark)	NE	NE		24	580.0

Bioassays were carried out in quadruplicate with stationary phase test cultures, grown under standard conditions in GP medium. Bioassays were incubated at 18°C; 100 μmol $m^{-2}s^{-1}$ and 10:14 h dark/light. NE: no effect of microalgae on bioassay organisms; A: morbid, no deaths.

Erythrocyte assay

Initial assays of fish erythrocytes demonstrated that the Fish rbc diluent was more suitable for use in the salmon blood assay than Alsever's diluent, as some haemolysis of erythrocytes occurred with the Alsever's diluent in the controls, leading to an unacceptably high background reading on the spectrophotometer. Results of the following experiments are based on assays carried out with the Fish rbc diluent. Haemolytic activity, determined as a percentage of saponin induced haemolysis, was detected in phosphate limited *Prymnesium parvum* (F.H.Chang) culture extracts (45.6%, standard deviation 6.7%). Lower percentages were recorded for *Chrysochromulina polylepis* and *C.quadrikonta* (Figure 5.7). Duplicate extracts of axenic and non-axenic phosphate limited *C.quadrikonta* cultures gave varied results, with differences in means of up to 10.2%. The differences between the axenic and non-axenic

cultures were not significant. In several endeavours to replicate this data, the controls (uninoculated culture medium) gave readings of from 20% up to 79%, so that whilst haemolytic activity of 90 to 100% was indicated for both *P.parvum* and *C.polylepis*, these results were discarded. However observations of the erythrocytes under the microscope showed that the cells treated with the control were intact and cells treated with saponin were completely disintegrated. Ninety percent of cells treated with either *P.parvum* or *C.polylepis* appeared as "ghosts", i.e. with their shape intact, but their contents gone. The balance (≈10%) remained intact (Figure 5.8).

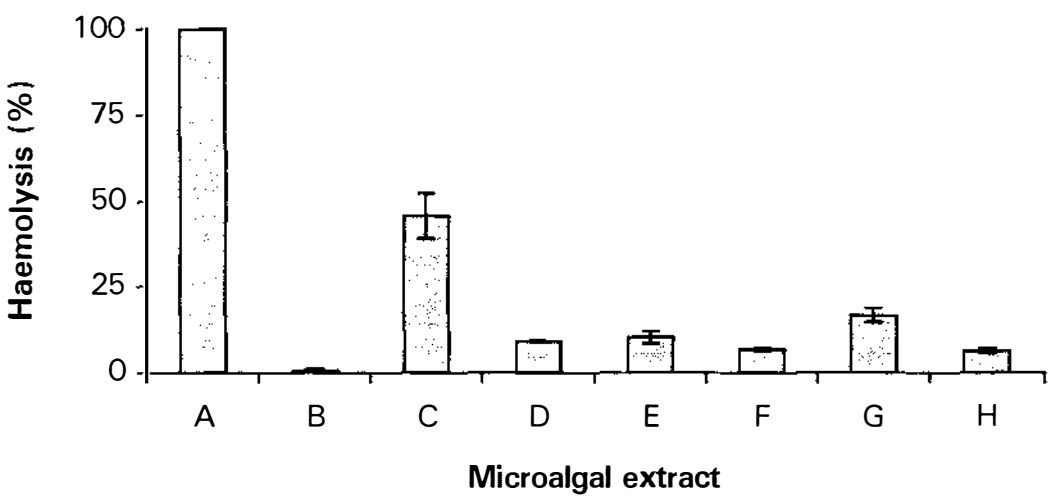


Fig. 5.7 Haemolysis of salmon erythrocytes by microalgal culture extracts. Results are expressed as percentages of saponin induced haemolysis and are means of triplicates (std.dev. indicated). A, saponin; B, ethanol:diluent (1:4); C, *Prymnesium parvum* (-P); D, *Chrysochromulina polylepis* (-P); E-F, *C.quadrikonta* (-P,axenic); G-H, *C.quadrikonta* (-P). Cell numbers at time of extraction were *P.parvum* 4.0 x10^8 cells l^{-1}, *Chrysochromulina* sp. 1.0 x10^8 cells l^{-1}. -P: phosphate limited culture.

