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Diversity, ecology and domoic acid production of *Pseudo-nitzschia* spp. in Scottish waters

Johanna Fehling

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To my parents

ABSTRACT

Some diatoms of the genus *Pseudo-nitzschia* produce the toxin domoic acid (DA). Accumulation of DA in shellfish has led to harvesting closures in western Scottish waters since 1999. This thesis investigated the diversity, ecology and distribution of toxic and non-toxic *Pseudo-nitzschia* species in western Scottish waters and physiological aspects of growth and toxin production dynamics of *P. seriata*. The temporal and spatial distribution of phytoplankton was analysed in two separate field studies. 1) Temporal changes were followed by sampling a site in coastal Scottish waters weekly to fortnightly over a period of three years. 2) The spatial distribution of the phytoplankton community was investigated by sampling a transect across-the-shelf. Within both studies, physical, biological and chemical parameters were measured and correlated to temporal and spatial distribution patterns in the phytoplankton community, indicating seasonality, and differences in the distribution of toxic and non-toxic *Pseudo-nitzschia* species between coastal and offshore waters. From those samplings 59 clonal cultures of *Pseudo-nitzschia*, comprising 7 species (2 of them toxic), were established. Strains were identified via classic morphological and genetic techniques. Phylogenetic relationships were established between Scottish *Pseudo-nitzschia* strains. *P. seriata* was identified for the first time in Scottish waters as a DA producer. Laboratory experiments with cultured strains showed a) enhanced toxin production by *P. seriata* under silicate (Si) and phosphate (P) limitation, with higher DA production under Si than under P limitation b) similar cell yields of *P. seriata*, when grown in nitrate or ammonia based media c) a preference for spring light conditions (short day length) in a non-toxic *P. delicatissima* strain and summer light conditions (long day length) for a toxic *P. seriata* strain, expressed by enhanced biomass yield under the respective light condition. It was also shown that the presence of bacteria enhanced the growth of single *P. seriata* cells.

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1 Chapter1: General introduction

1.1 Phytoplankton

Marine phytoplankton are drifting, photosynthetic, microscopic, commonly single-celled algae that thrive in the photic zones of the oceans. Cell sizes of phytoplankton are found in a range of 2 μm to 2 mm, with most of the cells reaching 10-50 μm (cf. Horner 2002). It is conservatively estimated that there are about 500 genera and 4000 species of phytoplankton (Sournia et al. 1991). Some of the main classes in phytoplankton are the Bacillariophyceae (diatoms), Dinophyceae (dinoflagellates), Raphidophyceae (raphidophytes), Prymnesiophyceae (prymnesiophytes, haptophytes) Dictyochophyceae (silicoflagellates) and Cyanophyceae (cyanobacteria). As the primary producers of the marine food web they are the basis of all animal production in the sea. They play an essential role in the global biogeochemical cycle by producing about a quarter of the world's oxygen, by their utilisation of carbon in photosynthesis and their production of volatile compounds (e.g. dimethyl sulfide) (cf. Horner 2002).

Light and inorganic nutrients are required for phytoplankton growth. Diatoms for example, build their cell walls from dissolved silicon (DSi) taking it up in the form of orthosilicic acid or "silicate" (Si). The total concentration of silicate present in a water mass may determine the upper limit of the size of a diatom bloom (Paasche 1973), although nitrogen (N) limitation is also possible. The yield of non-diatom phytoplankton depends more on the concentrations of N and phosphorus (P). Furthermore, decreasing Si:N or Si:P ratios could increase the remaining of N and P to non-diatom- phytoplankton (e.g. flagellates) and could favour a higher biomass of these organisms (Sommer 1994). Increasing nutrients can lead to increased biomass and eutrophication (e.g. Parsons et al. 1984). Hence changes in nutrient concentration may influence both species abundance and species succession.

Marine phytoplankton are a primary food source for filter-feeding bivalve shellfish as well as larvae of commercially important crustaceans, hence proliferation of planktonic algae is, in most cases, beneficial for the aquaculture. However, when forming a harmful algal

bloom (HAB), toxic and non-toxic microalgae may then have a negative effect on the environment, economy and human health (Daranas et al. 2001).

1.2 Harmful algal blooms

Visible blooms of microalgae and their consequences have long been recognised and feared by humans, even receiving mention in the bible as for example the first plague visited on Pharaoh before the exodus of the Israelites from Egypt, ca. 1290 BC (cited by Morris 1999):

"...and all the water that was in the Nile was turned to blood. And the fish in the Nile died; and the Nile became foul, so that the Egyptians could not drink water from the Nile; and there was blood throughout all of the land of Egypt! "(Exodus 7:20-21).

This is probably the first record of a red tide or form of HAB. While this event might have led to fish kills in the fresh water river Nile, there is another indication in the new testament of the bible of an event leading to mortality of animals in the sea (Revelation 16: 3): "...the second angel poured out his bowl on the sea and it turned into blood, like that of a corpse, and every living creature that was in the sea died."

The term "harmful algal bloom" is used to describe the often visible blooms of algae that can kill fish, make shellfish poisonous and cause numerous other effects on wildlife, humans and economy (Hoagland et al. 2002). If toxins produced by harmful algae are potent enough or accumulate over time in the food web, small numbers of organisms, that may not be concentrated enough to discolour the water, may be sufficient to cause ecosystem damage (Smayda 1997). Recently, observations of HABs in aquatic systems have increased on a global scale (e.g. Anderson 1989; Smayda 1990; Hallegraeff 1993; Anderson et al. 2002) affecting species interactions, aquatic animal health and population growth, ecology, human health, ecosystem integrity and major industries and economies (see Landsberg 2002 for review). The global expansion of HABs has been linked to eutrophication as a result of nutrient overloading from sewage, atmospheric deposition, groundwater flow, as well as agricultural and aquaculture runoff and discharge (Anderson et al. 2002).

HABs can be caused by a variety of cyanobacteria and microalgae, most of which are planktonic. However, the term of HAB can also include blooms of macroalgae (Hoagland

et al. 2002). HABs can cause mortality or physiological impairment of other organisms by two general mechanisms: 1. non-chemical and 2. chemical attributable to physical-chemical reactions, phycotoxins or other metabolites (Smayda 1997). Non-chemical mechanisms can be implemented through starvation, mechanical irritation, physical impairment (extra-cellular polymer excretions that lead to a viscosity or gelatinous barrier), or ambush feeding. Chemical effects can be caused through anoxia, NH_4 toxicity (e.g. blooms of the dinoflagellate *Noctiluca*) or, as in shellfish poisoning events, phycotoxins (Smayda 1997). Mortality through phycotoxins can occur through direct ingestion of the toxic species, upon exposure to secreted toxins, or from transfer by vectors through the food chain (Smayda 1997). In marine systems, about 90 species of microalgae are, to date, known to be harmful by producing phycotoxins, of which 70% are dinoflagellates, 13% diatoms, 9% haptophytes and 8% raphidophytes (IOC taxonomic reference list 2002).

1.3 Shellfish poisoning

Probably since the beginning of civilisation, the consumption of shellfish has been known to have sometimes resulted in sickness or even death in humans. Recognition of the existence of shellfish toxicity then resulted in new folklore and religious customs which preached abstinence from shellfish consumption at particular times of the year (Wright 1995). Today it is known that HABs associated with phycotoxins causing shellfish poisoning are mainly caused by dinoflagellates (so far 62 species are stated as harmful, IOC 2002). Five types of shellfish poisoning are known to occur world-wide due to HABs: paralytic, diarrhetic, neurotoxic, azaspiracid and amnesic shellfish poisoning (PSP, DSP, NSP, AZP and ASP respectively). PSP, DSP NSP and AZP are caused by dinoflagellates belonging to the genera *Alexandrium* (PSP), *Gymnodinium* (PSP) *Karenia* (NSP), *Pyrodinium* (PSP), *Dinophysis* and *Prorocentrum* (DSP) and the species *Peridinium crassipes* (AZP) (e.g. Hallegraeff 2003; James et al. 2003).

Amnesic shellfish poisoning is caused by diatoms of the genus *Pseudo-nitzschia* Peragallo. Some diatom species of this genus are able to produce domoic acid (DA), a potent neurotoxin (Bates 2000).

1.4 Domoic acid producing *Pseudo-nitzschia* spp

The genus *Pseudo-nitzschia* Peragallo comprises about 30 species (including species variants), of which today at least ten are confirmed to produce DA in the field or culture (Bates 2000): *P. australis* Frenguelli, *P. calliantha* Lundholm, Moestrup et Hasle sp. nov. (Lundholm et al. 2003), *P. delicatissima* (Cleve) Heiden, *P. fraudulenta* (Cleve) Hasle, *P. multiseriata* (Hasle) Hasle, *P. multistriata* (Takano) Takano, either *P. pseudodelicatissima* (Hasle) Hasle emend. Lundholm, Hasle et Moestrup or *P. cuspidata* (Hasle) Hasle emend. Lundholm, Moestrup et Hasle, *P. pungens* (Grunow ex Cleve) Hasle, *P. seriata* (Cleve) H. Peragallo, and *P. turgidula* (Hustedt) Hasle. Strains of the same species can vary in their toxin production (Villac et al. 1993a, b; Villareal et al. 1994; Rhodes et al. 1998; Smith et al. 2001). Within species some strains do not seem to produce DA, while others do (e.g. Lundholm et al. 1994; Bates et al. 1989 in *P. seriata*, Villac et al. 1993a in *P. australis*; Villareal et al. 1994 in *P. multiseriata*; Rhodes et al. 2000 in *P. multistriata*; Rhodes et al. 1996, Trainer et al. 1998a in *P. pungens*; Rhodes et al. 1998 in *P. fraudulenta*).

1.4.1 Distribution of some of the main *Pseudo-nitzschia* species

The distribution of the main toxic and non-toxic *Pseudo-nitzschia* species was reviewed by Hasle (2002) (see Figures 1.1 and 1.2). Most of the *Pseudo-nitzschia* species seem to be cosmopolitan, apart from *P. seriata* (northern hemisphere) and *P. turgidula* (mainly southern hemisphere, but with some reports from the northern hemisphere). The distribution of individual and potential toxic species is discussed in detail below.

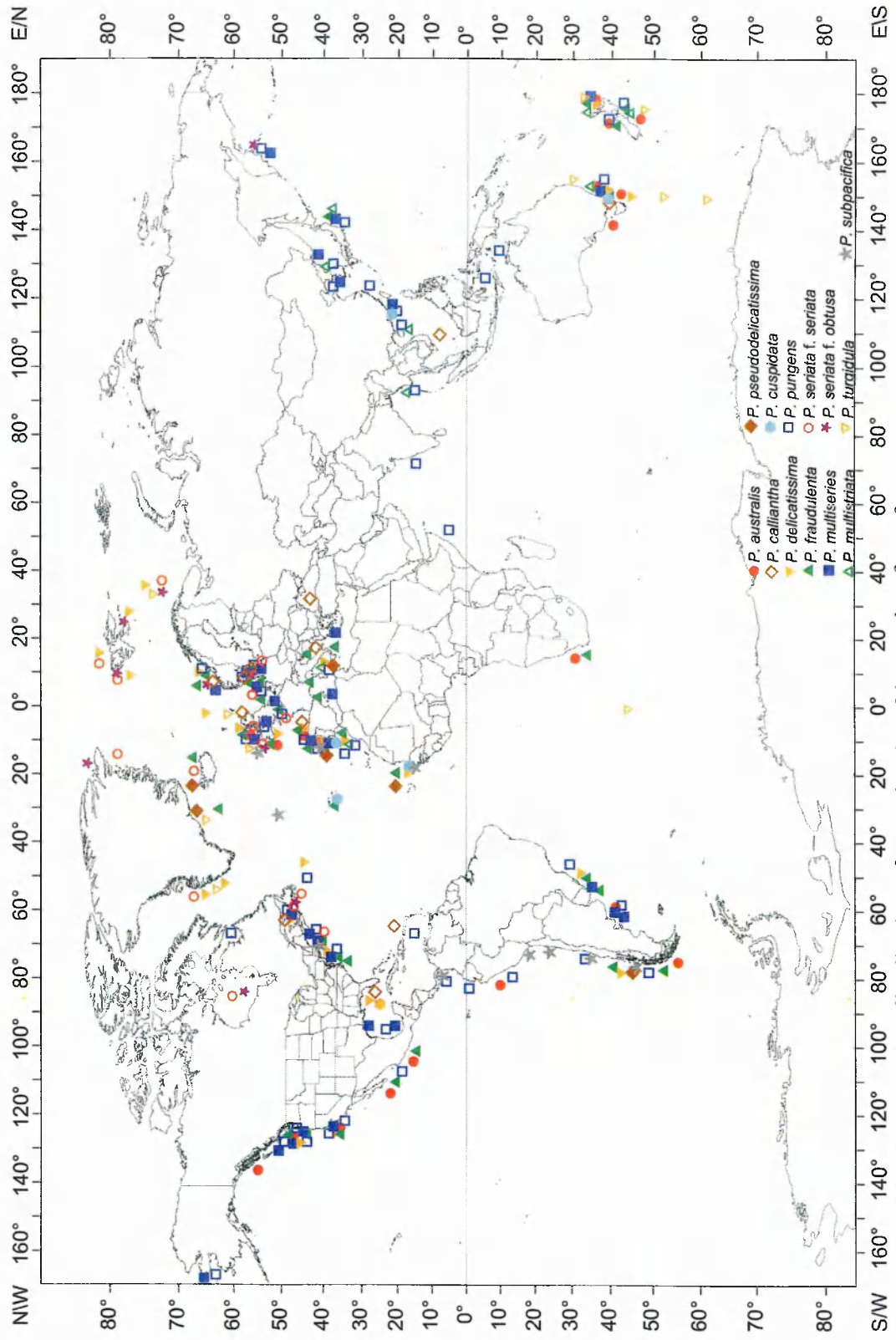


Fig. 1.1 World-wide recorded distribution of *Pseudo-nitzschia* species, for references see text.

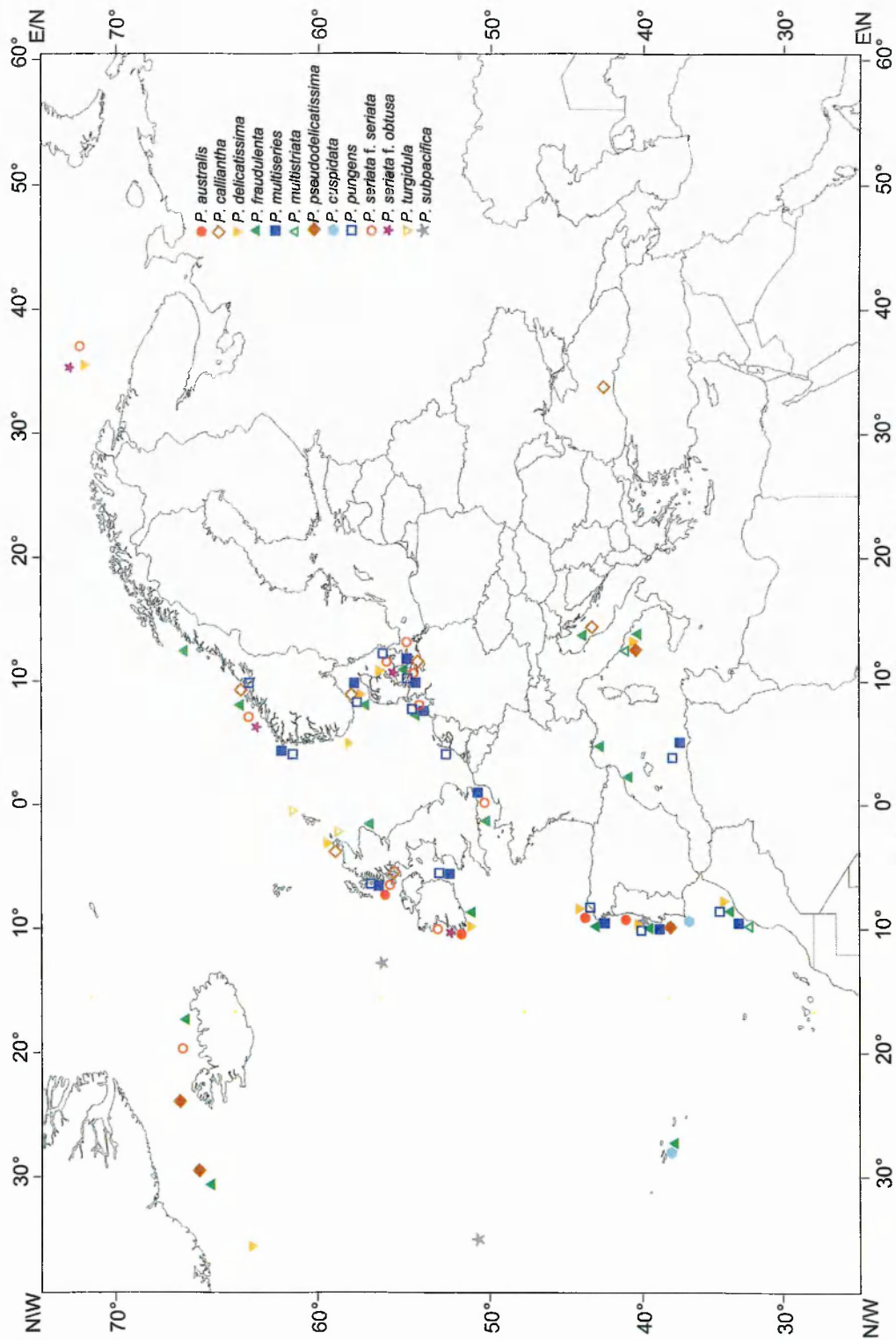


Fig. 1.2 European distribution of *Pseudo-nitzschia* species, for references see text.

1.4.1.1 *P. australis* Frenguelli

P. australis was initially thought to be restricted to the southern hemisphere (e.g. in Hasle 1965 as *Nitzschia pseudoseriata*), but it seems also to be abundant in the Atlantic from western Scottish waters (Campbell et al. 2001; Gallacher et al. 2001; the present work) to Argentinean waters (Ferrario et al. 1999), with reports from Irish waters (Cusack et al. 2000), north-west Spain (Miguez et al. 1996; Fraga et al. 1998), Portuguese coastal waters (Moita & Villarinho 1999) and the west coast of South Africa (Hasle 1972). In the Pacific, *P. australis* is known to occur along the west coasts of the North and South American continents, including the Gulf of Alaska (Hasle 2002), western Washington (Horner & Postel 1993) and Oregon waters (Fryxell et al. 1997), the Californian (e.g. Buck et al. 1992; Villac et al. 1993a, b; Scholin et al. 2000), Mexican (Hernández-Becerril 1998), Peruvian (Hasle 1965) and Chilean (Rivera 1985) coasts. It has also been found in Australian (Lapworth et al. 2001) and New Zealand waters (Hasle 1965; Hasle 1972; Rhodes et al. 1998).

1.4.1.2 *P. calliantha* Lundholm, Moestrup et Hasle

P. calliantha was recently erected as a new species (Lundholm et al. 2003). It had previously been recorded as toxic *P. pseudodelicatissima* in the Bay of Fundy, Canada (Martin et al. 1990, 1993) and in Danish waters (Lundholm et al. 1997; Skov et al. 1997). Some *P. pseudodelicatissima* reports were re-investigated from the literature and by direct observation, and subsequently delineated as *P. calliantha* (Lundholm et al. 2003). The type material of *P. calliantha* Lundholm, Moestrup et Hasle sp. nov. was collected in Danish waters (Lundholm et al. 2003), but the species appears to be cosmopolitan, as it has also been found in the Skagerrak, the Trondheimsfjord (Norway), some Scottish locations such as the Orkney Island (the present work) and west Loch Tarbert. Other European waters where *P. calliantha* is found are the Baltic Sea (Hasle et al. 1996), Spanish waters, the Black and Adriatic Seas (Lundholm et al. 2003). Furthermore it is known from Canadian waters (Martin et al. 1990), the Gulf of Mexico,

off Bermuda, along the Chilean coast, and in Vietnamese waters and near Sydney, Australia (Lundholm et al. 2003).

1.4.1.3 *P. delicatissima* (Cleve) Heiden

P. delicatissima has been observed in the Atlantic from 80°N, near Svalbard (Quillfeldt von 1996), to Brazil at ca. 32°S (Villac & Tennebaum 2001), including Norwegian coastal waters (Hasle 1965, Hasle et al. 1996), Danish (Skov et al. 1999), Scottish (Gallacher et al. 2001; personal observations) and Irish waters (Cusack et al. 2000). It is known from the Skagerrak (Hasle et al. 1996), north-western Spanish (Fraga et al. 1998) and Portuguese waters (Moita and Vilarinho 1999), the Gulf of Naples (Sarno & Dahlman 2000), off north-west Africa (Hasle 1965), along the Atlantic Moroccan coast (Akallal et al. 2002), and the west coast of Greenland (Quillfeldt von 1996). It has been observed in the Gulf of St. Lawrence, Canada (Couture et al. 2001), the Bay of Fundy, Canada (Kaczmarek et al. 2004), the Gulf stream (Kaczmarek et al. 1986) and the Gulf of Mexico (Parsons et al. 1999). In the Pacific it is distributed from Monterey Bay, California (Villac et al. 1993a) to Chilean waters (Rivera 1985) and has been found in Australian (Lapworth et al. 2001) and New Zealand waters (Rhodes et al. 1998).