Discussion

The *Artemia salina* bioassay (Persoone and Wells, 1987) was found to be preferable to the other assay systems tested, because of its simplicity, ease of replication, the availability of dried cysts and the low cost. The bioassay has previously been used for demonstrating the mitigation of

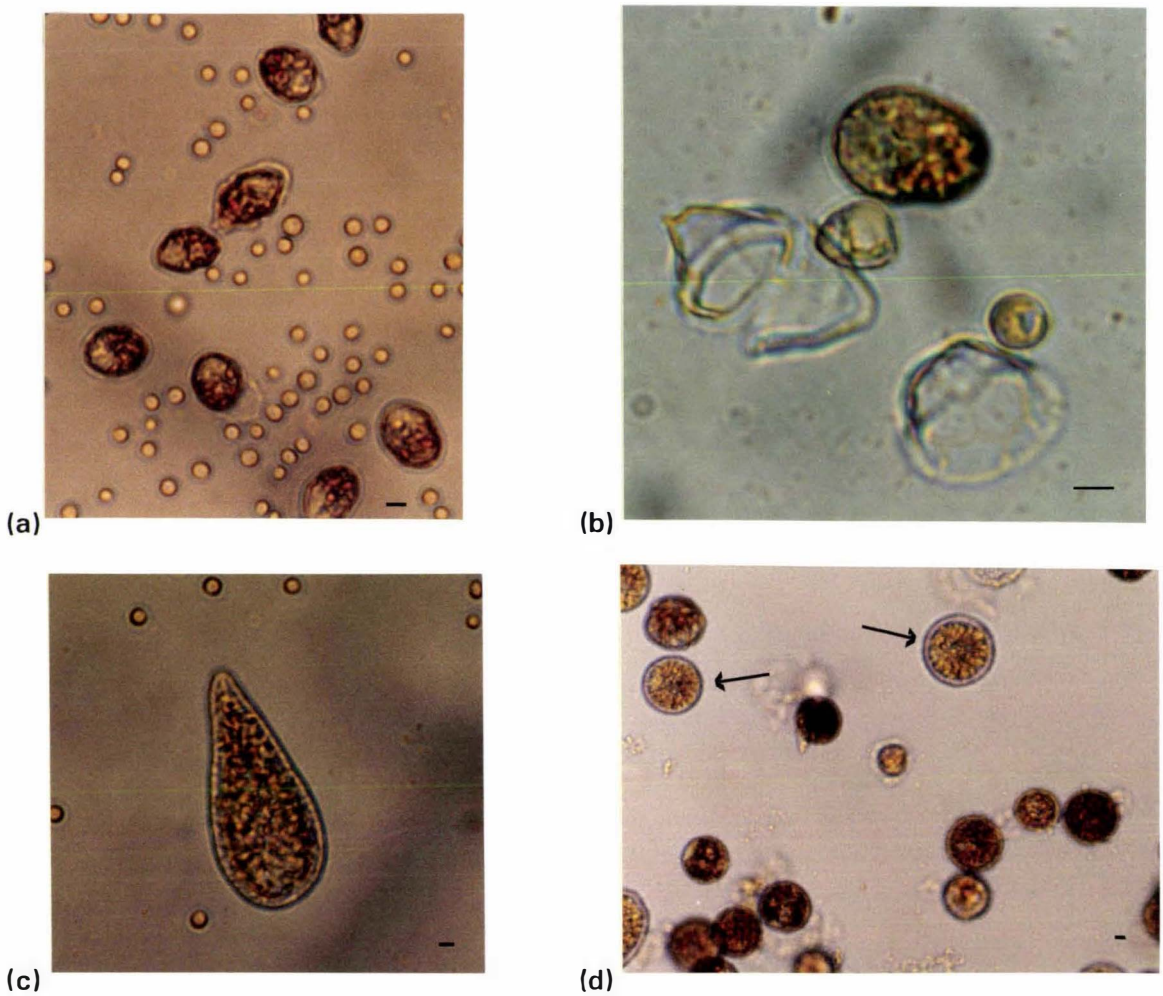


Fig. 5.6 Microalgal bioassays: (a) Healthy cells of *Heterocapsa triquetra*; (b) loss of thecae following incubation of *H.triquetra* with *Prymnesium parvum*; (c) healthy cell of *Chattonella antiqua;* (d) *C.antiqua* (golden-brown cells arrowed) rounding up prior to disintegration following incubation with *Alexandrium ostenfeldii* (dark brown cells). Bar = 5 μm.

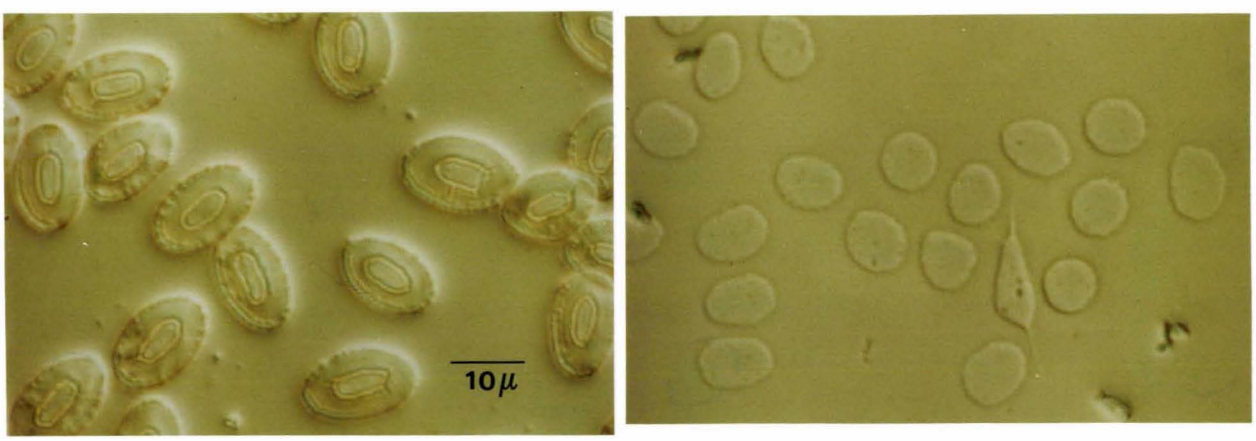


Fig. 5.8 Salmon erythrocytes: (a) prior to and (b) after addition of *Prymnesium parvum* (CSIRO) culture extract.

brevetoxin (produced by the dinoflagellate *Gymnodinium breve*) with the addition of the allelochemical "aponin" (Eng-Wilmot and Martin, 1981). It has also been used for assessing PSP (paralytic shellfish poison) in *Alexandrium tamarense* (Betz and Blogoslawski, 1982).

The characteristic morbidity elicited in *Artemia salina* by toxic raphidophytes distinguishes the response to this class of microalgae from the uncompromising death response elicited by prymnesin, amphidesmin (produced by *Amphidinium carterae*) (Ikawa and Sasner, 1975) and okadaic acid (produced by *Prorocentrum lima*) (Shimizu, 1987).

Unfortunately there are no such characteristic responses to help distinguish between the latter groups.

However two New Zealand strains of the dinoflagellate *Alexandrium ostenfeldii* (from Timaru and Kaitaia), each with a different saxitoxin profile (Lincoln MacKenzie, Cawthron Institute, Nelson, New Zealand , personal communication), and a toxic strain of *A.minutum* (from Croisilles Harbour), were ineffective against *Artemia salina.*

The *Artemia* bioassay has recently become a standard tool for detecting toxic activity in prymnesiophytes in Scandinavia (Edvardsen and Paasche, 1992), replacing cod and human erythrocyte assays (Meldahl *et al.*, 1993). The time required to carry out the bioassay, which could be a drawback, can be reduced if a source of hatched nauplii are maintained.

Paua larvae were investigated because of a potential supply and proved ideal for detecting the excreted toxin, prymnesin, which was effective even when the microalgal cells were excluded. The assay was not suitable as a general toxicity screening method; endotoxic microalgae tested such as *Prorocentrum lima* (strain 514) were excluded by the shellfish larvae and therefore did not elicit a response.

Microalgae have advantages as bioassay organisms. Stationary phase cultures are as susceptible to ichthyotoxins as are exponential phase cultures, so no lag period prior to readiness of the bioassay organism is required, and toxic effects can be rapid. *Heterocapsa triquetra* did not give as clear a response as *Chattonella antiqua*, although it has been used

as a rapid check for prymnesiophyte toxicity in Scandinavia (Moestrup and Arlstad, 1993). Death of *H.triquetra* often includes the loss of the theca (cellulosic coat), which is easily detected, but many cells die and settle on the bottom of the wells with no obvious change in morphology. If test wells are treated with Lugol's iodine and cells then counted to obtain quantitative data, discrimination between cells that were live or dead prior to fixation is difficult. Only where there is an appreciable difference in cell numbers between test and control is this useful, as is caused by the DSP (diarrhetic shellfish poison) producer, *Prorocentrum lima.*

The *C.antiqua* bioassay gave clearer results for the dinoflagellate *Alexandrium ostenfeldii,* but only for the most toxic of the prymnesiophytes. *C.antiqua* was unaffected by *P.lima.* Therefore for general screening of toxic microalgae, such as testing of bloom samples, the *Artemia* bioassay is at present the most comprehensive. For screening for highly toxic prymnesiophytes, the *Chattonella* bioassay is a quick, sensitive and quantitative tool. *C.antiqua* and *H.triquetra* assays could be run in tandem to distinguish between DSP and PSP producers, although confirmation by mouse bioassay or HPLC would still be required.