1.4.1.4 *P. fraudulenta* (Cleve) Hasle

P. fraudulenta usually appears to be nontoxic, apart from one strain from New Zealand in which low amounts of DA were detected by immunoassays (Rhodes et al. 1998). Its distribution ranges from the Norwegian coast (Hasle et al. 1996) to Argentinean waters (Lange 1985). It has been found in the Norwegian Sea and the Denmark Strait (Hasle 1965), Icelandic and Norwegian waters, the Skagerrak (Hasle et al. 1996), Danish (Lundholm et al. 1994), Scottish (Gallacher et al. 2001; personal observations) and Irish waters (Cusack et al. 2000). Furthermore, it was found in the North Sea, the English Channel (Hasle 2002), along the northern Spanish (Miguez et al. 1996) and Portuguese (Moita & Vilarinho 1999) coasts, in the Mediterranean Sea (Hasle 1972), around the Azores (Cleve 1902), in Atlantic Moroccan waters (Akallal et al. 2002) and the west

coasts of north and south Africa (Hasle 1965). From the eastern coast of the North America it is known from the Bay of Fundy, Canada (Kaczmarska et al. 2004), Rhode Island, USA (Hargraves et al. 1993), Maryland, USA (Hasle 1965), and North Carolina, USA (Hustedt 1955). From the southern American coast it has been reported from Brazil (Odebrecht 2001). In the Pacific *P. fraudulentus* was found along the west coast of the north and south American continents, from the Washington coast (Hasle 1972) to Chile (Rivera 1985), including Oregon and Californian waters (Villac et al. 1993a) and the Pacific coast of Mexico (Hernández-Becerril 1998). There are reports from Japanese (referenced in Hasle 2002), Australian (Hallegraeff 1994) and New Zealand (Hasle 1965; Rhodes 1998) waters.

1.4.1.5 *P. multiseriatus* (Hasle) Hasle

Similar to *P. fraudulentus*, *P. multiseriatus* has been observed in the Atlantic from Norwegian waters (Hasle 2002) to Argentina (Ferrario et al. 1999). It has been reported from Danish waters (Skov et al. 1999), the Skagerrak, Baltic and North Sea, the English Channel (Hasle et al. 1996), western Scottish (Gallacher et al. 2001) and Irish waters (Hasle et al. 1996). Further observations were made along the North coast of Africa (Skov et al. 1999) and in Moroccan waters (Akallal et al. 2002). In the western Atlantic, *P. multiseriatus* has been found in the Gulf of St. Lawrence, Canada (Bates et al. 1989) the Bay of Fundy, Canada (Kaczmarska et al. 2004), along the coasts of Rhode Island (Hargraves et al. 1993) and Maryland (Hasle 1965). It is known from the Gulf of Mexico (Fryxell et al. 1990), the Uruguayan coasts (Hasle 1965) and southern Brazil (Odebrecht et al. 2001). In the Pacific it has been found from the west coast of the Bering Sea (Orlova et al. 2000) to New Zealand (Rhodes 1998), including the coasts of British Columbia (Forbes & Denman 1991), Washington (Horner & Postel 1993), California (Villac et al. 1993a, b), Kamchatka (Orlova et al. 2000), the Sea of Japan (Orlova et al. 2000), Korean coastal waters (Fryxell et al. 1990), Japanese (referenced in Hasle 2002), and Hong Kong waters. Observations of *P. multiseriatus* were made in Chinese waters (Dickman & Glenwright 1997), as well as along the eastern Australian coast (Lapworth et al. 2001).

1.4.1.6 *P. multistriata*(Takano) Takano

P. multistriata has so far only been observed in the Gulf of Naples (Mediterranean Sea) (Orsini et al. 2002), along the Moroccan Atlantic coast (Akallal et al. 2002) and in the Pacific from Japanese (referenced in Hasle 2002) to New Zealand (Rhodes et al. 2000) waters including the Chinese Sea, parts of the Thailand coast and Australian waters (Hasle 2002).

1.4.1.7 *P. pseudodelicatissima* (Hasle) Hasle, *P. cuspidata* (Hasle) Hasle *emend.* Lundholm, Moestrup et Hasle

P. pseudodelicatissima has recently been re-examined (= *P. pseudodelicatissima* (Hasle) Hasle *emend.* Lundholm, Hasle et Moestrup) by Lundholm et al. (2003). The original type form of *P. pseudodelicatissima* (then called *Nitzschia delicatula* Hasle) was known from scattered localities in the North Atlantic (17° to 67°N), Canadian and Chilean waters (Hasle 1965). Further records of those strains identified as *P. pseudodelicatissima*, but that might have been other species of the *P. pseudodelicatissima*/*P. cuspidata* complex (e.g. *P. cuspidata*, *P. calliantha* or *P. caciantha*; Lundholm et al. 2003) are referenced in Hasle (2002). Species representing the re-examined *P. pseudodelicatissima* were found in the Denmark Strait, near Iceland, the Portuguese coast and off Napoli (Italy) (Lundholm et al. 2003).

P. cuspidata, which previously might have been mistaken for *P. pseudodelicatissima*, has been observed near the Canary Islands, south of Portugal, off the coast of North Africa, in Hong Kong, Sydney and the Gulf of Mexico (Lundholm et al. 2003). Either *P. cuspidata* or the re-examined *P. pseudodelicatissima* has produced domoic acid in a study by Pan et al. (2001), but the identity of the culture was not clear.

1.4.1.8 *P. pungens* (Grunow ex Cleve) Hasle

P. pungens has been found in the Atlantic from the Norwegian Trondheimsfjord (Hasle et al. 1996) to Argentinean waters (Ferrario et al. 1999). Records include Danish (Lundholm et al. 1994) waters, the Skagerrak (Hasle et al. 1996), western Scottish

waters (Gallacher et al. 2001; personal observations) and the Irish, the North and Baltic Seas. It has been found in the English Channel, north-west Spain and Portuguese coastal waters, the Mediterranean (Hasle 1972, 1995; Moita & Vilarinho 1999), Atlantic Moroccan waters (Akallal et al. 2002) and along the west coast of Africa (Hasle 1965). Records for Canadian waters include the Hudson Strait (Hasle 1972) the Bay of Fundy, Canada (Kaczmarska et al. 2004) and the Gulf of St. Lawrence (Bérard-Therriault et al. 1999). In the northern United States waters it was found along the coasts of Rhode Island and Maryland (Hasle 1972). It has been observed in the Gulf stream (Kaczmarska et al. 1986), Gulf of Mexico (Hasle 1972, Fryxell et al. 1990), the Caribbean Sea (Hasle 2002) and along the coast of Brazil (Odebrecht et al. 2001; Villac & Tennebaum 2001). In the Pacific records of *P. pungens* include waters from the Bering Sea (Orlova et al. 2000) to Chile (Rivera 1985), with finds in British Columbia (Forbes & Denman 1991), Washington (Hasle 1972; Horner & Postel 1993; Villac et al. 1993a; Stehr et al. 2002), Oregon (Fryxell et al. 1997) and Californian waters (Villac et al. 1993a; Lange et al. 1994). Further observations were made along the Pacific coast of Mexico (Hernández-Becerril 1998), the Gulf of Panama (Hasle 1965), off Ecuador (Hasle 2002), Peru and Chile (Hasle 1965; Rivera 1985), off Kamchatka (Orlova et al. 2000), the Sea of Japan (Orlova et al. 1998), Korean (Fryxell et al. 1990; Cho et al. 2001) and Chinese coastal waters, the Chinese Sea (in Hasle 2002) and Hong Kong (Dickman & Glenwright 1997). *P. pungens* was also found along the African and Indian coasts of the Indian Ocean (Hasle 1972), the Gulf of Thailand (Hasle 1965) and Indonesian waters (Sidabutar et al. 2000). It is known to occur in Australian waters (Hallegraeff 1994), as well as waters around New Zealand (Rhodes et al. 1998). Only *P. pungens* from New Zealand produced low amounts of DA in culture so far, DA toxicity has not been observed in *P. pungens* from other waters (Rhodes et al. 1996).

1.4.1.9 *P. seriata* (Cleve) H. Peragallo

Pseudo-nitzschia seriata, including the subspecies f. *seriata* and f. *obtusa*, has so far only been reported from the northern hemisphere, from the northern Atlantic (north of Svalbard) (Quillfeldt von 1996) to the Grand Banks south of Newfoundland (Hasle

1972), including the Barents Sea, west and north of Svalbard (Quillfeldt von 1996), Norwegian coastal waters (Hasle et al. 1996), Icelandic waters (Hasle 2002), the Norwegian Sea (Hasle 1965), Danish (Lundholm et al. 1994), Scottish (Gallacher et al. 2001; personal observations) and Irish (Cusack et al. 2000) waters. It has been observed in the North and Baltic Seas (Hasle 1972), the English Channel, and the west coast of Greenland. It was found in the Canadian Arctic (Quillfeldt von 1996), Hudson Strait (Hasle 1965), the Gulf of St. Lawrence (Couture et al. 2001), and the western Atlantic including parts of the east coast of the USA (Hasle 2002). Some scarce records from the northern Pacific (Hasle 2002) do exist for *P. seriata* f. *obtusa*, but not for *P. seriata* f. *seriata*. However, *P. seriata* f. *obtusa* is been found at most locations where *P. seriata* f. *seriata* is observed (Hasle 2002).

1.4.1.10 *P. subpacifica* (Hasle) Hasle

P. subpacifica is not regarded as a toxin producing species, but as it was observed within this study (as *P. cf. subpacifica*) in North Atlantic waters, some of its previous sparse records are stated. It was first described as *Nitzschia subpacifica* by Hasle (Hasle 1965), found in the Chesapeake Bay (USA), north-west Africa, the Gulf of Panama and near Portugal in the North Atlantic. It has been observed in the Bay of Fundy, eastern Canada (Kaczmarek et al. 2004). Its presence in the north-western Atlantic might be due to transport by the Atlantic Current (Hasle 1965), which is likely to explain its presence at 57°30'N 12°15'W (station F, *RV Discovery* cruise 257, chapters 2 and 3). In the Pacific, *P. subpacifica* was identified from Chilean waters between 18°20'S and 43°S (Rivera 1985). *P. subpacifica* has a very similar morphology to *P. heimii*, although it is smaller but slightly wider (Hasle et al. 1996), it is regarded as a warm water species (Skov et al. 1999).

1.4.1.11 *P. turgidula* (Hustedt) Hasle

For *P. turgidula* some questionable reports exist for the northern hemisphere in the Barents Sea, north-east and west of Greenland (Quillfeldt von 1996), off Shetland (Hasle

1965) and in Scottish waters (Gallacher et al. 2001). It has been reported from the southern Atlantic (Hasle 2002) and is found in Australian (Hallegraeff 1994) and New Zealand waters (Rhodes 1998). One New Zealand strain has tested positive for DA production (Rhodes et al. 1996).

1.5 Domoic acid

Domoic acid (DA), the toxin that is produced by *Pseudo-nitzschia*, is a naturally occurring, water-soluble, neuroexcitatory amino acid that mimics the excitatory neurotransmitter L-glutamic acid (Fig. 1.3).

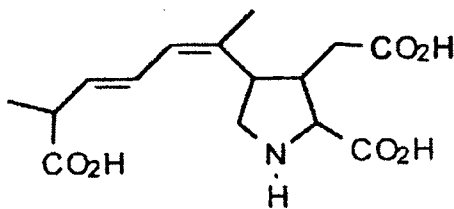


Fig. 1.3 Structure of the domoic acid molecule.

It strongly binds to glutamate receptors in the brain where it causes nerve cells to transmit impulses continuously until the cells die (cf. Horner & Postel 1993). It was identified as the toxin responsible for the first recorded outbreak of ASP in Prince Edwards Island (PEI), Canada in 1987 (Wright et al. 1989), where over 100 people became ill and 3 died after the consumption of DA contaminated blue mussels (*Mytilus edulis*) (Fritz et al. 1992). The irreversible binding of DA to glutamate receptor sites, causes destructive neuronal depolarisation (Debonnell et al. 1998) with the effect of permanent short-term memory loss in mammals (Perl et al. 1990; Todd 1993). In humans, symptoms of DA intoxication can occur within 30 min to 24 h after consumption of contaminated shellfish. The symptoms may include vomiting, diarrhoea, abdominal cramps and headache in mild cases, while in severe cases excessive bronchial secretions, difficulty in breathing, loss of equilibrium, permanent short-term memory loss, coma or death may occur; the memory loss is irreversible, an antidote to DA is not known (cf. Horner & Postel 1993). The name "amnesic" shellfish poisoning was given because patients with memory loss remember

things that happened before they became ill, but not what happened afterwards (Horner & Postel 1993).

Before the ASP event in 1987, the first reported sources of domoic acid were macro-algae of the family Rhodomelaceae: *Alsidium corallinum* C. Agardh (Impellizzeri et al. 1975), *Chondria armata* Okamura (Takemoto & Daigo 1958) and *C. baileyana* Montagne (Laycock et al. 1989; Wright et al. 1989). The former two species occur in warmer waters (Japan and the Mediterranean), the latter in Canada (Todd 1990). The name domoic acid was given from the Japanese word 'domoi' for seaweed. DA was also known as a folk medicine in Japan to treat intestinal pinworm infestations in very small doses (Altwein et al. 1995). The only other diatoms that are, to date, known to produce domoic acid are *Amphora coffeaeformis* (Agardh) Kuetzing (Maranda et al. 1990) and the recently described *Nitzschia navis-varingia* (Lundholm & Moestrup 2000), which was isolated from a shrimp pond in Vietnam and produced DA in culture (Kotaki et al. 2000).

DA is formed by the condensation of an activated citric acid cycle derivative (probably glutamate arising from an alpha-ketoglutarate in the Krebs cycle) with geranyl (probably as geranyl pyrophosphate, which is originally from acetyl-CoA) (Douglas et al. 1992). For the biosynthesis of DA from those two precursors present in algal cells, one or two enzymes might be required (Plumley 1997) plus substantial quantities of ATP and NADPH (Douglas et al. 1992). Douglas et al. (1992) had suggested a direct coupling of proline and DA metabolism. Following that hypothesis Smith et al. (2001) tested the association between DA metabolism with proline metabolism in *Pseudo-nitzschia* species by measuring free amino acids in 5 species and 20 strains of *Pseudo-nitzschia*. They found that proline was lower in cells accumulating high levels DA. This led to the conclusion that proline may function as an upstream precursor for DA. From the amino acid profile in *Pseudo-nitzschia* species Smith et al. (2001) additionally found an accumulation of taurine pools (ca. 50% of total free amino acids) in DA producing *Pseudo-nitzschia* species. Taurine is a non-protein amino acid and was not detected in other phytoplankton, hence Smith et al. (2001) suggested it may provide a biomarker for potentially toxic bloom events.

1.5.1 Stability of Domoic acid

DA is assumed to be relatively stable under dark and cold conditions. When kept in the dark at -12°C , it was reported to be stable in acetonitrile/water (1:9, v/v) (Ravn 1995). Bates et al. (2004) kept DA stock solutions in distilled water for up to 2 years in darkness in a refrigerator ($3-4^{\circ}\text{C}$) without its degradation. However, when exposed to light, DA was observed to degrade. DA in culture filtrate from a mid-exponential *P. multiseriis* culture, kept under axenic conditions in flasks under continuous light at $\sim 100 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and ca. 20°C , showed a 68% decline from its initial mean DA concentration after 12 days. Flasks that additionally contained bacteria and were kept in the light, showed a 40% decline, while flasks containing DA kept in darkness displayed only a 17% decline. This indicated that the bacteria present in that study were not directly influencing the elimination of DA, whereas light did. From their results Bates et al. (2004) suggest that the DA concentration in experimental cultures, that were exposed to similar light and temperature conditions, (e.g. Bates et al. 1991) may have been underestimated. They also emphasise that the potential for DA photodegradation in the sea has to be considered.

Investigating biodegradation processes and disposal of DA, Stewart et al. (1998) isolated bacteria from blue mussels (*Mytilus edulis*) and soft-shell clams (*Mya arenaria*). When supplied with low concentrations of growth factors, the bacteria exhibited growth and biodegradation of DA. Molluscs that are known to retain DA for lengthy periods (as e.g. *Placopecten magellanicus* and *Modiolus modiolus*) only occasionally yielded bacteria with this capability. Stewart et al. (1998) suggested that autochthonous bacteria maybe be significant factors in the elimination of DA, however, further studies would be required to confirm their findings.

All DA production experiments in this thesis (see chapter 4) were conducted with xenic cultures (bacteria present). For those experiments the identity of bacteria was not determined and it is not certain if bacteria altered the DA concentration in the samples. However, to minimise any potential effects of bacteria or light on the DA samples, samples were immediately frozen and kept in the dark prior to analysis to avoid biodegradation of DA.

1.5.2 Domoic acid and the food chain

DA may enter the food chain from diatoms via filter feeders such as molluscan shellfish (e.g. Addison & Stewart 1989; Nijjar et al. 1991; Novacek et al. 1992; Wohlgeschaffen et al. 1992; Drum et al. 1993; Horner et al. 1993; Langlois et al. 1993; Mackenzie et al. 1993; Jones et al. 1995; Douglas et al. 1997; Stewart et al. 1998; Vale & Sampayo 2001; Blanco et al. 2002a, b). In scallops (*Pecten maximus*) DA mainly accumulates in the digestive gland, mantle tissue and gills, but also in gonad and adductor tissue (Campbell et al. 2001; Hess et al. 2001; Blanco et al. 2002a, b). Apart from shellfish, some crustaceans such as copepods (e.g. *Acartia tonsa*, *Temora longicornis* and *Pseudocalanus acuspes*) (Turner & Tester 1997; Lincoln et al. 2001), krill (Bargu et al. 2002, 2003) the sand crab (*Emerita analoga*) (Ferdin et al. 2002; Powell et al. 2002) or Dungeness crab (*Cancer magister*) (Lund et al. 1997) may function as vectors for DA. Also finfish e.g. juvenile sardines (*Sardina pilchardus*) (Vale & Sampayo 2001), sanddabs (*Citharichthys* spp.) (Lefebvre et al. 2002), northern anchovy (*Engraulis mordax*) (McGinness et al. 1995; Lefebvre et al. 2001) and mackerel (*Scomber japonicus*) (Sierra-Beltrán et al. 1997) accumulate the toxin. The vectors may transfer DA within the food chain to sea birds such as brown pelicans (*Pelecanus occidentalis*) (Work et al. 1993a, b; Sierra-Beltrán et al. 1997) and Brandt's cormorants (*Phalacrocorax penicillatus*) (Work et al. 1993a) or to marine mammals, such as the California sea lion (*Zalophus californianus*) (Lefebvre et al. 1999; Scholin et al. 2000), and the blue whale (*Balaenoptera musculus*) (Lefebvre et al. 2002), which as a consequence of ingesting the toxin may beach and in most cases die.

1.5.3 The role of domoic acid

HAB toxins are considered as secondary metabolites, compounds that do not fulfil a role in intermediary metabolism (Plumley 1997). From the microbiological view, secondary metabolites are compounds produced when normal, balanced growth ceases. The synthesis of a given secondary metabolite is often restricted to a specific phylogenetic group or even a single species (for references see Plumley 1997). The role of those substances may be intrinsic, e.g. to protect the organism from UV light or to store intracellular nutrients, or extrinsic, e.g. to be toxic to predators, to be an allelopathic

substance, to promote symbiotic relationships, or as a siderophore to scavenge metals (Plumley 1997). However, the precise evolutionary pressure driving the synthesis of each HAB toxin remains enigmatic, toxins of different species are chemically distinct, and hence the evolutionary pressure of each toxin might be species-specific (Plumley 1997).

The physiological role of domoic acid in algal metabolism remains obscure (Maldonado et al. 2002). Toxigenic *Pseudo-nitzschia* species have been found to produce greater amounts of DA when, triggered by for example silicate limitation, cell division slows down or ceases (Bates et al. 1998). It is hypothesised that DA may simply serve as a way of eliminating excess photosynthetic energy when cells are no longer able to grow optimally (see Mos 2001). The relationship between environmental conditions and DA production by *Pseudo-nitzschia* spp. is still unclear (Buck et al. 1992; Garrison et al. 1992; Lange et al. 1994). Rue & Bruland (2001) suggest that the tricarboxylate amino acid structure of DA resembles that of known iron-complexing agents, such as mugineic acid, a phytosiderophore, produced by terrestrial plants. This similarity in chemical structure of DA to other phytosiderophores suggests a role of DA as a trace metal chelator. Rue & Bruland (2001) revealed that DA can strongly bind iron and copper. They concluded that *Pseudo-nitzschia* spp. may produce the toxin to selectively bind trace metals in order to either increase the availability of an essential micro-nutrient (e.g. iron), or to decrease the availability of a potential toxic trace metal (e.g. copper). Rue & Bruland (2001) suggested that DA released to the water column during *Pseudo-nitzschia* blooms could potentially alter the chemical speciation of iron and copper in seawater, in other words its role would be the acquisition or detoxification of trace metals in seawater. In another study the relationship between trace metal availability and DA production of two toxigenic *Pseudo-nitzschia* species was examined (Maldonado et al. 2002). They observed the production and active release of DA by the cells in response to stress caused by Fe-deficiency or Cu-toxic conditions, suggesting that DA functions as an organic metal-complexing ligand that is released by the toxigenic algae under metal stress.

Smith et al. (2001) suggest that taurine and DA may fulfil the homeostatic role of proline. Proline, which is essential in protein biosynthesis, is often assigned to have an

osmoregulatory role. As an upstream precursor for DA, it is negatively associated with DA.

1.5.4 Domoic acid detection methods

After the first reported ASP outbreak in Canada in 1987 (see section 1.6, Bates et al. 1989; Perl et al. 1990; Todd 1993) chemical analysis soon revealed that toxicity of the mussels was not due to one of the then known shellfish toxins or anthropogenic xenobiotics. Extracts of contaminated mussels were injected intraperitoneally into mice that then showed reproducible symptoms as scratching and eventual death (Wright et al. 1989). Within five days Wright et al. (1989) identified DA as the responsible toxin, separating it with techniques including high-performance liquid chromatography (HPLC), high-voltage paper electrophoresis and ion-exchange chromatography, and characterising it by spectroscopic techniques, including ultraviolet, infrared, mass spectrometry and nuclear magnetic resonance .

Due to the risk of ASP, many countries with shellfish fisheries monitor their seafood products for shellfish toxins. The current safe limit for DA in shellfish of $20 \mu\text{g} \cdot \text{g}^{-1}$ shellfish tissue is below the detection limit ($> 50 \mu\text{g} \cdot \text{g}^{-1}$) of the mouse bioassay and therefore does not make this detection method feasible (Wright 1995). Improved technologies and animal ethics have now led to replacement of the mouse bioassays. This method has already been banned from some countries, including Germany, with bans soon following in other European Union member states.

Methods that are today commonly applied to test shellfish and phytoplankton extracts can be classified into chromatographic analysis, immunological analysis, receptor binding assays, capillary electrophoresis, cytotoxicity/cell culture assays and algal monitoring (Garthwaite 2000).

1.5.4.1 Chromatographic analysis

As mentioned in section 1.5.4, HPLC with ultraviolet (UV) diode array detection (DAD) was the first method used to study the 1987 ASP outbreak in Canada. After extracting the homogenised shellfish tissue in distilled water, the extract was purified by solid phase

Chapter 1

extraction (SPE). Reversed-phase HPLC coupled with DAD was then applied to determine the DA concentration in the mussel extract. The detection limit of DA in the extract solution depends on the sensitivity of the UV detector and in this case was about 10-80 ng · mL⁻¹. However, the detection limit of DA in the shellfish tissue depends on the extraction and cleanup methods and for this method was about 1µg DA · g⁻¹ shellfish (Quilliam et al. 1989).

To lower the detection limit a derivatization method was developed and liquid chromatography (LC) with fluorescence detection was applied (Pocklington et al. 1990). As DA lacks chromophores a derivatization of DA is required to detect it with fluorescence (Garthwaite 2000). HPLC of the fluorenylmethoxycarbonyl (FMOC) derivative is a highly sensitive method to measure trace levels of DA in plankton cultures, plankton net tows and seawater itself, with a detection limit of 15 pg DA · mL⁻¹ seawater (described by Pocklington et al. 1990). Early use of this method verified that *P. multiseriis* (then called *Nitzschia pungens* f. *multiseriis*) produced domoic acid and was the causative organism of the 1987 ASP event in Canada (Bates et al. 1989). However, this method only detects DA in seawater and phytoplankton samples or cultures, not in seafood tissue. Analysis of shellfish tissue in some cases interferes with analysis reagents which can lead to chromatographic misinterpretations, hence for shellfish tissue the FMOC derivatization cannot be applied. An extraction method involving 50% aqueous methanol and a cleanup based on strong-anion exchange (SAX) was developed and applied to seafood tissue prior to DA determination by LC with UV DAD. The method was successfully applied for DA determination in mussels, razor clams, crabs and anchovies, reaching a detection limit between 20-30 ng · g⁻¹ (Quilliam et al. 1995).

More recently HPLC of the 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivative has been used with fluorescence detection to measure DA in *P. multiseriis* cultures, a detection limit of 1 pg DA · mL⁻¹ culture was achieved (Sun & Wong 1999). Another derivatization method which is applicable for DA from shellfish tissue and phytoplankton samples was developed using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), with a detection limit < 1ng DA · mL⁻¹ (James et al. 2000).

Liquid chromatography coupled with mass spectrometry (LC/MS) has been developed for DA determination in seafood samples. Furey et al. (2001) used electrospray ionisation to perform multiple tandem MS experiments and achieved DA detection limits with LC-MS between 0.02 and 0.008 $\mu\text{g DA} \cdot \text{mL}^{-1}$ seafood extract.

1.5.4.2 Immunoassays

Antibodies can be used to detect domoic acid, for example by applying an enzyme linked immunosorbent assay (ELISA). The antibodies recognise specific toxic structures and bind to them. Toxin levels more than 500 times below the maximum permitted DA concentration in shellfish can be detected (Garthwaite 2000). Antibodies for DA developed by Garthwaite et al. (1998) have been successfully used in a highly specific ELISA to classify *Pseudo-nitzschia* species as toxic in New Zealand waters (Rhodes et al. 1998).

The MIST Alert™ for ASP is another rapid commercial antibody-based test (Jellett Biotek Limited, Dartmouth, Canada). It was first developed for PSP toxins (Jellett et al. 2002). It is reportedly easy to use and can be quickly applied on shellfish extracts by the shellfish farmer to test phytoplankton and shellfish extract samples. Tests have demonstrated its reliability and reproducibility in toxin detection, successfully identifying DA in samples containing the regulatory limit for ASP of 20 $\mu\text{g} \cdot \text{g}^{-1}$ shellfish flesh (Mackintosh & Smith 2002).

1.5.4.3 Capillary electrophoresis and electrochromatography

Possible alternatives to HPLC for analysis of DA are capillary electrophoresis (CE) and capillary electrochromatography (CEC) (Piñeiro et al. 1999; Bartle et al. 2001). In CE and CEC the sample is driven through the column by applying an electric field, rather than by applied pressure as in HPLC. Based on the different mobilities of polar substances (such as DA) in an electric field, depending on their molecule size and charge, those substances are separated with CE or CEC and then analysed by photodiode array detection. Both

techniques have been recently developed and applied for DA and other shellfish toxin detection (Martins et al. 2002; Gago-Martínez et al. 2003).

1.6 Chronology of ASP events/ mass occurrences of *Pseudo-nitzschia* spp.

For an overview of reported ASP events and occurrences of toxic *Pseudo-nitzschia* species see Table 1.1.

The first observed ASP event occurred during November /December 1987 in the bays of PEI, Canada. Deaths, illness and short time memory loss occurred in people after the consumption of blue mussels (*Mytilus edulis*) (Bates et al. 1989). The outbreak resulted in at least three human deaths and 107 cases of gastrointestinal illness and ASP (Perl et al. 1990; Teitelbaum et al. 1990; Todd 1993). The edible mussel tissue contained up to 900 $\mu\text{g DA} \cdot \text{g}^{-1}$ wet weight (Addison & Stewart 1989), which represented an extremely high dose of toxin for the consumers. This event also led to the identification of the neurotoxin DA as a harmful algal toxin (Wright et al. 1989). The diatom *Pseudo-nitzschia multiseriis* (Hasle), then called *Nitzschia pungens* Grunow f. *multiseriis* (Hasle 1995), was identified as the causative domoic acid producer, that had contaminated the blue mussels (Subba Rao et al. 1988b; Bates et al. 1989). The Health and Welfare Ministry of Canada established a regulatory guideline of 20 $\mu\text{g DA} \cdot \text{g}^{-1}$ shellfish tissue for human consumption from estimates during the event of amounts ingested by patients (in Villac et al. 1993a). The detailed chronology of this first ASP event is described by Addison & Stewart (1989). Subsequently there were blooms of *P. multiseriis* at PEI in the following three years, but because of an intensive monitoring programme, human intoxication was prevented (Smith et al. 1990a, b; Villac et al. 1993a).

Table 1.1 ASP events and mass occurrences of *Pseudo-nitzschia* spp. in chronological order

Time	Location	Species	Effect	Reference
Nov./Dec. 1987	PEI, Canada	<i>P. multiseriata</i>	DA contaminated <i>Mytilus edulis</i> , 107 cases of human illness, 3 human deaths	Bates et al. 1989
Aug.- Oct. 1988	Bay of Fundy, Canada	<i>P. calliantha</i>	DA in <i>Mytilus edulis</i> and <i>Mya arenaria</i> , closure of harvest areas	Martin et al. 1990; Lundholm et al. 2003
Sep. 1991	Monterey Bay, California, USA	<i>P. australis</i>	DA contaminated anchovies, seabird deaths	Buck et al. 1992; Work et al. 1993b
Oct. 1991	Washington coast (Pacific)	<i>P. multiseriata</i> , <i>P. australis</i>	high DA levels in <i>Siliqua patula</i> , closure of harvest area, 21 cases of human illness,	Wekell et al. 2002; Drum et al. 1993
summer 1992	Pudget Sound, Washington, USA	<i>Pseudo-nitzschia</i> sp.	DA in <i>Mytilus edulis</i> and <i>Crassostrea gigas</i>	Horner & Postel, 1993
autumn 1994	Hood Canal, Washington, USA	<i>P. multiseriata</i> , <i>P. australis</i> , maybe <i>P. pungens</i>	high DA levels in <i>Mytilus edulis</i>	Horner et al. 1997
Jan. 1993	Danish waters	<i>P. seriata</i>	DA production in lab cultures, no ASP event	Lundholm et al. 1994
Nov. 1993 - Jul 94	Dutch wadden sea	<i>P. multiseriata</i>	DA production in lab cultures, no ASP event	Vrieling et al. 1996
Sep. 1994	Galicia, Spain	<i>P. australis</i>	DA in <i>Mytilus galloprovincialis</i>	Miguez et al. 1996
1993 - 1997	Northland, New Zealand	<i>P. australis</i>	DA in <i>Pecten novaezelandiae</i> , also in <i>Crassostrea gigas</i> , <i>Tiostrea chilensis</i> , <i>Austrovenus stutchburyi</i> , closure of scallop fishery	see Rhodes et al. 1998
Dec. 1994	Marlborough Sound	<i>P. pungens</i>	<i>Perna canaliculus</i> , two weeks closure of harvesting site	Rhodes et al. 1998
Dec. 1995- Jan 96	Baja California peninsula, Mexico	<i>Pseudo-nitzschia</i> sp.	DA contamination of <i>Scomber japonicus</i> , death of ~ 150 <i>Pelecanus occidentalis</i>	Sierra-Beltrán et al. 1997
Jan.- Feb. 1997	Gulf of California, Mexico	<i>P. australis</i>	DA contamination of <i>Sardinops sagax</i> , seabird (766) and sea mammal deaths (182)	Sierra-Beltrán et al. 1998
May -Jun 1998	central Californian coast, USA	<i>P. australis</i> , maybe also <i>P. pseudodelicatissima</i>	DA contamination of <i>Engraulis mordax</i> , > 400 <i>Zalophus californianus</i> deaths	Scholin et al. 2000
Oct. 1998	Washington coast, USA	<i>P. pseudodelicatissima</i>	high DA levels in <i>Siliqua patula</i>	Adams et al. 2000
Oct. 1998	Chinhae Bay, South Korea	<i>P. multiseriata</i>	high DA levels in shellfish	Cho et al. 2002
1998	French Atlantic coast	<i>P. multiseriata</i>	DA production in lab culture, no ASP event	Amzil et al. 2001
1999, 2000	Scotland	<i>P. australis</i>	DA contaminated <i>Pecten maximus</i> , to date largest shellfish closures	Campbell et al. 2001; Gallagher et al. 2001
1999	Ireland	<i>P. australis</i>	DA contaminated <i>Pecten maximus</i> , shellfish closures	McMahon & Silke 2000
Mar. 2000 - 2001	Portugal	<i>Pseudo-nitzschia</i> spp.	DA contamination of <i>Mytilus edulis</i> , <i>Cerastoderma edule</i> , <i>Scrobicularia plana</i> , <i>Venerupis pullastra</i> , <i>Ostrea edulis</i> , <i>Ensis</i> spp., <i>Ruditapes decussata</i>	Vale & Sampayo, 2001
1998 - 2000	Gulf of St. Lawrence, PEI, Canada	<i>P. seriata</i>	DA contamination of molluscs, shellfisheries closures, DA production in lab culture,	Couture et al. 2001
2002-2003	Californian coast, USA	<i>P. australis</i>	sea mammal and sea bird deaths	news press (see text for refs.)

From August to October 1988, DA amounts greater than the acceptable level were detected in blue mussels (*Mytilus edulis*) and soft-shell clams (*Mya arenaria*) in the southwestern Bay of Fundy, New Brunswick, Canada, resulting in the closure of shellfish harvesting areas (Martin et al. 1990; Martin et al. 1993). The causative organism was then identified as *Nitzschia pseudodelicatissima*, which is today known as *P. pseudodelicatissima*. Following morphological investigations on the *P. pseudodelicatissima/cuspidata* complex in a recent study Lundholm et al. (2003) found that their *P. pseudodelicatissima* strain was identified as the new species *P. calliantha*.

The first reported ASP event in the USA led to seabird deaths and seafood quarantines and was associated with *P. australis*. It occurred along the Californian coast in Monterey Bay, in September 1991 (Buck et al. 1992; Work et al. 1993b). More than a hundred brown pelicans (*Pelecanus occidentalis*) and Brand's cormorants (*Phalacrocorax penicillatus*) died from DA intoxication after feeding on DA contaminated northern anchovies (*Engraulis mordax*) (Fritz et al. 1992). It was an unusual event because this time the vector was not shellfish but finfish (Work et al. 1993a). *P. australis* was found in the anchovies' stomach contents and was suspected to be the cause of the DA intoxication, as it dominated the phytoplankton community in Monterey Bay at that time. Using laboratory isolates, *P. australis* was confirmed to be the DA-producing organism responsible (Garrison et al. 1992).

In October 1991 DA levels up to $154 \mu\text{g} \cdot \text{g}^{-1}$ wet weight were found in razor clams (*Siliqua patula*) along the Pacific coast of Washington, USA (Drum et al. 1993; Wekell et al. 2002), while *P. multiseriis* and *P. australis* were abundant. As a consequence the recreational and commercial harvest of the clams had to be closed until May 1992. However during that autumn 21 people showed gastrointestinal symptoms after the consumption of razor clams, more than half of them also had neurological symptoms, but no deaths were reported (Horner & Postel 1993). At the same time Dungeness crabs (*Cancer magister*) from coastal water of Washington, Oregon and California were also found to be DA contaminated and the commercial fishery was closed for several weeks (Horner & Postel 1993). It was suggested, that the ASP event in California in September 1991 and the DA contamination of razor clams and Dungeness crabs in Washington might

have been part of a widespread bloom of *P. australis*, starting in California that could have been carried to Washington by the currents (Horner et al. 1997).

In the following summer (1992) trace amounts of DA were detected in blue mussels (*Mytilus edulis*) and oysters (*Crassostrea gigas*) in northern Puget Sound, Washington, USA (Horner & Postel 1993). Along the coasts of California, Oregon and Washington from 1991-1993, DA was measured in doses above the regulatory limit in seafood including anchovies, razor clams, crabs and spiny lobsters. Of all tested species anchovies were found to be the most highly contaminated (Altwein et al. 1995). It was estimated that the HAB event was mainly affecting the razor clam recreational fishery and tourism. In Oregon and Washington it brought a loss for the economy of about \$700 thousand in 1991 and \$7 million in 1992 (Hoagland et al. 2002).

After a persistent six week bloom of *P. pungens*, *P. multiseriis* and *P. australis* in Hood Canal, western Washington, USA in autumn 1994, $\sim 14 \text{ g DA} \cdot \text{g}^{-1}$ were measured in the phytoplankton and $\sim 10 \mu\text{g DA} \cdot \text{g}^{-1}$ wet weight in mussels. This was the first time since 1991, when the monitoring began, that relatively high DA levels were found in mussels and in inland waters of western Washington (Horner et al. 1997).

The first evidence of DA in phytoplankton outside North America was found in European, Danish waters. Cultures of *P. seriata* that were isolated from the Baltic Sea in January 1993, produced DA. However an ASP event did not occur (Lundholm et al. 1994).

The first European strain of *P. multiseriis* was isolated from the Dutch Wadden Sea between November 1993 and July 1994. Although the *P. multiseriis* culture produced DA under laboratory conditions, no ASP event occurred (Vrieling et al. 1996).

Following a *P. australis* bloom in Galicia, north-west Spain (Miguez et al. 1996) in September 1994, DA was first detected in mussels (*Mytilus galloprovincialis*) in European waters. The DA content of the mussels was below the legal limits for DA concentration in shellfish. However it was the first record of *P. australis* and DA in European waters and the north-east Atlantic, although the capability of the European *P. australis* to produce DA was then not proven. Shortly after the bloom (October 1994) *P.*

australis and some other species were successfully cultured from Galician waters and DA production confirmed (Fraga et al. 1998),

In New Zealand, DA was first detected in Northland in 1993, in scallops (*Pecten novaezealandiae*) that had ingested *P. australis* (Rhodes et al. 1998). A year later, the maximum DA contamination in Northland was recorded in scallop digestive glands ($600 \mu\text{g} \cdot \text{g}^{-1}$), coinciding with the presence of *P. australis* in seawater samples (Rhodes et al. 1996). Between 1993 and 1997, scallop harvesting sites had to be closed every early austral summer (November-December) in Northland, with *P. australis* suspected to be the causative organism. In December 1994, a Greenshell mussel (*Perna canaliculus*) harvesting site was closed for two weeks in Marlborough Sound, New Zealand, due to elevated DA levels in the mussels, this was linked to *P. pungens*. A single site was closed in October 1995, due to $22 \mu\text{g} \cdot \text{g}^{-1}$ in Greenshell mussels (Te Araroa Beach, New Zealand). However, no ASP events or more shellfisheries closures have occurred until 1998 in this location (Rhodes et al. 1998).

About 150 brown pelicans (*Pelecanus occidentalis*) were killed by domoic acid intoxication at the tip of the Baja California peninsula (Pacific coast of Mexico) in late December 1995 to January 1996. The seabirds had been feeding on mackerel (*Scomber japonicus*) that was contaminated by a domoic acid producing *Pseudo-nitzschia* sp. (Sierra-Beltrán et al. 1997). A year later, during January-February 1997, 766 common loons (*Gavia immer*) and 182 sea mammals (4 different species) were found dead in the Gulf of California. *P. australis* frustules were found in stomachs of common dolphin (*Delphinus capensis*) and in sardines (*Sardinops sagax*) that had been ingested by some of the dolphins. The presence of DA in mammal tissues was confirmed and *P. australis* was thought to be the causative organism. This was the first report of *P. australis* in the Gulf of California, Mexico (Sierra-Beltrán et al. 1998).

In May and June 1998 the first ASP event that killed marine mammals in the USA was documented in sea lions (*Zalophus californianus*) along the central Californian coast (Monterey Bay, USA). Over 400 sea lions died after ingesting anchovies (*Engraulis mordax*) that had been feeding on a *P. australis* bloom, which reached levels up to $7\text{-}32 \text{ pg DA} \cdot \text{cell}^{-1}$ (in one sample even $75 \text{ pg DA} \cdot \text{cell}^{-1}$). During the bloom in May about 1.3

$\times 10^6$ cells \cdot L⁻¹ of *P. australis* were counted in water samples. The water was enriched with silicate from terrestrial freshwater runoff, that might have enhanced the yield of the bloom (Lefebvre et al. 1999; Scholin et al. 2000).

Following the DA poisoning of the sea lions in summer 1998 at Monterey Bay, California, record levels of DA were found in razor clams (*Siliqua patula*) along the Washington coast, USA, in October 1998. The toxin was this time not associated with *P. australis*, but with a bloom identified as *P. pseudodelicatissima* which then occurred at an abundance of 1.0-1.5 $\times 10^6$ cells \cdot L⁻¹ (Adams et al. 2000).

In October 1998, DA contamination was reported from Asian waters, in southern Korea (e.g. Chinhae Bay). DA concentrations just below the international acceptance level of 20 μ g \cdot g⁻¹ were detected in shellfish and associated with *P. multiseriis*. *P. multiseriis* is not the only occurring *Pseudo-nitzschia* species in Korean waters, but is regarded to be the most likely DA producer (Cho et al. 2002). *P. multiseriis* had previously been found in Japanese waters (e.g. Ofunato Bay), but only in small numbers and no ASP or ASP-like poisoning had been reported from that area (Kotaki et al. 1999).