None of the above assays are specific for prymnesin. A more selective assay is the erythrocyte assay, which quantifies the haemolytic activity of hydrophobic culture extracts. This test fails to assess other potential effects, such as neurotoxicity. It also has such inherent problems as the difficulty of maintaining ready supplies of erythrocytes (although human erythrocytes can be used) (A-S Meldahl, Norwegian Defence Research Establishment, Norway, personal communication) and the susceptibility of erythrocytes to solvents. There is also the problem of well documented non-toxic species causing haemolysis at high concentrations and/or long incubation times (B.Edvardsen, University of Oslo, Norway, personal communication).

A recent phenomenon overseas is the apparent acquisition of toxic activity by benign species *in vivo,* for example, the *Chrysochromulina* blooms in Scandinavia since 1988 (Manton and Parke, 1962; Skjoldal and

Dundas, 1989; Tangen, 1991); New Zealand aquaculturalists will need to be on the alert for similar trends.

Research is being carried out into the development of genetic probes and immunochemical assays for these microalgae. Such methods will eventually give rapid and specific identification, and will, hopefully, differentiate between toxic and non-toxic strains. Currently, however, the microalgal and brine shrimp assays are a standard procedure, and have been the means of detection of shellfish toxins and ichthyotoxins in bloom samples.

CONCLUSION

The prymnesiophytes are a constant and, on occasion, major component of New Zealand's marine microflora. It is probable that the vast blooms formed by *Emiliania huxleyi* and *Gephyrocapsa oceanica* (Coccolithophorales) observed in New Zealand's coastal waters in recent years are linked to such seasonal environmental forcing factors as sea water temperatures, nutrient upwelling and light intensity. It is also possible that these blooms have a role in determining species succession by conditioning the sea water with their secretions. Most of this study has been carried out during an unusually protracted El-Niño phase of the Southern Oscillation Index and it will be interesting to determine the changes in species dominance and succession when sea temperatures finally rise.

The prymnesiophytes appear to be a ubiquitous group: *Chrysochromulina acantha, C.hirta*, *C.pringsheimii* and *C.quadrikonta* (Prymnesiales) are new records for New Zealand, but all are now known from widely separated geographical sites. It is probably only time before species currently recorded in other temperate and sub-tropical regions are found in the southern Pacific. The New Zealand strain of the novel quadriflagellate species *C.quadrikonta* (isolated from Nelson Harbour in 1991) has been described and a bloom of this species recorded in the Hauraki Gulf in 1994.

Some differences in morphology have been noted between northern and southern hemisphere prymnesiophyte isolates; the southern hemisphere coccolithophores have heavier calcification than their northern counterparts and several of the *Chrysochromulina* species have slightly larger cell sizes and longer and more robust unmineralised spine scales than their type species. It is also clear that electron microscopy alone is

inadequate for identification of prymnesiophyte species and it is probable that in some instances spine scales that were previously identified as belonging to *C.ericina* were in fact from *C.quadrikonta*; live cells are desirable for definitive identifications.

There is debate as to whether the genera *Chrysochromulina* and *Prymnesium* should remain separate or be integrated, but studies in which fluorescently-tagged lectins showed differential binding for the two genera support the current separation.

The *Chrysochromulina* species investigated in this study have had their growth optima characterised and they fell into two groups on the basis of temperature preference; a temperate and a sub-tropical group. Most species preferred slightly brackish conditions, similar to coastal waters (only *C.camella* preferred sub-tropical oceanic conditions) and most grew at low light intensities, which could give this genus a competitive advantage over other microalgal species.

Toxic prymnesiophytes have become a major problem to aquaculture in Scandinavian waters, particularly with the occurrence of toxicity in previously benign species. New Zealand has the potential for similar problems with its burgeoning aquaculture industry. Known ichthyotoxin producers in this region include *Prymnesium calathiferum* and *C.quadrikonta* (although toxicity in the latter has only been detected by erythrocyte bioassay and then at very low levels in stationary phase cultures). Toxicity bioassays have been developed to detect the presence of prymnesin in bloom samples and in *in vitro* cultures. The use of *Artemia salina* is useful as a general screening method for ichthyotoxicity, while selected microalgae and shellfish larvae can be used to discriminate between different toxins, including the exotoxin prymnesin.

One of the outcomes of this study has been the realisation of the dearth of knowledge about other nanoflagellate groups in New Zealand coastal waters; the application of fluorescent probe techniques to these groups, which like the prymnesiophytes are difficult to identify under the light microscope, will prove invaluable.

The increased knowledge of the New Zealand prymnesiophytes gained in this study will continue to be built on, with long term monitoring of sites around the New Zealand coastline in place and the testing for ichthyotoxicity in new species now a standard procedure.

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