Again in Europe, this time along the French Atlantic coasts, DA was detected in shellfish in 1998. The potential DA producers *P. pseudodelicatissima* and *P. multiseriis* were identified by SEM from water samples. Only *P. multiseriis* was cultured and positively tested for DA (Amzil et al. 2001).

In Scottish waters *P. australis* was suspected to be the source of the DA contamination of king scallops (*Pecten maximus*) in 1999 and 2000 (Gallacher et al. 2001), which resulted in the, to date, largest area of fisheries closures due to a harmful algal bloom (Campbell & Kelly 2001). At times of shellfish closures *P. australis* and other *Pseudo-nitzschia* species were abundant (Gallacher et al. 2001). Two *P. australis* cultures were established in August 1999 and it was shown that they were capable of producing DA (Campbell et al. 2001). At the same time, in 1999, DA amounts above the regulatory limit of 20 μ g \cdot g⁻¹ wet weight were also found in king scallops in Ireland (McMahon & Silke 2000), coinciding with findings of *P. australis* in the phytoplankton community (Cusack et al. 2002).

In Portuguese shellfish the presence of DA is a recurrent event and affects the shellfish resources several times a year, mainly in spring and autumn (Vale & Sampayo 2001). In early March 2000 a maximal DA concentration of $90 \mu\text{g} \cdot \text{g}^{-1}$ was detected in a mussel sample, but DA contamination in shellfish has been observed from 1995. Apart from blue mussels (*Mytilus edulis*) other shellfish that are known to regularly become contaminated with DA in Portuguese waters are common cockle (*Cerastoderma edule*), peppery furrow shell (*Scrobicularia plana*), carpet shell (*Venerupis pullastra*), oyster (*Ostrea edulis*), razor clam (*Ensis* spp.) and clam (*Ruditapes decussata*). However toxic diatom blooms at the Portuguese coast seem to be a recurrent phenomenon of relatively short duration, ASP symptoms in humans have not yet been reported, but might pass unnoticed in the wildlife (Vale & Sampayo 2001).

Between 1998 and 2000, at the same time as the shellfish closures in north-western Europe, *P. seriata* was suspected to be the cause of DA contamination in eastern Canadian waters. Since 1997 the presence of DA in molluscs in the Gulf of St. Lawrence, PEI, Canada, had been monitored by the Canadian Food Inspection Agency. They found substantial amounts of DA in scallops and shellfish-fisheries had to be closed between 1988 and 2000. The DA contamination of the molluscs coincided with *P. seriata* blooms. One *P. seriata* strain was successfully cultured and tested positive for DA production (Couture et al. 2001).

In the past few years frequent spring blooms of *P. australis* have lead to reoccurring domoic acid contamination of wildlife in California, USA. In 2002 alone, 685 sea lions were killed by domoic acid in Santa Barbara and Ventura county, with another 518 stranded. Concurrently approximately 75 dolphins died with 23 showing domoic acid poisoning symptoms (Gollan 2003, Schultz 2003). Between April and June 2003, 104 sea lions died and 177 had fallen ill, 43 dolphins died and five had stranded along the Californian coast, due to a domoic acid outbreak (Gollan 2003). *P. australis* was again thought to be the causative organism with DA accumulating in sardines and anchovies that fed on the algae (Thomas-Anderson 2003).

These events show that ASP is a serious threat for wildlife and humans and strongly impacts the shellfish harvesting industry. There might be further implications on tourism (e.g. beached sea lions and pelicans might keep tourists away). Of all *Pseudo-nitzschia* species only *P. multiseriata*, *P. australis*, *P. seriata* (*P. seriata* group) and *P. calliantha* have been confirmed as causes of ASP events or shellfish closures. Reports of DA contamination due to *P. pungens* and *P. pseudodelicatissima* should be re-confirmed by cultivation and DA testing of the species.

1.7 Identification

1.7.1 Diatoms

Diatoms (division Chromophyta, class Bacillariophyceae) include two orders, the Biddulphiales, or Centrales and the Bacillariales (= Pennales) (cf. Hasle & Syvertsen 1996). The division into those two orders is based on their symmetry. While centric diatoms have radial symmetry with surface patterns arranged in relation to a central point, pennate diatoms have longitudinal symmetry with surface patterns arranged in relation to a line (e.g. Fig. 1 in Horner 2002). The genus *Pseudo-nitzschia* Peragallo belongs to the order Pennales. There are about 1400-1800 diatom species within the marine phytoplankton, 850-1000 of them are centric, about 500-800 pennate (Sournia et al. 1991). They occur in all oceans (in the plankton, benthos, sediments, sea ice) and are the most abundant organisms in the phytoplankton community in terms of biomass and species. From their pigments (chlorophyll *a*, *c2*, sometimes *c1* or *c3*, beta-carotene, fucoxanthin, diatoxanthin, diadinoxanthin) the cells may appear yellow, yellowish green, golden brown or dark brown. Diatoms range from 5 to 200 µm; some cells may reach up to 4 mm in length. One of their main characteristic is the siliceous cell wall, which consists of two halves, the slightly smaller hypotheca and the larger epitheca. The hypotheca fits into the epitheca, like the two halves of a petri-dish (see Fig. 1.4A). Both halves are marginally connected by girdle bands. Diatoms occur in various shapes, which vary between species. Additionally many cells have processes, spines, ridges and

elevations, which together with the highly variable pore patterns of the cell wall (Fig. 1.4B) are used to delineate genera and species.

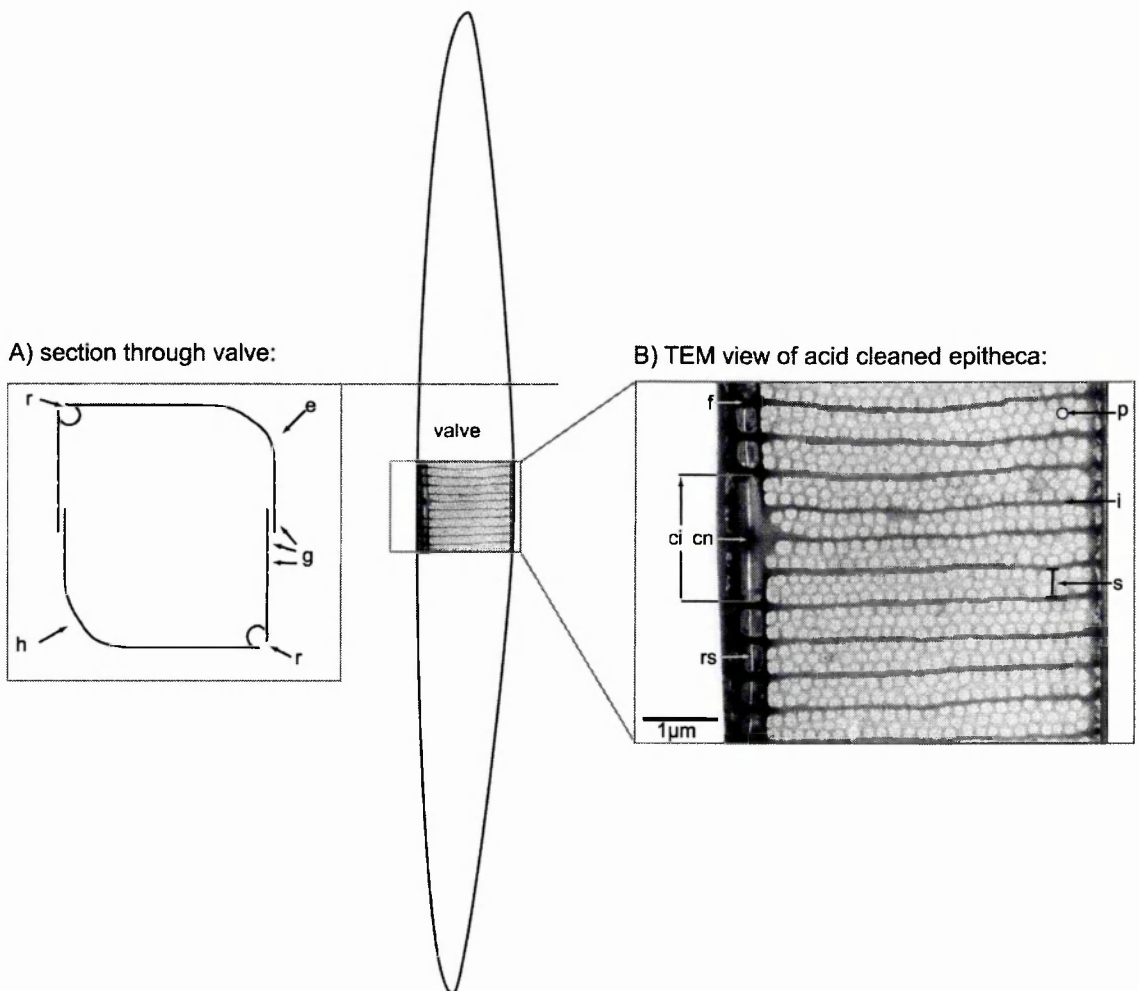


Fig. 1.4 Schematic representation of identification features for *Pseudo-nitzschia* cells. A) Section through valve shows epitheca and hypotheca; e= epitheca; g = girdle, with girdle bands; h = hypotheca; r = raphe. B) TEM view of the valve of *P. fraudulenta* with fine structural features used for species identification; ci = central interspace; cn = central nodulus; f = fibula; i = interstria; p = poroid; rs = raphe slit; s = stria.

Cells are solitary or form colonies. Colonies are formed when cells stay linked after cell division (e.g. Fig. 1.5). The shape of colonies also varies between genera and species and can be used as an additional feature for identification. Diatom reproduction is primarily asexual by cell division, which happens through the formation of new siliceous components inside the parental cell. Each daughter cell receives one half of the parent cell theca, which becomes the new epitheca, and forms a hypotheca. Through this process the diatom cell becomes increasingly smaller, for the genus *Pseudo-nitzschia*, in general, the length is reduced while the width stays about the same. The average cell size of a diatom

population decreases to about one third of their maximum size, before it has to be restored through sexual reproduction (cf. Horner 2002).

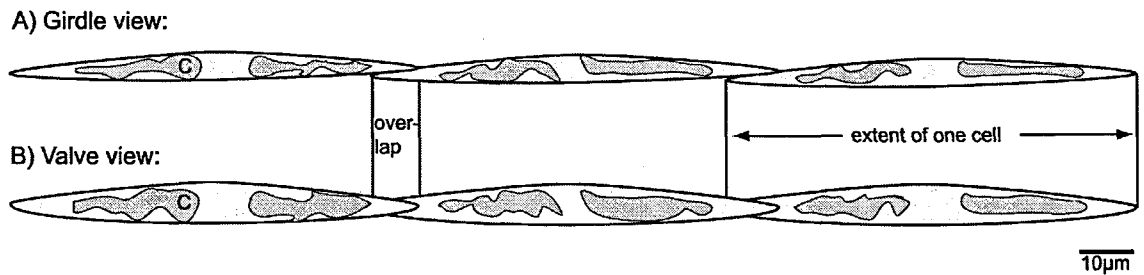


Fig. 1.5. Chain of *Pseudo-nitzschia* cells in girdle (A) and valve (B) view; c = chloroplast.

1.7.2 *Pseudo-nitzschia*

Diatoms that now belong to the genus *Pseudo-nitzschia* were formerly within the genus *Nitzschia* Hassall 1854. After recognising that diatoms like *Nitzschia seriata*, *N. fraudulenta* and *N. sicula* had more sharply pointed tips that overlapped to form a chain, the genus *Pseudo-Nitzschia* was erected by H. Peragallo (Peragallo & Peragallo 1900). However, because of the partly reduced raphe and a partially retained motility, Hustedt reduced the genus to a section of the genus *Nitzschia* in 1958. After the ASP event in PEI, Canada, in 1987, attention was drawn back to the genus following the discovery that *Nitzschia pungens* f. *multiseries* was the source of DA (Bates et al. 1989). Hasle (1994) recognised *Pseudo-nitzschia* as different from the genus *Nitzschia*. The re-erection was based on morphological characters and the fact that *Pseudo-nitzschia* species are marine and planktonic (the genus *Nitzschia* comprises both planktonic and benthic species).

1.7.2.1 Morphological characteristics

With light microscopy (LM), some primary morphological characters of *Pseudo-nitzschia* spp. can be observed. Usually *Pseudo-nitzschia* spp. cells are arranged in "stepped colonies" (Fig. 1:5). A chain of *Pseudo-nitzschia* cells can move, while single cells within the colony cannot (as they can in *Bacillaria* Gmelin which is of the same family).

Valves of *Pseudo-nitzschia* cells are shallow, flattened or smoothly curved, not undulated. The extremely eccentric raphe, is not elevated above the level of the valve as in *Nitzschia*. For species delineation in *Pseudo-nitzschia*, the valve outlines from both valve and girdle view (Fig. 1.5) must be described. This may be achieved with a light microscope and requires measurement of width, length and the overlap of cells. Width information divides *Pseudo-nitzschia* species into the *Pseudo-nitzschia seriata* group (*Nitzschia seriata* complex), containing species with frustule widths $> 3 \mu\text{m}$ in valve view, and the *Pseudo-nitzschia delicatissima* group (*Nitzschia delicatissima* complex), including species with valve widths $< 3 \mu\text{m}$ (Hasle 1965; Hasle & Syvertsen 1996).

Producing permanent slides (e.g. with Naphrax, N. Lundholm, personal communication) after removing the organic material of the frustules can allow further identification with the LM. For example one may distinguish between *P. australis* and *P. fraudulenta*, as with LM, cleaned *P. fraudulenta* valves often show the central interspace (Fig. 1.4B), while *P. australis* lacks a central interspace. In some cleaned samples one may distinguish *P. multiseriata* from *P. pungens*, because, in correctly prepared samples, the poroids (Fig. 1.4B) can be seen in *P. pungens* valves under 1000 x LM magnification, but not in *P. multiseriata* (N. Lundholm, personal communication). *P. seriata* and *P. australis* are indistinguishable by LM, as they have a very similar shape and symmetry, and both lack a central interspace. Using electron microscopy (EM), it is possible to identify *Pseudo-nitzschia* cells to species level. Removal of organic material (see chapter 3), is also a requirement for any fine structure study of the frustules with electron microscopy. While EM is more expensive and takes more preparatory effort than LM, it is much more reliable than LM identification, and considered necessary for critical species identification (Skov et al. 1999). Transmission electron microscopy (TEM) is generally more useful than scanning electron microscopy (SEM). For SEM the cells need to be coated (with gold-palladium) resulting in an image that represents the surface of that coat. With TEM, the electron beam passes through the pores and openings in the frustule resulting in an image that reveals many of the fine structural features more clearly. For example the structure of the poroid hymen and the band stria require a resolution that cannot be achieved using

SEM (Lundholm et al. 2003). Tables 3.3 and 3.4 (chapter 3, section 3.3.2) show species specific measurements of fine structural features that are used for species delineation.

Morphological features (Fig. 1.4B) that can only be seen with EM, and that are necessary for identification to species level, are:

- The central interspace. This feature can sometimes be recognised in the LM, but not as clearly as in EM, it is present in some species, but absent in others.
- The rows of poroids per stria.
- The number of poroids in 1 μm
- The number of fibulae and interstriae in 10 μm .
- The shape of the valve ends and additionally the structure of the poroid hymen and the cingular bands (poroids and stria) can also be studied for species delineation.

The identification of *Pseudo-nitzschia* and other microalgae using LM and EM is time-consuming, requires substantial expertise, and is still often difficult and ambiguous when the cells have to be discriminated to species level (e.g. Miller & Scholin 1996). Hence other methods of species identification, for example the use of genetic methods are becoming more common.

1.7.2.2 Genetic Characters

For genetic identification and phylogenetic studies on *Pseudo-nitzschia* spp., genes or partial genes from the nuclear rDNA (ribosomal RNA genes) have been used (Fig. 1.6). The rDNA operon is typically present in a high copy number (e.g. Baldwin et al. 1995) in the eukaryotic genome, and this is also the case for *Pseudo-nitzschia* (Cangelosi et al. 1997). The high copy number promotes detection, amplification, cloning and sequencing of the rDNA. The rDNA also undergoes rapid concerted evolution, that promotes uniformity among repeat-units (Baldwin 1995). The rDNA operon consists of the small subunit (SSU, 18S) gene, the internal transcribed spacer (ITS) gene and the large subunit (LSU, 28S) gene. The ITS region is bisected into ITS1 and ITS2 by the 5.8S gene, a highly conserved region (Palumbi 1996). While the SSU and LSU are moderately

variable between species, the non-coding ITS1 and ITS2 genes exhibit more genetic variation. Just before the SSU, at the 5' end of the rDNA operon lies the external transcribed spacer (ETS) (Fig. 1.6).

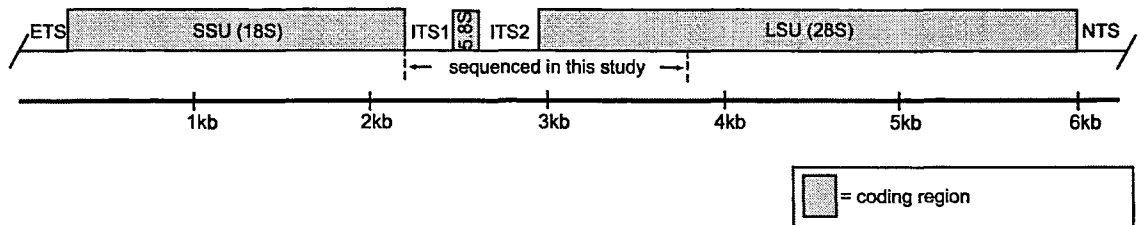


Fig. 1.6 The nuclear rDNA operon (modified after Gerbi 1985). ETS = external transcribed spacer; ITS = internal transcribed spacer; kb = kilo base; LSU = large subunit; NTS = non-transcribed spacer; SSU = small subunit.

Douglas et al. (1994) sequenced SSU rRNA genes from *P. pungens*, *P. multiseriis* and *P. australis* and other marine diatoms. Differences in the nucleotide sequences between *P. pungens* and *P. multiseriis* permitted them to design PCR (polymerase chain reaction) primers that allowed discrimination between the two species, that were, at that time, seen as two subspecies of *P. pungens* (f. *pungens* and f. *multiseriis*). They inferred from the differences between SSU rDNA sequences, that *P. pungens* f. *pungens* and *P. pungens* f. *multiseriis* were separate species. In phylogenetic analyses *Pseudo-nitzschia* was clearly distinguished from the other marine diatoms (Douglas et al. 1994). This was supported by morphological data as well.

Scholin et al. (1994) based a phylogenetic analysis on partial LSU sequences to discriminate between the toxic and non-toxic *Pseudo-nitzschia* species *P. australis*, *P. delicatissima*, *P. americana*, *P. pungens* f. *pungens* (= *P. pungens*) and *P. pungens* f. *multiseriis* (= *P. multiseriis*).

Partial LSU sequences were again used to relate the, then newly described, species *Pseudo-nitzschia galaxiae* (Lundholm & Moestrup 2002), *P. brasiliiana* and *P. linea* as well as the *Pseudo-nitzschia americana* complex (Lundholm et al. 2002b) and in another study *P. multistriata* (Orsini et al. 2002) to other *Pseudo-nitzschia* species.

Also based on partial LSU rDNA sequences, a phylogeny of the family Bacillariaceae (including the genera *Bacillaria*, *Cylindrotheca*, *Fragilariopsis*, *Neodenticula* and *Pseudo-nitzschia*) with emphasis on *Pseudo-nitzschia* was inferred (Lundholm et al. 2002a).

With LSU rDNA sequences Stehr et al. (2002) revealed the similarity of some *Pseudo-nitzschia* species (*P. multiseriis*, *P. pungens*, *P. australis* and *P. heimii*) from Washington waters to species from Californian waters, while others, *P. delicatissima* and *P. pseudodelicatissima* were distinct. However, when that distinction was made the *P. pseudodelicatissima* complex had not been resolved (later resolved by Lundholm et al. 2003) and in hindsight they may have been different species.

Manhart et al. (1995) used the SSU, ITS1 and parts of the 5.8S to confirm the morphological distinction between *P. multiseriis* (Hasle) Hasle and *P. pungens* (Grunow ex Cleve) Hasle. Cangelosi et al. (1997) aligned ITS2 and ETS1 sequences of *P. multiseriis*, *P. pungens* and *P. australis* to find genetic differences between those species. They suggested the use of the ITS region as targets for molecular probe identification of *Pseudo-nitzschia* species.

As yet, Lundholm et al. (2003) are the only authors to have used the ITS region in *Pseudo-nitzschia* to establish a phylogeny that includes 16 of the main *Pseudo-nitzschia* species. With ITS sequences and phylogenetic analysis they resolved the position of the *P. delicatissima* complex within the genus *Pseudo-nitzschia*.

While previous studies used either the SSU, ITS or LSU, a useful approach for species-level comparison and phylogenetic analysis would be the combination of genes. This approach was taken in the present study (chapter 3), combining ITS, 5.8S and LSU sequences.

1.7.2.3 Immunological and Biochemical Characters

Based on rDNA sequences, molecular probes have been developed to distinguish between *Pseudo-nitzschia* species from culture and field samples. Whole cell hybridisation allows *in situ* identification and enumeration of microorganisms such as *Pseudo-nitzschia* (for review see Amann et al. 1995). With this technique rRNA in the cell is specifically detected within morphologically intact cells by rRNA-targeted fluorescently labelled oligonucleotide probes. To speed up and ease identification of *Pseudo-nitzschia* species from cultures, whole-cell (*in situ*) hybridisation with species-specific LSU rRNA-targeted oligonucleotide probes was used to successfully distinguish between *P. australis*, *P.*

pungens, *P. multiseriis*, *P. fraudulenta*, *P. heimii*, *P. delicatissima*, *P. pseudodelicatissima* and *P. americana* (Miller & Scholin 1996). This technique was also used to enumerate cultured and wild *Pseudo-nitzschia* cells (Miller & Scholin 1998). Intact cells that retain the probes are visualised using epifluorescence microscopy.

In contrast to whole cell (in situ) hybridisation, sandwich hybridisation requires the homogenisation of living cells to liberate their cell contents (referenced in Scholin et al. 1996). Both techniques were applied by targeting oligonucleotide probes towards the same key LSU rRNA sequences to discriminate *P. australis* from cultures (Scholin et al. 1996, 1997) and natural populations (Scholin et al. 1997) from its closely related congeners. Scholin et al. (1997) suggest that sandwich hybridisation potentially offers the most rapid and simple means to identify *P. australis* in its natural environment when screening large numbers of environmental samples. It also offers a faster mode of sample processing than whole-cell (in situ) hybridisation because of its high amenability to automation (Scholin et al. 1999). Depending on the probes used and species studied, cross-reactions can occur (e.g. Miller & Scholin 1998; Parsons et al. 1999), which result in one probe labelling more than one species. The detection of *Pseudo-nitzschia* in field samples with LSU rRNA targeted oligonucleotide probes has been widely applied in field studies from different locations, (e.g. Rhodes et al. 1998; Trainer et al. 1998b; Bates et al. 1999; Parsons et al. 1999; Scholin et al. 1999; Cho et al. 2001, 2002).

Some other methods that have been used to identify *Pseudo-nitzschia* species are immunochemical techniques such as immunofluorescence (Bates et al. 1993a), electro-immunoblotting (Ross & Bates 1996) and lectin binding assays (e.g. Rhodes 1998; Fraga et al. 1998; Cho et al. 2001, 2002). In immunochemical techniques cells are first incubated in a primary antiserum and then with a fluorescently tagged secondary antiserum directed against the primary antibody (referenced in Bates et al. 1993), in this case *Pseudo-nitzschia* cells. The target cells can then be visualised by immunofluorescence. Both immunochemical methods have been applied to discriminate between *P. pungens* and *P. multiseriis*.

Lectins are proteins or glycoproteins of non-immune origin, each different lectin binds to a specific sugar or group of sugars. The different species-specific binding patterns of

fluorescent-conjugated lectins (FTIC-conjugated lectins) to sugars on algal surfaces can be used to differentiate between microalgae species (see Aguilera & González-Gil 2001). FTIC-conjugated lectins have been applied to *Pseudo-nitzschia* species in Spain (Fraga et al. 1998), New Zealand (Rhodes 1998) and Korea (Cho et al. 2001, 2002). Results showed that the application of fluorescently labelled lectins facilitates the identification and quantitative estimation of several *Pseudo-nitzschia* species in the field and therefore provides a promising tool for monitoring programmes.

Scottish *Pseudo-nitzschia* populations are composed of mixed species (chapter 2 and chapter 3), hence techniques as described above would be useful in distinguishing between the species, identifying potentially harmful blooms.

1.8 Toxin production of *Pseudo-nitzschia* species in culture

1.8.1 Growth cycle and DA production

Previous studies investigated the growth dynamics and DA production of a few *Pseudo-nitzschia* species in regard to factors such as light, temperature and inorganic nutrient concentration. Those studies investigated the toxin production dynamics with the aim to predict and potentially mitigate ASP events. *Pseudo-nitzschia* cultures first had to be established from field samples and maintained under laboratory conditions. Hence, so far only a few *Pseudo-nitzschia* species have been studied under a few conditions. Those species include *P. multiseriata*, *P. australis*, *P. cf. pseudodelicatissima* and *P. seriata*.

Most studies of DA production have focused on *P. multiseriata*, as it was the first *Pseudo-nitzschia* species shown to produce DA, and was isolated into culture soon after the ASP event in Canada 1987. It has been shown to produce DA in batch cultures only in the late exponential and stationary phases of the cell growth cycle, when cell division either declines or ceases (e.g. Bates et al. 1998; Pan et al. 1998). DA production and the growth rate appear to be inversely correlated (Pan et al. 1996a); when cell division terminates in stationary phase DA production is greatly enhanced (Pan et al. 1996b). It is believed that

this is attributable to stress such as phosphate or silicate limitation (Pan et al. 1998 and references therein).

Toxin production by *P. australis* has been tested in two studies. In the first, two strains from the California coast were thought to produce DA in mid-exponential phase (Garrison et al. 1992). However, due to a high inoculum, it could not be ruled out that high numbers of late stationary phase cells, could have led to carry over of DA to the experiment. The second study of one Irish Sea isolate of *P. australis* showed a similar pattern of toxin production to *P. multiseriis* when grown in batch culture under very low light conditions ($12 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$); no DA was detected until late stationary phase (Cusack et al. 2002). In contrast, when the same isolate was grown at a higher irradiance ($115 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), DA production began in late exponential phase. In a study on *P. cf. pseudodelicatissima* (Pan et al. 2001), which can be identified as either *P. pseudodelicatissima* or *P. cuspidata* (Lundholm et al. 2003), cellular levels and net production of DA were highest in the early exponential phase, while population growth rate was high and cell concentration was low. This pattern of DA production differed from that of other *Pseudo-nitzschia* species.

Toxin production of *P. seriata* is even less well understood. In the only study with *P. seriata* where toxin was tested (Lundholm et al. 1994), traces of DA were observed during the exponential growth phase and highest amounts of DA in the whole culture (cells + medium) in late stationary phase. Toxin production appeared to be temperature dependent with higher amounts of DA produced at 4°C than at 15°C.

1.8.2 Influence of nutrients on DA production

Although nutrient limitation would have occurred in the above studies, the limiting nutrient was not defined. All studies specifically investigating the effects of limitation by a particular nutrient have been carried out on *P. multiseriis*, mainly in batch culture.

1.8.2.1 Nitrogen

DA is an amino acid and therefore requires nitrogen (N) as one of its key elements for synthesis. However, as N contributes only 4.5% of the molecular weight of DA, the purpose of DA production by the cell is unlikely to be the storage of N (Bates et al. 1991). Bates et al. (1991) demonstrated the N-requirement for DA production in an experiment with a xenic batch culture of *P. multiseriis*. At the onset of stationary phase, the culture started producing DA. Cellular DA levels remained relatively constant due to concurrent release of DA into the medium. Later during stationary phase, when N was absent, DA production ceased, but resumed when N was added back to the medium. The authors concluded that DA production requires cessation of cell division and the availability of nitrogen.

Certain ammonium concentrations (220-440 μM) may enhance DA production. When *P. multiseriis* cultures were exposed to equivalently high ammonium and nitrate concentrations the cellular DA production was enhanced by two- to fourfold. The increased DA production at these ammonium concentrations can be interpreted as either a physiological stress imposed on diatom growth due to ammonium toxicity or the use of a more energetically favourable form of nitrogen for the synthesis of primary amino acids and DA (Pan et al. 1998). However, such high ammonium concentration are unlikely to occur in nature.

1.8.2.2 Silicate and Phosphate

In contrast to nitrogen, silicate (Si) and phosphate (P) limitation are thought to result in toxin production in stationary phase. This is shown in experiments with *P. multiseriis* (Bates 1998 and references therein; Bates et al. 1991; Pan et al. 1996a, b, c). This will be discussed in detail in sections 4.1.2 and 4.4.1 (chapter 4, experiment A)

1.8.3 Temperature and DA production

When growing *P. multiseriis* in batch cultures at temperatures ranging from 5°C to 25°C, Lewis et al. (1993) observed higher cell concentrations at lower temperatures, but also a

lower division rate and DA production rate with lower temperature. High temperature conditions such as 25°C might have imposed physiological stress on the cells that enhanced DA production. Low temperatures, may also have implemented a form of physiological stress, but not in the way that it would have increased the rate of DA production.

Interestingly, temperature seemed to affect the morphology of the frustules (Lewis et al. 1993). At 15°C and below, *P. multiseriis* cells showed 3-4 poroids per stria as common for the species, but at 25°C only 2-3 poroids per stria were formed. Lundholm et al. (1997) observed a similar reduction in rows of poroids from four to two (or sometimes three) in *P. seriata* growing at 15°C instead of 4°C. These observations are important, as taxonomy and species identification is often based specifically on these fine-structure morphological features (see section 1.7.2.1).

P. multiseriis was shown to continue growth and domoic acid production when the temperature was changed from 13° to 5° or 0°C and from 5° to 0°C (Smith et al. 1993), indicating that this species can exist and produce DA in winter conditions. Similarly, *P. seriata* from Danish waters produced higher levels of DA when exposed to 4°C than when grown at 15°C (Lundholm et al. 1994). This might suggest that DA production was a response to temperature stress. However, *P. seriata* is assumed to be a cold water species and known to occur at low temperatures (Smith et al. 1993), and hence should not show signs of stress at low temperature.

A study with the domoic acid producing *P. calliantha* (as *P. pseudodelicatissima*) showed the effects of various temperatures and salinities on the growth rate, valve morphology and toxicity (Lundholm et al. 1997). All cultures expressed an optimal salinity of 25 for growth at all temperatures, while cells did not grow at salinity of less than 10. Growth was enhanced at higher temperatures. Domoic acid was detected, but its production could not be related to the external factors. Effects on the valve morphology (e.g. a change in the poroid pattern) were not detected.

The effect of temperature on DA production of other toxin producing *Pseudo-nitzschia* species is still unknown.

1.8.4 Light and DA production

While the photo period appears to influence DA production (e.g. Bates et al. 1991), it is not clear, whether light intensity plays an important role (e.g. Lewis et al. 1993; Cusack et al. 2002). The influence of day length will be discussed in section 4.1.4 (chapter 4, experiment C).

The effect of ultraviolet radiation on *P. multiseriis*, *P. pungens* and *P. fraudulenta* was studied by Hargraves et al. (1993). While the growth of non-toxic strains of *P. fraudulenta* and *P. pungens* was significantly (the former) and slightly (the latter) inhibited, *P. multiseriis* appeared to have acquired a tolerance against UV. The DA data were ambiguous, UV exposure reduced the cellular DA content in 35-day old cultures, but the effect was not clear in 63-day old cultures. The authors concluded that, in regard to increasing UV light as a result of global ozone depletion, *P. multiseriis* apparent UV-resistance may have implications on phytoplankton species compositions in coastal waters.

1.8.5 Trace metals and DA production

A few studies on *P. multiseriis* have investigated the influence of trace metals on DA production. Zhiming & Subba Rao (1998) showed that increasing Germanium (Ge), in form of germanic acid $\text{Ge}(\text{OH})_4$, inhibited growth. Exposed to a Ge:Si ratio of 35, *P. multiseriis* cells ceased to produce DA.

In contrast to Ge, enrichment with lithium (Li) significantly sustained and enhanced the DA production in *P. multiseriis* (Subba Rao et al. 1998). The Li supply triggered by a massive freshwater-runoff, over and through an adjacent dump, following a drought may have played a role in the bloom formation and enhancement of toxin production of *P. multiseriis* in Cardigan Bay, PEI, Canada in 1987.

The effect of iron on domoic acid production of *P. multiseriis* was studied by Bates et al. (2001). Cultures exposed to an increased iron concentration produced 5-10 times more chlorophyll *a* (chl *a*) than cultures that were grown without additional iron. Cultures with added iron showed increased DA production in stationary phase, while iron-stressed cultures showed very little increase in DA levels. It was suggested that DA has iron

chelating abilities (Rue & Bruland 2001; Maldonado et al. 2002). But results indicated that DA is not produced as a chelator to increase the availability of iron (Bates et al. 2001). As described by Bates et al. (2001) iron is a key component of enzymes essential for nitrogen uptake and production of precursors for DA biosynthesis. Decreased cellular chl *a* levels reduce the capacity of capturing photosynthetically active radiation and lead to a deficit in energy for all processes in the cell. Therefore the cause of the decrease in DA production may be explained by the fact that iron-deficit conditions have a limiting effect on the two resources that are a requirement for DA biosynthesis, nitrogen uptake and energy.

All of the above described laboratory studies showed that DA is produced when cell division rate slows because of nutrient limitation, or through the effects of temperature and irradiance. However, these effects do not explain clonal variability in toxin production. One other factor that might play an important role in this regard are bacteria (see section 4.1.5 and 1.8.6 below).

1.8.6 Interactions between bacteria and harmful algae

The studies reviewed above were conducted with xenic (bacteria containing) cultures. Bacteria are recognised to play an important role in the biology and ecology of unicellular eukaryotic algae due to the interaction between them (Doucette 1995).

1.8.6.1 Bacterial influence on domoic acid production

Douglas & Bates (1992) verified the toxin production of *P. multiseriis* in absence of bacteria (following treatment with gentamicin, penicillin and streptomycin). The division rate of the axenic culture was comparable with that reported for xenic cultures, and DA was similarly produced once cell division had ceased. The axenic culture achieved similar biomass, and toxin per cell yields, to corresponding xenic cultures (Subba Rao et al. 1988b; Bates et al. 1989; Bates et al. 1991). The results provided the first evidence, that *P. multiseriis* produces DA in the absence of other microorganisms.

The above studies did not preclude the possibility that bacteria may enhance the growth and toxin production of *P. multiseriis*. Comparison of two clones of *P. multiseriis* isolated from PEI, Canada, in axenic cultures, indicated slightly higher cell division rates and a two to three weeks longer viability of the xenic culture (Douglas et al. 1993). Both cultures produced DA, and the toxin level was up to 20 times greater in the xenic culture, compared to the axenic one.

In a further study Bates et al. (1995) found evidence that the presence of bacteria in *P. multiseriis* cultures had a marked impact on toxicity of the algae. The reintroduction of bacteria isolated from xenic *P. multiseriis* cultures to axenic ones enhanced the DA production by 2 to 95-fold, while division rate and cell yields were not substantially affected. Introduction of bacteria isolated from a non-toxic *Chaetoceros* sp. culture also enhanced DA production per cell (by 115-fold), showing that the bacteria have not necessarily to be isolated from a toxic culture to enhance toxin production. There was no evidence of intracellular bacteria in *P. multiseriis* cells from axenic cultures, but various species of bacteria were isolated from xenic cultures. The authors concluded that DA production in *P. multiseriis* is enhanced by several bacterial species and also bacteria are important, but not essential for DA production. Together, all studies with axenic and xenic *P. multiseriis* strains showed that the degree of enhancement in DA production varied considerably with both the diatom and the bacterial strain tested (Bates et al. 1995). As none of the bacterial strains were so far known to be capable of autonomous DA production, it appears that the bacteria invoked enhanced toxicity is a result of an indirect contribution, such as the synthesis of DA precursor molecules (Doucette 1995).

Osada & Stewart (1997) confirmed the above findings for *P. multiseriis* and extended them to show that the addition of the bacterial products proline and glutamic acid enhanced the algal growth considerably.

1.8.6.2 Evidence for bacterial toxin production

The role of bacteria in phycotoxin production, whether it is autonomous bacteria toxigenesis or algal toxicity as the result of bacterial interaction, is considered in Doucette (1995).

Smith et al. (2002) found bacteria isolated from a toxic strain of *P. multiseriis* and from mussels (*Mytilus edulis*), to produce trace amounts of DA when grown in marine broth without the algae. Toxin production was enhanced in the presence of silt particles, suggesting that the adhesion to the particles might play a role in bacterial toxin production. However the results have yet to be confirmed (E. Smith, personal communication).

Testing the hypothesis that free-living bacteria, rather than *P. multiseriis* cells are the source of DA in stationary phase, Bates et al. (2004) concluded that bacteria were not able to autonomously produce DA. Their results confirmed the previous conclusions (Douglas et al. 1993; Bates et al. 1995) that the observed increase of DA after reintroduction of bacteria to axenic *P. multiseriis* cultures was not due to autonomous production of DA by those bacteria.

1.9 The ASP situation in Scottish waters

Although there are no reported cases of marine wildlife deaths, no reports of human sicknesses or human death following ASP, toxic *Pseudo-nitzschia* species are known to be abundant and are responsible for vast shellfish closures in Scottish waters (Campbell et al. 2001; Gallacher et al. 2001).

Following the EC shellfish hygiene directive 91/492/EC and the Food Safety (Live Bivalve Molluscs and other Shellfish) Regulations in 1992, a phytoplankton and shellfish monitoring programme for HABs and phycotoxins was initiated for Scotland in 1995, undertaken by the Marine Laboratory Aberdeen on behalf of the Scottish Office Agriculture Environment and Fisheries Department (SOAEFD). In 1996, 23 sites were monitored around the Scottish coast, including the Orkney Islands and Shetland, selected on the basis of their importance as shellfish growing harvesting sites. Phytoplankton and shellfish samples were taken by volunteer helpers and shellfish farmers weekly, fortnightly or monthly. Toxic phytoplankton species were counted and identified (to genus level) by light microscopy. The genus *Pseudo-nitzschia* was found in 71% of all samples. This monitoring program gave the first indication of ASP in Scotland. On 6 June 1996 a maximal concentration of 3.5×10^6 *Pseudo-nitzschia* spp. cells per litre was counted from a site off Shetland and DA was detected in mussel samples (Kelly &

Macdonald 1997). It is not known what species that bloom consisted of, as cells were not identified to group or species level.

From 1997 onwards, the funding for the monitoring programme was provided by the Ministry of Agriculture, Fisheries and Food. In 1997 the monitoring sites were increased to 26 and a level of 1.5×10^5 cells \cdot L⁻¹ of *Pseudo-nitzschia* spp. was set as the threshold above which shellfish samples should be taken for DA testing. This level was only once exceeded in that year, when more than 2×10^5 cells \cdot L⁻¹ of *Pseudo-nitzschia* spp. were observed on the Scottish east coast (Stonehaven) (Kelly & Fraser 1998).

In 1999 the threshold level for DA testing in shellfish was lowered to 5×10^4 cells \cdot L⁻¹ of *Pseudo-nitzschia* spp.. The sampled sites were increased to 31 and *Pseudo-nitzschia* spp. were observed at all sites and were present in 71% of the samples, with a maximum concentration on 16 July 1998 of more than 3×10^6 cells \cdot L⁻¹ at a site around the Orkney Islands. DA was detected on 57 occasions with a maximum level of 70 mg \cdot g⁻¹ (Kelly & Fraser 1999), which clearly exceeded the EC Shellfish Hygiene Directive (that the maximum value of DA in the whole animal or any edible part separately should not exceed 20 μ g \cdot g⁻¹). Kelly & Fraser (1999) observed a general trend of elevated DA levels in shellfish occurring about two to three weeks after detection of maximum *Pseudo-nitzschia* concentrations.

In 1999, 32 sites were sampled around the Scottish coasts and 30 in 2000 (Bresnan et al. 2002). To monitor harmful algae species from some offshore locations, infrequent samples were taken *ad-hoc* by chartered vessels. Maximum *Pseudo-nitzschia* spp. cell densities of more than 2.3×10^6 cells \cdot L⁻¹ were recorded from Loch Etive (west coast) in 1999 and a maximum of 1.6×10^5 cells \cdot L⁻¹ from Shetland in 2000. From offshore samples a maximum density of 2×10^5 cells \cdot L⁻¹ was observed in 2000 in the Minch west of the Isle of Skye. During both years toxin accumulation occurred extensively in offshore scallops on the west coast, Moray Firth and Orkney. DA levels above 20 μ g \cdot g⁻¹ were found in scallop gonads from some west coast sea lochs (Bresnan et al. 2002). The contamination of king scallops (*Pecten maximus*) prompted a widespread closure of the king scallop fisheries across areas of northern and western Scotland starting in July 1999. Closures persisted for more than 10 months and covered a maximum of 49,000 km². To

date, this represents the largest fisheries closures world-wide due to a harmful algal bloom (Campbell & Kelly 2001; Campbell et al. 2003). Two *P. australis* cultures were established and shown to be capable of producing DA (Campbell et al. 2001), however, it was not known if other species were responsible for toxin production during that period. Between spring 2001 and spring 2002 maximum *Pseudo-nitzschia* spp. concentrations of 5.1×10^5 cells \cdot L⁻¹ from inshore and 1.6×10^5 cells \cdot L⁻¹ from offshore sites were reported. Ten samples were analysed and based on morphological characters some *Pseudo-nitzschia* species were identified, as *P. australis*, *P. cf. delicatissima*, *P. fraudulenta*, *P. cf. heimii*, *P. multiseriata*, *P. pungens*, *P. cf. pseudodelicatissima* and *P. seriata* var. *obtusata* (Bresnan 2003). However, none of the species were isolated and cultured, the essential requirement for toxin analysis, experimental work, identification by genetics and phylogenetic analysis. Furthermore, as identifications were mainly based on LM (and limited TEM), some of the records may be questionable.

1.9.1 Implications of ASP for the Scottish economy

Information on the economic impact of ASP in Scotland is given by EKOS (2002). Over the last decade the scallops fishery has become increasingly important with the Scottish coast providing extensive scallop grounds. Scallops are mainly harvested by dredging, small quantities are landed by divers (Campbell et al. 2001). From 8,900 tonnes in 1991, the landings into Scotland by UK vessels have been steadily grown to over 16,600 tonnes in 1998. Following ASP and consequent shellfish closures landings fell to under 13,500 tonnes in 1999 and below 13,000 in 2000, before they recovered again in 2001 to 15,300 tonnes. The value of landings was equivalent to £19.5 million (EKOS 2002).

From 300 scallop-divers a few years ago only 150-200 are in business today (EKOS 2002). Some small volumes of queen scallops (*Aquipecton opercularis*) and king scallops (*Pecten maximus*) are farmed. The farmed shellfish production in Scotland is dominated by mussels (*Mytilus edulis*) and oysters (*Ostrea edulis*), which can also be affected by ASP, although those bivalves do not retain the toxin for as long periods as scallops (Novaczek et al. 1992; Blanco et al. 2002a, b).

During 2001 the number of days shellfish areas were closed was reduced, but the number of closed areas was increased. In 2002 only 18 boxes (one box representing an area of 15 x 15 nautical miles), compared to at times 56 boxes in 1999 (from Figure 1 in Gallacher et al. 2001), had to be closed for the king scallop fishery. Currently, the trend in banned areas is decreasing (EKOS 2002).

1.10 Current state of research in *Pseudo-nitzschia* and objectives of this study

Although DA is regularly detected in Scottish shellfish, it is not known which *Pseudo-nitzschia* species occur in Scottish waters and which of those present, are toxic or potentially toxic in this region. Information on the seasonal occurrence of *Pseudo-nitzschia* species and their spatial distribution across the shelf is lacking.

Species identifications from previous studies were mainly based on LM and some samples were inspected by TEM. No genetic identification of Scottish *Pseudo-nitzschia* strains had been attempted. Genetic identification by sequencing parts of the rDNA operon is necessary for the future development of molecular probes which could then simplify and speed up species identification in monitoring programs.

Only two *P. australis* and one *P. pungens* strains had been cultivated from Scottish waters, but apart from the confirmation of *P. australis* as a DA producer, no other studies of DA production have been undertaken. To investigate the dynamics of DA production laboratory experiments are necessary. While experiments had previously been undertaken with *P. multiseriata* (see section 1.9.2), no data existed about the effects of nutrient limitation on other *Pseudo-nitzschia* species in either Scotland or world-wide.

The objectives of this study were therefore:

- To identify *Pseudo-nitzschia* species present in western Scottish waters using morphological and genetic approaches.
- To determine if *Pseudo-nitzschia* species abundances follow a seasonal cycle and are in any way annually predictable.

Chapter 1

- To assess the spatial distribution of *Pseudo-nitzschia* species across the Scottish continental shelf.
- To establish uni-algal cultures of potentially toxic *Pseudo-nitzschia* species.
- To screen these strains for DA production.
- To investigate the dynamics of DA production in *Pseudo-nitzschia* under P and /or Si limitation.
- To investigate relationships between *Pseudo-nitzschia* species and associated marine bacteria.

1.10.1 Work undertaken within this study

To assess, isolate and cultivate toxic and non-toxic *Pseudo-nitzschia* species in western Scottish waters and to study their ecophysiology, growth dynamics, DA production behaviour and their genetic characteristics, the following steps were taken:

- A phytoplankton monitoring programme was instigated at a coastal station (LY1) in the Lynn of Lorne near SAMS in November 2000. This examined seasonal changes of the phytoplankton community, nutrient concentrations and hydrographic parameters over 33 months (still ongoing). The aim was to resolve which *Pseudo-nitzschia* species occur in Scottish waters and under what environmental conditions. Other diatom and dinoflagellate species were included into the monitoring to reveal seasonal patterns and patterns of co-occurrences with *Pseudo-nitzschia* species (chapter 2). From net samples single cells or chains of cells were isolated and clonal cultures established (chapter 3).
- A transect of seven stations through parts of the Lynn of Lorne and Loch Creran, including the LY1 permanent monitoring site, was sampled on three occasions in summer 2002 in order to monitor the phytoplankton distribution and chemical and physical parameters, to determine if the LY1 site was representative of locations north and south of it (chapter 2).

- To obtain an insight on the cross shelf distribution of *Pseudo-nitzschia* species, samples were taken on a transect from the Isle of Mull (western Scotland) to the open Atlantic (the "Ellett Line", see chapter 2). Chemical and physical parameters were recorded. The horizontal and vertical distribution of *Pseudo-nitzschia* and other phytoplankton species was investigated (chapter 2). Some *Pseudo-nitzschia* cells were isolated and cultured for genetic identification (chapter 3).
- Isolated and cultured *Pseudo-nitzschia* spp. were identified via classic morphological and genetic techniques. This work led to the records for Scottish waters, of the toxic species, *P. seriata* f. *seriata*. A phylogenetic analysis of *Pseudo-nitzschia* species was carried out to assess the genetic relationship among Scottish strains (chapter 3).
- The domoic acid production of *P. seriata* isolated from Scottish waters was studied under Si and P limitation in laboratory batch cultures (chapter 4).
- To investigate the impact of different nitrogen sources on the growth of *P. seriata*, laboratory cultures grown with either nitrate or ammonium as an N-source were compared (chapter 4).
- Observations from field monitoring, indicated a spring bloom of *P. delicatissima* and high cell numbers of the *P. seriata* group in summer. Therefore *P. delicatissima* and *P. seriata* were grown in laboratory batch cultures under spring (short light phase) and summer (long light phase) light conditions to compare their growth rates (chapter 4).
- Single *P. seriata* cells were incubated at the presence and absence of bacteria and bacterial exudates. The algal growth was monitored to test if bacteria or their exudates impact algae division rates. Three bacterial strains associated with *P. seriata* were isolated and characterised to provide some ground work for further studies to investigate the influence of those bacteria on diatom DA production (chapter 4).

2 Chapter 2: Temporal and spatial phytoplankton distribution

2.1 Introduction

2.1.1 Succession of phytoplankton

Temporal changes and seasonal patterns in the phytoplankton community composition have been studied extensively in many areas of the world (e.g. Karentz & Smayda 1984; Maddock et al. 1989; Lange et al. 1992; Smith & Hobson 1994; Reid et al. 1995). Cleve (1903) concluded from his phytoplankton observations that each current-system carries its own plankton, when currents mix with other currents, the phytoplankton assemblage becomes modified. This implies that the seasonal variability of the phytoplankton assemblage of a certain locality would be influenced in its composition solely by the transport of water masses of different origin, with no reference to ecological requirements (Lange et al. 1992). In response to Cleve (1897), Gran (1915) suggested that changes in the phytoplankton community were not just caused by a change of water masses, but would depend on changes occurring within that water mass. These views were synthesised by Braarud et al. (1953), suggesting that changes in the phytoplankton community at one locality may be due partly to succession of species within a water mass. A change in the phytoplankton assemblage due to transport of water masses through the observed area would be a 'sequence of plant societies'.

There are many examples of phytoplankton succession during an annual cycle. One is given by Drebes (1974) in the introduction to his phytoplankton identification key, where he described the seasonal cycle of the temperate phytoplankton in North Sea waters around the German island Helgoland: A typical spring bloom lasting throughout March and April was instigated due to the balance of enhanced light availability and reduced turbulence. Once the nutrients were diminished by their uptake by phytoplankton, algal growth ceased. Additionally, grazing by zooplankton was reducing

the phytoplankton number. During summer a smaller phytoplankton population was maintained by recycled nutrients, due to remineralisation of organic matter, by for example zooplankton metabolism. A second bloom was occasionally observed in autumn, when turbulent mixing regenerated nutrients from deeper water layers and light availability was still sufficient. The bloom persisted until the beginning of winter, when short light periods and mixing of the algae into deeper aphotic layers of the water column prevented their growth. During winter a complete mixing of the water column added nutrients back to surface waters in sufficient concentrations to start the seasonal cycle again, once the light and physical conditions were again favourable for algal growth. In general, many phytoplankton species were part of the plankton community throughout the year, particular species appeared mainly at particular times of the year, being adapted to either warm or cold water conditions.

Succession of phytoplankton and/or associated parameters (such as chl *a*) in Scottish waters has previously been studied (Tett & Wallis 1978; Jones 1979; Tett et al. 1981a, b; Lewis 1985; Tett et al. 1986; Tett 1992).

This study aimed to investigate seasonal succession of phytoplankton in Scottish west coast waters at site LY1 in the Lynn of Lorne (Firth of Lorne). Within the analysis of seasonal succession potentially harmful phytoplankton were monitored with special focus on the diatom genus *Pseudo-nitzschia* and other members of the phytoplankton assemblage. A knowledge of seasonality of harmful algae can help predict blooms and in case of *Pseudo-nitzschia* species, prepare communities for ASP events.

2.1.2 Spatial distribution of phytoplankton in regard to shelf seas and open ocean

Phytoplankton data have been collected along cruise tracks, mapping phytoplankton distributions for large areas (e.g. Parsons et al. 1984). From such studies it is apparent

that there is small-scale and a larger-scale patchiness in the spatial distribution of species.

Differences in the composition of the phytoplankton assemblages can be observed in different oceanic areas. Shelf seas differ in many respects from open oceanic waters (Barnes & Hughes 1988), being generally well mixed by wind action and richer in nutrients. Coastal waters also receive the discharge of ground water and drainage of river water, containing on average twice as much nutrients per unit volume as sea-water (Barnes & Hughes 1988). This nutrient richness within shelf seas leads to elevated phytoplankton biomass and production. Although the shelf seas occupy just about 10% of the world ocean, neritic phytoplankton contributes about a quarter of the phytoplankton primary production (Ott 1996). In contrast, the surface water of the open ocean is in general relatively nutrient-poor and stable.

The phytoplankton of both, shelf seas and open ocean is generally dominated by diatoms and dinoflagellates (Ott 1996). However, due to differences in nutrient richness and hydrographical factors, differences in cell densities and taxa between coastal and oceanic sites are common. An example of a phytoplankton group that is typically found in offshore and oceanic waters are the coccolithophorids (Prymnesiophyceae) (Smayda 1958).

2.1.3 *Pseudo-nitzschia* field studies

Other field survey studies have investigated the temporal and spatial distribution of *Pseudo-nitzschia* species in coastal waters in different parts of the world. Those areas included North America (e.g. Martin et al. 1990; Horner & Postel 1993; Lange et al. 1994; Bates 1997; Fryxell et al. 1997; Parsons et al. 1999; Stehr et al. 2002; Trainer et al. 2002; Couture et al. 2001; Kaczmarska et al. 2004), South America (e.g. Rivera 1985), Australia/ New Zealand (e.g. Hallegraeff 1994; Rhodes 1998) Asia (Cho et al.

2002) Africa (Akallal et al. 2002) and Europe (e.g. Hasle et al. 1996; Cusack et al. 2000; Beliaeff et al. 2001; Orsini et al. 2002).

2.1.4 Objectives

In this study the phytoplankton assemblage, with special focus on *Pseudo-nitzschia* species, was studied taking into account two aspects: I. the temporal phytoplankton distribution at the coastal site LY1 and II. the spatial distribution across the shelf towards the open Atlantic.

2.1.4.1 Aims of LY1: temporal study

The coastal site LY1 was monitored in a high temporal resolution over a period of nearly three years, with a number of aims:

- to identify seasonal trends (species succession) in the phytoplankton assemblage,
- to examine associations between phytoplankton, physical (temperature, salinity, density) and chemical parameters (dissolved inorganic phosphate, silicate, nitrate and ammonium concentrations),
- to assess the occurrence of *Pseudo-nitzschia* species to identify potential toxin producers,
- to provide toxic and non-toxic *Pseudo-nitzschia* species for cell isolation and cultivation,
- to conduct laboratory experiments investigating their growth and toxin dynamics,
- to determine the suitability of LY1 as a sampling site in representing the local coastal waters, through sampling a transect from Loch Spelve to Loch Creran.

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2.1.4.2 Aims of the Ellett Line cruise: spatial study

During a cruise with *RV Discovery* (D257) in autumn 2002, shelf and open ocean stations along the Ellett Line were sampled:

- to investigate the spatial (horizontal and vertical) distribution of *Pseudo-nitzschia* and other phytoplankton species,
- to compare the coastal and open ocean phytoplankton assemblages,
- to relate the spatial distribution of the phytoplankton assemblage to physical and chemical factors.

2.1.5 Study sites

2.1.5.1 Phytoplankton monitoring and Spelve-Creran transect

Figure 2.1 shows the location of the phytoplankton monitoring site LY1, that was sampled fortnightly during winter and weekly at other times, and the other stations along the transect from Loch Spelve to Loch Creran (hereafter referred to as the Spelve-Creran transect), that were sampled on three occasions in summer 2002. The exact positions and water depth at stations are given in table 2.1 (section 2.2.1.1).

Site LY1, is located in the Lynn of Lorne, which is part of the Firth of Lorne (separating the Island of Mull and Ardnamurchan from the main part of Argyll). The depth was approximately 52 m. LY2, LY3 and 700 lie on a line south of LY1, at approximately 3.3 to 6 km distance from each other. From their location, LY1 and LY2 might have been directly influenced by outflow from the sea lochs Etive and Creran. C2 was situated north of LY1, close to the entrance of Loch Creran. Stations C3 and C5 were in the main basin of Loch Creran.

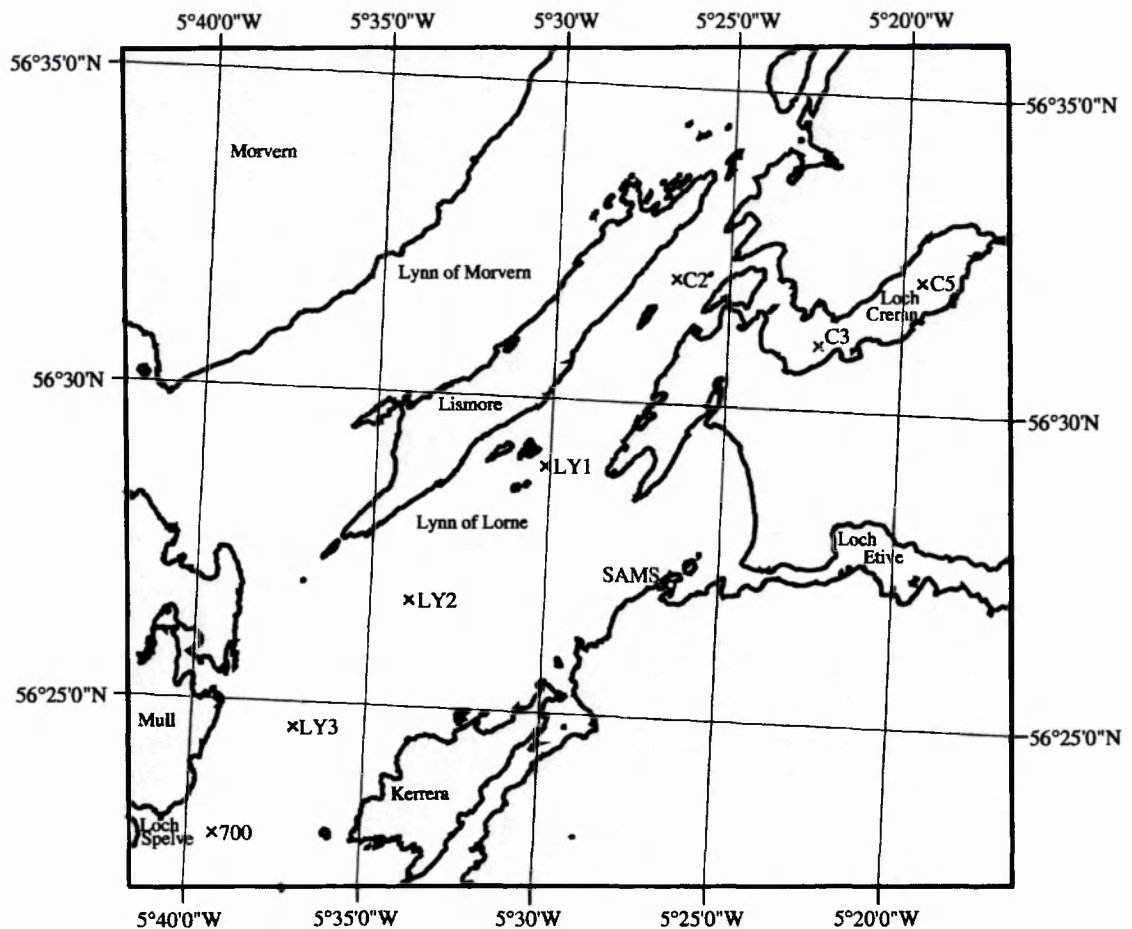


Fig. 2.1 Location of the phytoplankton monitoring station LY1 and stations 700 to C5 along the Spelve-Creran transect.

Loch Creran is situated approximately 15 km north of Oban. According to Landless & Edwards (1976) it is a typical fjordic Scottish sea loch regarding its dimensions, freshwater input and tides. It consists of an upper and lower (main) basin. Samples were taken in the lower basin (Fig. 2.1). The lower basin is approximately 11 km long and 1.5 km wide with a maximum depth of 53 m (Milne 1972). In most places within the lower basin the water depth does not exceed 20 m. However, in two areas water depth greater than 20 m can be found (Jones 1979). The area with greatest depth occurs at the western end of the lower basin, the second location of deeper water is found around station C5, which lies in the centre of the loch. Loch Creran connects with the Lynn of Lorne by a 200 m wide channel, separated by a sill of minimum 5 m water depth (Tett & Wallis

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1978). The main freshwater source is the river Creran, which enters at the head of the upper basin.

2.1.5.2 Ellett Line transect

Figure 2.2 shows the cross-shelf transect that was sampled during cruise D257 in September/ October 2000 with *RV Discovery*. Sampling sites and surface current patterns (Ellett 1979; McKay et al. 1986) that affected those sites are indicated on the map.

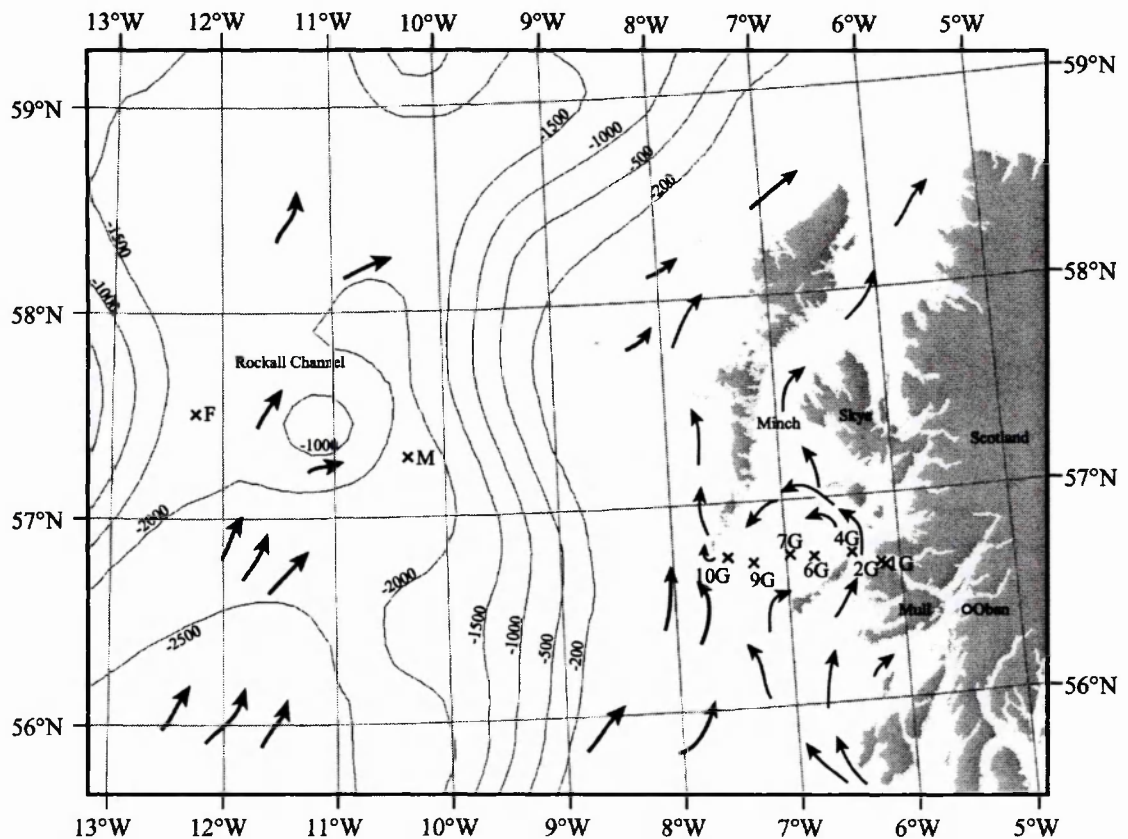


Fig. 2.2 Ellett Line stations (F, M, 10G, 9G, 7G, 6G, 4G, 2G, 1G) and circulation patterns of the Scottish coastal current and the North Atlantic surface water. Circulation patterns redrawn after Ellett 1979 and McKay et al. 1986).

The north-western Scottish shelf area is bounded approximately by latitudes 55°30'N to 59°N and longitudes 6°W to 9°W (Fig. 2.2), the beginning of the shelf edge is indicated

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in Fig. 2.2 by the 200 m contour line. In this study coastal stations east of Barra Head (1G to 10G) were sampled and compared with stations M and F. While 1G to 10G mostly represented Scottish coastal water, M and F were influenced by the North Atlantic current.

The maximum depth at the shelf stations was about 190 m (10G), M and F were considerably deeper with 2150 and 1800 m (for approximate maximal depth at each station see Tab. 2.2, section 2.2.1.2).

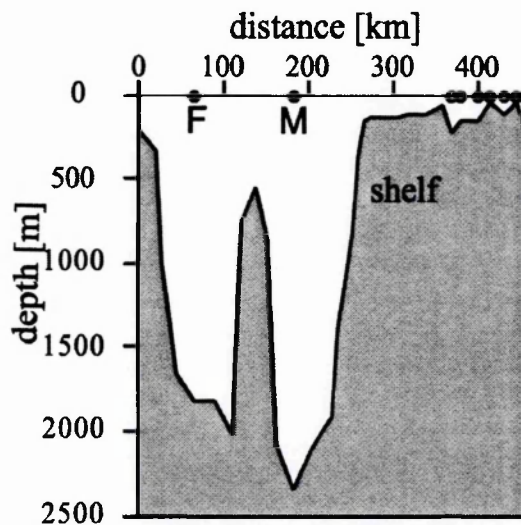


Fig. 2.3 Schematic diagram showing the depth at stations and their distribution across the shelf and open ocean. A circle indicates a sampled station. F and M were the open ocean stations.

Fig. 2.3 shows the distance between stations and the depth at stations. It illustrates the difference between the stations due to their location either in the open ocean (F and M) or on the shelf, near the Scottish coast (10G to 1G).

2.1.6 Previous work conducted in the study area

2.1.6.1 LY1, Loch Creran

Most of the previous studies of Firth of Lorne stations including LY1 were undertaken from 1979 to 1981 (Grantham 1983a, b; Grantham et al. 1983) and investigated temperature, salinity, nutrients and chl *a* concentrations in a low temporal resolution. Past studies conducted in Loch Creran also focussed on chl *a* and nutrient cycles (Tett & Grantham 1978; Tett & Wallis 1978; Jones 1979) and phytoplankton ecology (Jones 1979; Tett et al. 1981a, b; Tett et al. 1985; Lewis 1985; Harris 1995).

For the Firth of Lorne area Tett et al. (1981b) provided one of the few studies which included actual phytoplankton counts over the annual phytoplankton cycle. They monitored the phytoplankton community weekly at two locations in Loch Creran during 1979, along with physical and chemical properties, enumerating the main species of diatoms, dinoflagellates and other microflagellates. At that time the genus *Pseudo-nitzschia* had not been established (Hasle 1994). Hence, most of the species, which today belong to the *P. seriata* group were then enumerated as *Nitzschia seriata* (e.g. as described for Allen in Lange et al. 1994). Species today assigned to the *P. delicatissima* group, were then called *Nitzschia delicatissima*. Only since the ASP event in Prince Edwards Island, Canada in 1987 (Bates et al. 1989), has it been realised that some of the *Pseudo-nitzschia* species were capable of producing DA. This has resulted in emphasis on differentiation between *Pseudo-nitzschia* species. Before then, *Pseudo-nitzschia* species were even summarised with other species, that were then called *Nitzschia*. For example Tett (1973) and Tett et al. (1981b) presented cell count data of total '*Nitzschia*' species together with *Cylindrotheca closterium* (which was then called *Nitzschia closterium*). Hence, from early *Nitzschia* records it is not possible to make conclusions about the annual cycle of *Pseudo-nitzschia* spp. abundance. However, in their recent

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review Tett & Edwards (2002) present the cell count data on *N. seriata* and *N. delicatissima* from Loch Creran, that were summarised in Tett et al. (1981b).

2.1.6.2 Ellett Line transect

Since 1975, repeated physical measurements have been undertaken on a transect from the Scottish west coast to the open Atlantic (e.g. Ellett 1979; Ellett & Edwards 1983). The "Ellett Line" transect consists of a series of stations from the Scottish continental shelf, starting at the Isle of Mull, leading across the Minch towards Barra Head and across the shelf to Rockall. The time series was established by David Ellett and hence named after him. Most work on this transect investigated the current flows and water mass properties through the Rockall Trough. Only the study of Savidge & Lennon (1987) investigated the distribution of phytoplankton and the hydrography along the Ellett Line, in May and August 1983. They also enumerated the main phytoplankton species and summarised what might have been different *Pseudo-nitzschia* species as "*Nitzschia* spp.". However, it is not known if *Nitzschia* spp. stood for the *P. seriata* or *P. delicatissima* group or as in Tett et al. (1981b) comprised both plus other species. Only two other studies look at the phytoplankton in the area. Gowen et al. (1998) investigated the distribution of zoo- and phytoplankton in the region south of the Ellett Line, the southern Malin Shelf in August 1996. Moreover, abundance of *Pseudo-nitzschia* species was not presented. More recently Yallop (2001) analysed the phytoplankton assemblage from samples collected in close vicinity to the area studied in this thesis. He identified physical, chemical and biological parameters along the 20° median from 37 to 59°N and studied an eddy for several days at 59°N 20°W. As in this study, *Pseudo-nitzschia* species were analysed as *P. seriata* and *P. delicatissima* groups, but then combined with *Cylindrotheca closterium* and *Nitzschia longissima*. From his observations Yallop

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(2001) concluded that *Pseudo-nitzschia* species, together with *C. closterium* and *Thalassiosira oestrupii* were associated with waters of a mixed depth down to 20-40 m.

2.2 Material & Methods

2.2.1 Sampling locations

2.2.1.1 Coastal sites

The phytoplankton monitoring at a coastal site in the Lynn of Lorne at station LY1 (56°28.9N, 5°30.1W, 52 m depth) was instigated in November 2000 and data included in this study were obtained until the end of July 2003 (100 sampling occasions). LY1 was sampled fortnightly from November until April and weekly throughout the rest of the year. The position of the sampling site was chosen because of its coastal location, its representational properties of local waters (Grantham 1983a, b), its convenient accessibility (20 minutes steaming with *RV Seol Mara*) and because it had been sampled, with a low temporal resolution (two to four times a year), between 1979 and 1981 by Brian Grantham (physical data, nutrient and chl *a* analysis).

Table 2.1 Names and positions of stations sampled on a transect from Loch Spelve to Loch Creran on 18 July, 23 August and 13 September 2002.

Station	Position	approx. Depth [m]
700	56° 22.8 N, 05° 39.3 W	130
LY3	56° 24.6 N, 05° 37.1 W	200
LY2	56° 26.7 N, 05° 33.9 W	42
LY1	56° 28.9 N, 05° 30.1 W	52
C2	56° 31.9 N, 05° 26.05 W	33
C3	56° 31.0 N, 05° 22.4 W	48
C5	56° 32.1 N, 05° 19.4 W	25

To verify the representational properties of LY1 for the local waters, a transect of seven stations, including LY1, from Loch Spelve (Isle of Mull) to Loch Creran (see Tab. 2.1

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and Fig. 2.1 for positions and depths) was sampled on three occasions (on 18 July, 23 August and 13 September) during summer 2002.

2.2.1.2 Ellett Line transect

A transect of nine stations along the Ellett Line across the shelf and in the open Atlantic (stations and positions see Tab. 2.2 and Fig. 2.2) was sampled between 29 September and 4 October 2001 during cruise D257 (with *RV Discovery*) to assess the spatial abundance, distribution (vertical and horizontal) and composition of the phytoplankton community, with special focus on *Pseudo-nitzschia* spp.. Bad weather and ship time requirements prevented the sampling of more stations along the Ellett Line.

Table 2.2 Names, positions, dates and depths of stations sampled on a transect Mull (western Scotland) to Rockall (North Atlantic) on cruise D257 (*RV Discovery*), 21.09.-09.10.2001. Station 1G to 10G were situated on the shelf, while stations M and F were in the open ocean.

Station	Position	Date (2001)	approx. Depth [m]	Depths sampled [m]
1G	56° 40.03 N, 06° 08.21 W	29.09.	180	5, 15, 40, 80, 120, 171
2G	56° 41.08 N, 06° 09.25 W	29.09.	35	5, 15, 25
4G	56° 44.1 N, 06° 26.82 W	29.09.	96	5, 15, 30, 40, 60, 85
6G	56° 43.98 N, 06° 45.31 W	30.09.	41	5, 15, 30, 35
7G	56° 44.45 N, 06° 59.38 W	30.09.	135	5, 15, 40, 80, 100, 130
9G	56° 43.41 N, 07° 19.34 W	30.09.	146	5, 15, 40, 60, 80, 130
10G	56° 44.15 N, 07° 30.67 W	30.09.	220	5, 30, 50, 100, 150, 190
M	57° 17.96 N, 10° 22.94 W	03.10.	2200	5, 15, 30, 100, 1000, 2150
F	57° 30.5 N, 12° 14.96 W	04.10.	1804	5, 15, 30, 100, 850, 1800

2.2.2 Sample collection and analysis

2.2.2.1 LY1 and Spelve-Creran transect

Water samples for cell counts, chl *a* and inorganic nutrient analysis were collected in three casts from 10 m depth (LY1) or 5 m (on Spelve-Creran transect) with a 1 L Niskin bottle attached to a winch. LY1 was sampled at 10 m to sample a depth that is not much

influenced by a freshwater surface layer. The Spelve-Creran transect was sampled at 5 m due to shallow water depth in some parts of Loch Creran. Integrated net samples for further identification of plankton and isolation, cultivation and identification of *Pseudo-nitzschia* spp. (see chapter 3) were taken from 0-20 m depth with a 20 µm mesh hand plankton net (Hydro-Bios, Kiel). Temperature (T), and salinity (S) profiles of the upper 15 m of the water column were recorded with a CTD probe (Seabird 19). From T and S profiles the density (D) profile was derived. T, S and D data sets were kindly downloaded from the probe by SAMS staff from the Marine Technology group. C. Griffith kindly provided T, S and D raw contour plots for LY1, the Spelve-Creran and Ellett Line transects.

A 200 ml subsample of the Niskin bottle water sample was used for phytoplankton cell counts, and 50 ml of the plankton net sample was taken for electron microscopical identification of the *Pseudo-nitzschia* species composition in the field. These samples were preserved immediately with Lugol's Iodine (1% final concentration). The remaining water was transported back (in the dark and within one hour) to the laboratory, to be prepared for chl *a* and nutrient analysis.

Immediately after returning from the sampling site, 500 ml of the water sample were filtered in duplicate on 25 mm glass fibre filters (type A/E, Pall Corporation) and stored frozen at -20°C, to be later used for chl *a* analysis. Prior to the analysis filters were thawed, and pigments were extracted over night into 8 ml of 90% acetone. Filters were sonicated for one minute and after centrifugation (at 3000 rpm for 5 min) chl *a* was measured with a Turner TD-700 fluorometer.

Non-preserved net samples were immediately investigated under the microscope (Zeiss Anxiovert 100) for identification of taxa and isolation of mainly *Pseudo-nitzschia* cells for cultivation (see chapter 3).

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Samples for the determination of inorganic nutrient concentrations (silicate, phosphate and nitrate, ammonium was analysed starting on 28 February 2002) were collected from filtrate (A/E glass fibre filters), stored frozen at -20°C prior to analysis, and analysed by T. Brand (SAMS) with a LACHAT Quick chem 8000 autoanalyser using standard flow injection autoanalyser methods.

2.2.2.2 Ellett Line transect

At each Ellett Line station a CTD (conductivity, temperature, depth) profile was recorded, providing salinity, temperature, depth (in form of pressure) and density data. Water samples from six depths (see Tab. 2.2) were taken with 10 L water bottles attached to the CTD (Seabird 911), after observation of the CTD profile. The mixed layer, deep water and bottom water were sampled. Water samples were processed onboard the ship and later analysed as above. Plankton net samples were collected and analysed as described above.

2.2.3 Isolation and cultivation of *Pseudo-nitzschia* spp.

At LY1, from non-preserved fresh net samples, single *Pseudo-nitzschia* cells or chains were isolated and grown in culture, as described in chapter 3. Species were identified using morphological and molecular methods and maintained as described in chapter 3. From samples from cruise D257 *Pseudo-nitzschia* spp. were isolated in a similar way to those from LY1. However, on the ship, a Zeiss 4651251 light microscope was used for micropipette isolates and cultures were initially grown in a modified refrigerator with a varying temperature from 4 to 8 °C and a 15:9 light-dark cycle, until they were transferred to the CCAP culture facilities at the end of the cruise. Here they were re-

isolated, grown, identified and maintained together with the other *Pseudo-nitzschia* cultures as described in chapter 3.

2.2.4 Cell counts

Phytoplankton cells from all samples (LY1, Spelve-Creran transect and D257) were counted after the Utermöhl method (Utermöhl 1931) from 50 ml Lugol's Iodine fixed subsamples. Samples were settled in a 50 ml Utermöhl chamber, for 24 hours prior to the counts. Protists $> 5\mu\text{m}$ were counted using an inverted light microscope (Zeiss Anxiovert 100) at 200 x magnification. The area of at least half a chamber or 100 cells of the most abundant species were counted from each sample. Counting the whole chamber fixed the limit of detection of any species at $20 \text{ individuals} \cdot \text{L}^{-1}$. Included in the counts were common diatom, dinoflagellate and, if they were abundant, some ciliate species (only *Mesodinium rubrum* was included in the analysis). Most of the dinoflagellate species were summarised in two size classes (dinoflagellates $< 20 \mu\text{m}$ and $> 20 \mu\text{m}$), but species belonging to the genera *Ceratium*, *Dinophysis* or *Prorocentrum* were identified based on their morphology. Because of their great variety, *Chaetoceros* species were also enumerated in two size classes ($> 10 \mu\text{m}$ and $< 10 \mu\text{m}$, taking into account the body of the cell and not the setae). For morphological identification species keys by Tomas (1997) and Horner (2002) were used.

For *Pseudo-nitzschia* spp. the whole area of the chamber was inspected and all cells counted. Species were initially divided into three groups (Tab. 2.3). As it is not possible to confidently distinguish between *Pseudo-nitzschia* species within the *Pseudo-nitzschia* groups (*P. seriata* group: width $> 3 \mu\text{m}$, *P. delicatissima* group width $< 3 \mu\text{m}$ (Hasle 1965)) using the light microscope, groups one and two were combined for data analysis as the *P. seriata* group, leaving just two groups (*P. seriata* and *P. delicatissima*) for the

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data analysis (see Tab. 2.3). *P. americana* was counted separately, but was not recognised in samples prior to September (cruise D257) or December 2002 (LY1).

Table 2.3 Groups of *Pseudo-nitzschia* species that were initially counted, and then summarised for data analysis as the *P. seriata* and *P. delicatissima* groups after (Hasle, 1965).

<i>P. seriata</i> group		<i>P. delicatissima</i> group
Group 1	Group 2	Group 3
<i>P. australis</i> <i>P. seriata</i> <i>P. fraudulenta</i> <i>P. cf. subpacifica</i>	<i>P. pungens</i> <i>P. multiseriata</i>	<i>P. delicatissima</i> <i>P. pseudodelicatissima</i> <i>P. calliantha</i> other <i>Pseudo-nitzschia</i> species < 3 µm width

Sampling events where *Pseudo-nitzschia* spp. occurred in high diversity and cell density were identified by light microscopy. Net samples from those events were then inspected by transmission electron microscopy (TEM), to identify which *Pseudo-nitzschia* species commonly occurred in western Scottish waters. Most of the cultures were also inspected by TEM and additionally identified by genetic analysis. For information on species identification by TEM and genetic methods see chapter 3.

2.2.5 Statistical analysis of data sets

Data sets of phytoplankton abundance at LY1, along the Spelve-Creran transect and at the Ellett Line stations (cruise D257) were analysed with the multivariate statistical technique multidimensional scaling (MDS). This analysis illustrates similarities in the phytoplankton assemblage in different samples. Other multivariate techniques such as principal component analysis (PCA) and redundancy analysis (RDA) were applied to visualise the similarity between D257 stations regarding their environmental factors (with PCA) and to relate the biological factors (phytoplankton abundance) to the physical and chemical factors measured at LY1 and the D257 stations (with RDA). MDS and PCA were performed with the software package PRIMER™ (Plymouth

Routines in Multivariate Ecological Research, Plymouth, UK), RDA was carried out with the CANOCO software (Leps & Smilauer 2003). The analysis was performed in conjunction with T. Wilding (SAMS).

2.2.5.1 Multidimensional Scaling

First introduced by Shephard (1962) and Kruskal (1964), MDS (Clarke & Warwick 2001) constructs a map or configuration of the samples in a specified number of dimensions which attempts to satisfy all the conditions imposed by a rank (dis)similarity matrix. On the ordination the placement of samples, rather than representing their geographical location, reflects the similarity of their biological communities. The distances between samples on the ordination attempts to match the corresponding dissimilarities in community structure. Hence, nearby points have very similar communities, while samples which are far apart have few species in common or the same species at very different levels of abundance (Clarke & Warwick 2001).

At LY1 MDS was used to illustrate seasonality in the samples. Hence, abundance data for each species were summarised to express cell numbers for each month of the year. Mean monthly abundance values for the sampling period from November 2000 to July 2003 were calculated and used in the analysis.

For the Spelve-Creran transect MDS was applied to evaluate differences in assemblage composition at the different stations, in order to evaluate the representativeness of LY1, as a permanent monitoring station. First, species composition and abundance data for every sample (each of the three months) at each station were plotted. Then abundance data for each species for each station were calculated as the average of cell numbers in the three samples taken in July, August and September.

Along the Ellett Line the aim was to study differences in species abundance and composition between stations. The number of sampled depths and the actual sampled

depth was not the same at each station. Furthermore, most of the taxa were not present or occurred in only low numbers below 100 m depth. Hence, for each station only the mean of samples of the top 100 m of the water column, the approximate mixing depth, was used in the analysis.

For all sites, prior to the analysis, species abundance data were fourth root transformed, to down-weight the effect of highly abundant species. As cell numbers were taken into account and not biomass, a chain forming species with small cell size, as e.g. *Leptocylindrus minimus* would otherwise get a lot more weight than a species which consists of large, single cells, such as e.g. *Ditylum brightwellii*.

After transformation, MDS plots were constructed based on a Bray-Curtis dissimilarity matrix, which compares species pairs across each sample. The "stress" factor, shown on each MDS plot, is a measure of the degree to which the 2D or 3D representations of space portray the full dimensional space. It indicates the extent, to which the spatial configuration of points had to be stressed in order to obtain the data distances. A small value of "stress" factor (close to 0) is desirable. In general stress < 0.05 for ecological data gives an excellent representation of the data with no prospect of misinterpretation (Clarke & Warwick 2001).

2.2.5.2 Principal Component Analysis

The technique of principal component analysis (PCA) was first described by Pearson (1901). Similar to multidimensional scaling, PCA is a technique to approximate high-dimensional information in low-dimensional plots (Clarke & Warwick 2001). Data from a dissimilarity matrix of the environmental data sampled at different stations are used to define the positions of samples in relation to axes representing the full set of measured environmental parameters (Clarke & Warwick 2001). As in MDS ordination, the distance between two stations in the plot is a measure of their dissimilarity. The further

two points are apart, the more the environmental parameters at that station differ from each other. PCA uses Euclidean distances between samples while MDS used Bray-Curtis. Euclidean distance is appropriate where there are few zero values. PCA was used for multivariate analysis of the environmental data (temperature, salinity, density, phosphate, silicate and nitrate) along the stations of the Ellett Line transect. Again data from the upper 100 m water column were used after a square root transformation (the only option for root transformation in that programme) and normalisation across samples.

2.2.5.3 Redundancy Analysis

Redundancy analysis (RDA) was originally developed by Rao (1964). It can be described as a series of multiple linear regressions, using a linear model of relationships among environmental parameters and between the biological and environmental parameters. RDA may also be considered as a constrained extension of Principal Component Analysis (PCA) which identifies trends in the scatter of data points that are maximally and linearly related to a set of constraining (explanatory) variables (Makarenkov & Legendre 2002). RDA is used to investigate the strength of relationships between measured environmental factors (in this study: temperature, salinity, density, and inorganic nutrients) and individual species within a multivariate data set (Ter Braak & Smilauer 2002). As for PCA, RDA uses the Euclidean distance (Ter Braak & Prentice 1988). A Monte Carlo test is used to test the significance between environmental variables and the biotic composition.

RDA was carried out on the LY1 and D257 data sets to investigate which environmental factors significantly influenced species presence and abundance patterns. Prior to analysis, species abundance data were square root transformed to down-weight highly abundant species. At LY1, RDA was based on the absolute abundance of species to

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reflect changes in species numbers due to seasonality. For the D257 dataset species abundance was normalised (across samples) to analyse the relative abundance of each species in a given sample. The resultant ordination therefore reflects the relative species composition rather than absolute abundance differences between samples. For the D257 stations again the top 100 m of the water column were taken into account.

2.3 Results

2.3.1 LY1

2.3.1.1 Hydrography

2.3.1.1.1 Temperature

The temperature profile of the upper 15 m of the water column at LY1 from the period February 2001 to end of July 2003 is presented in Figure 2.4. Data from November 2000 to February 2001 were excluded, because they were measured with a hand-held probe that was unreliable and was replaced by a new probe. The profile shows temperature stratification, indicating that the water column was well mixed at LY1 during the sampled period.

A strong seasonal signal was observed with the coldest temperatures regularly found in March and the warmest in September. Between the seasons, a narrow temperature range in the 1 m surface water (6.4-14.5 °C) was observed as expected for Scottish coastal water (C. Griffith, personal communication). Some interannual variability was evident with a lower minimum temperature in the first winter (March 2001) than in early 2002 and 2003 and the highest annual temperature maximum in summer 2003 when 15 °C in 1 m depth (on 8 August 2003) were measured.

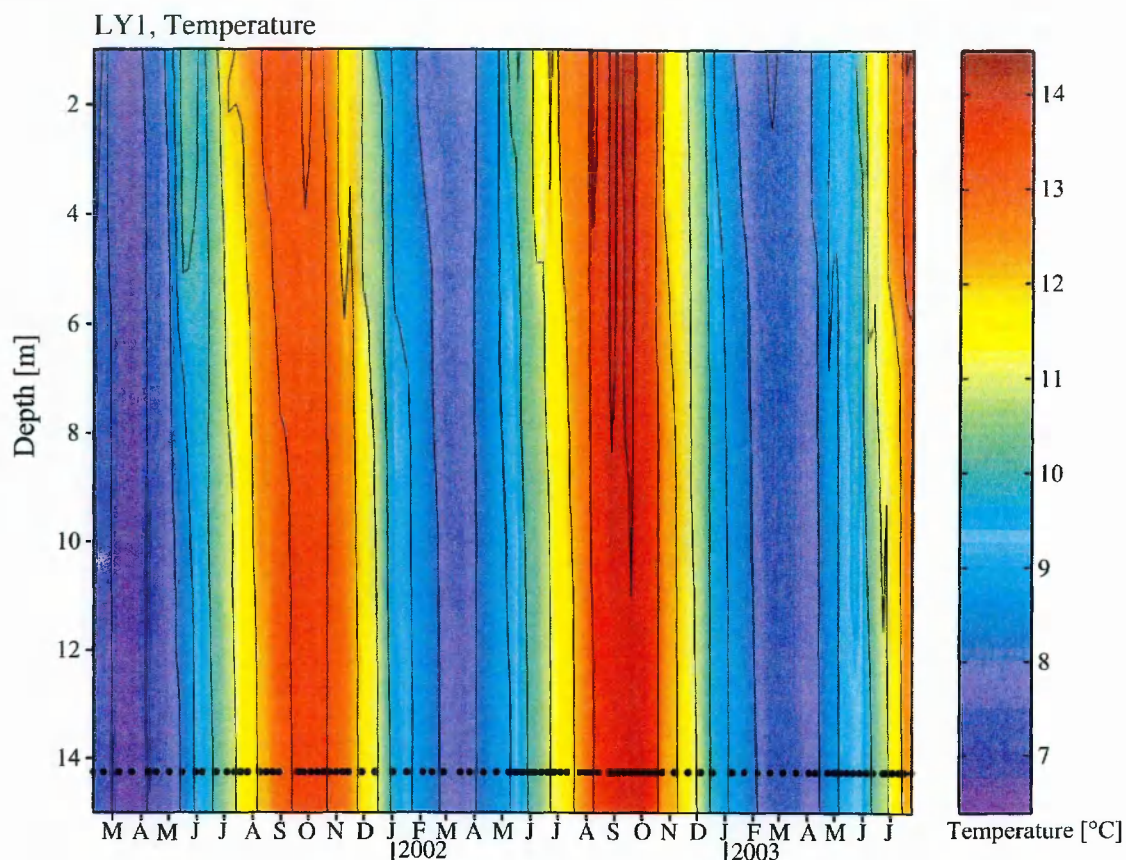


Fig. 2.4 Temperature profile of the 0-15m water column at LY1 from February 9 2001 until July 25 2003. Sampling events of which temperature data were included are indicated by a dot (•).

Samples for inorganic nutrient and phytoplankton analysis were taken from 10 m depth. Hence temperature, salinity and density data are presented for that depth in Figs. 2.5, 2.7 and 2.9.

Figure 2.5 shows the temperature data for 10 m depth at LY1 during the sampled period. The sinusoidal profile was expected, as water temperatures in these latitudes are typically increasing from spring towards autumn and decreasing in winter. The data reflect the strong seasonal signal that was observed in the upper 15 m of the water column (Fig. 2.4). Temperatures varied seasonally, reaching a minimal value in February and March and were highest during September and October. In general, the water temperature in 2001 was slightly colder than in 2002 and 2003.

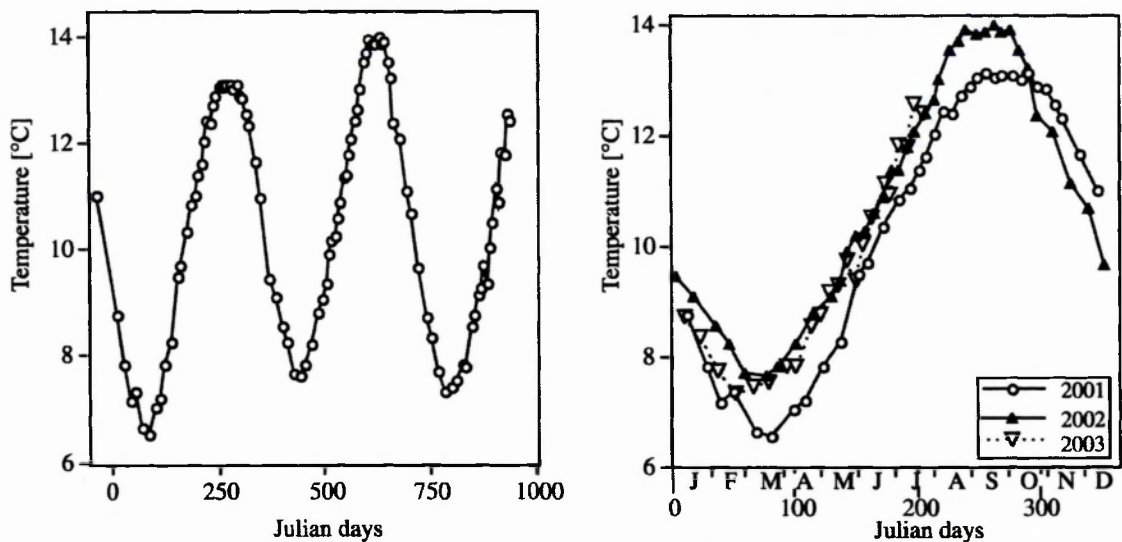


Fig. 2.5 Water temperature at LY1 in 10 m depth from January 2001 until July 2003.

2.3.1.1.2 Salinity

The salinity profile of the 15 m water column (Fig. 2.6) does not show the obvious seasonal patterns observed in temperature, although some trends can be recognised.

Fresh water influxes from Loch Etive and Loch Creran were observed in August and early November 2001, February and May to August 2002, February to April and from May 2003, leading to surface stratification in the upper 9 m. April and May were usually the months with the highest measured salinity, except for May 2003, reflecting the great amount of precipitation during that month (it rained for most of May 2003). The highest salinity at the surface was reached on 19 April 2001 (33.8) and the lowest of 23.3 on 15 February 2002.

In general the water at 10 m depth (from which phytoplankton and samples for inorganic nutrient analysis were taken) was not greatly influenced by a freshwater influx. However during months with high precipitation (for example November 2001 and late February 2002 and May 2003, see also Fig. 2.6) the freshwater influx led to the rapid decrease of salinity at that depth.

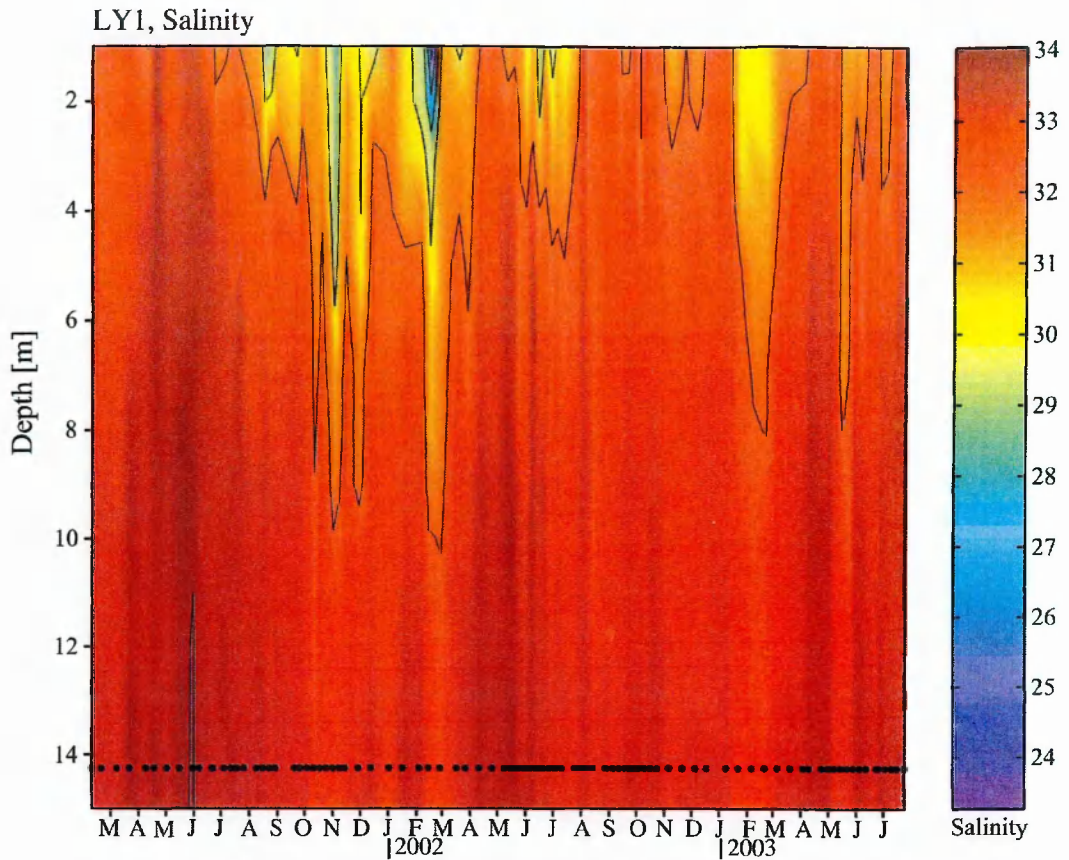


Fig. 2.6 Salinity profile of the 0-15 m water column at LY1 from 9 February 2001 until 25 July 2003. Sampling events of which salinity data were included are indicated by a dot (•).

As already noted for the upper 15 m of the water column, salinity in 10 m water at LY1 did not show the clear seasonal pattern as was observed in the water temperature (Fig. 2.7).

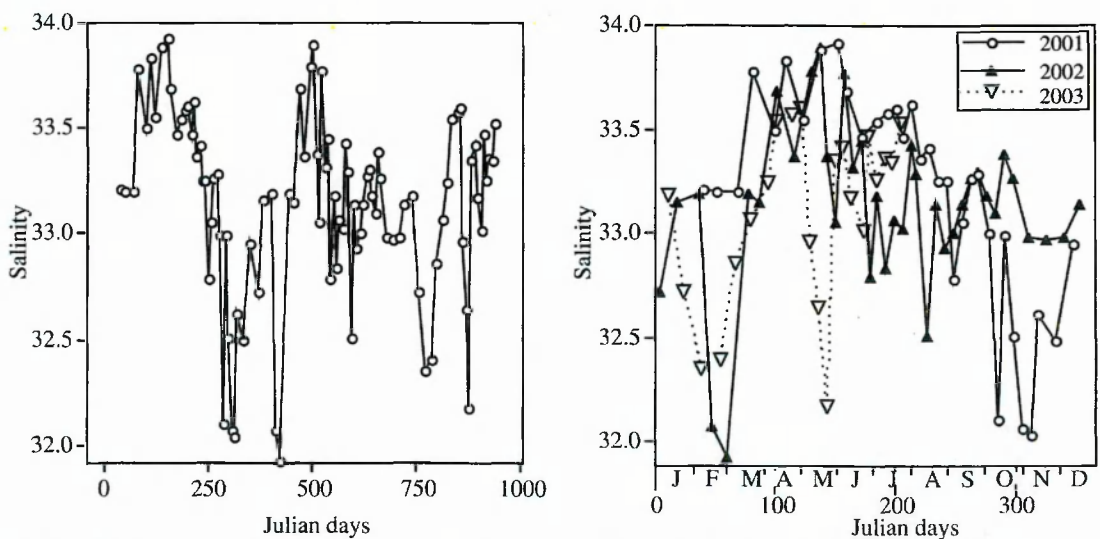


Fig. 2.7 Salinity of the water in 10 m depth at LY1 from January 2001 until July 2003.

Salinity in 10 m was very variable, although some trends were recognised, such as a general decrease from late May to November, with an increasing trend thereafter towards the next summer. Salinity ranged from 31.9 (28 February 2002) to 33.9 (1 June 2001) with high values usually found in May or early June and low values in November. Due to heavy and persistent precipitation, salinity minima were observed in early November 2001, at the end of February 2002 and in late May 2003.

2.1.1.1.3 Density

The density profile (Fig. 2.8) mirrors the salinity profile.

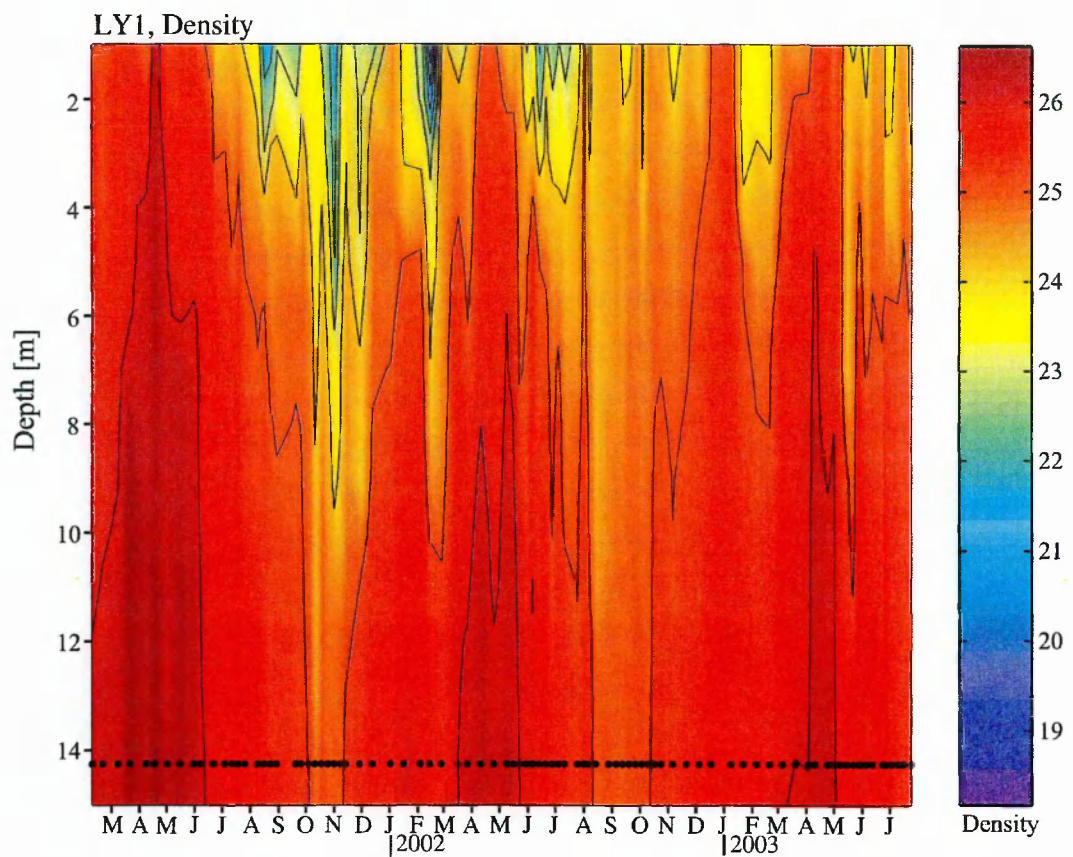


Fig. 2.8 Density [σ_t] profile of the 0-15 m water column at LY1 from February 2001 until July 2003. Sampling events of which density data were included are indicated by a dot (•).

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In general, the density profile shows the low stratification of the upper 15 m, with some surface stratification at periods of fresh water influx. Highest densities were found from March to May. From 8 m downwards a seasonal pattern, mainly influenced by the water temperature, with a slight interannual variability could be recognised: high densities from February to June 2001, March to June 2002 and in April 2003; low densities from October to December 2001 and August to mid-October 2002.

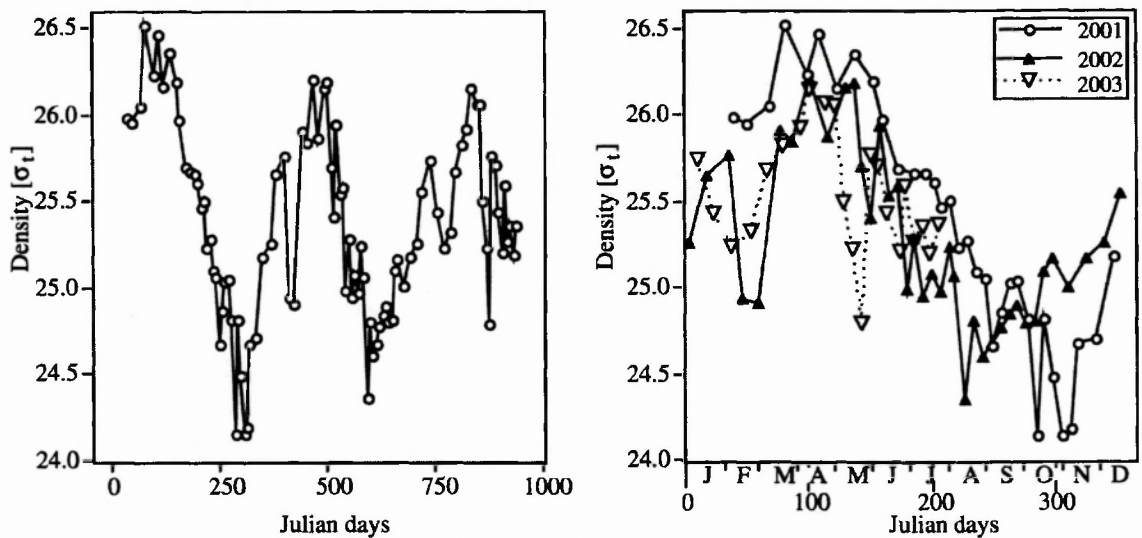


Fig. 2.9 Density [σ_t] of the water in 10 m depth at LY1 from January 2001 until July 2003.

Density values at 10 m showed seasonal trends that reoccurred in all years (Fig. 2.9). The density reached values between 26.5 (23 March 2001) and 24.1 (5 October 2001) with highest values observed from March to May and low densities in October, November 2001 and August 2002. High precipitation in May 2003 led to a rapid decrease in density until the end of that month.

2.3.1.2 Nutrients

2.3.1.2.1 Phosphate

The dissolved inorganic phosphate (DIP) concentration reflects a seasonal pattern with highest values in winter and lowest values in summer (Fig. 2.10). Interannual variability was recognised: the DIP concentration generally increased between the years, with lowest values in 2001 and highest in 2003. Values between May and July 2003 showed high variability. Over a seasonal cycle, the inorganic phosphate concentration rapidly decreased from March (2001, 2003) or April (2002) until the summer. This was followed by an increase between July and December. Phosphate concentrations then stayed relatively constant until February. A significant negative correlation between the DIP concentration and salinity was found (Spearman rank, $p < 0.0001$).

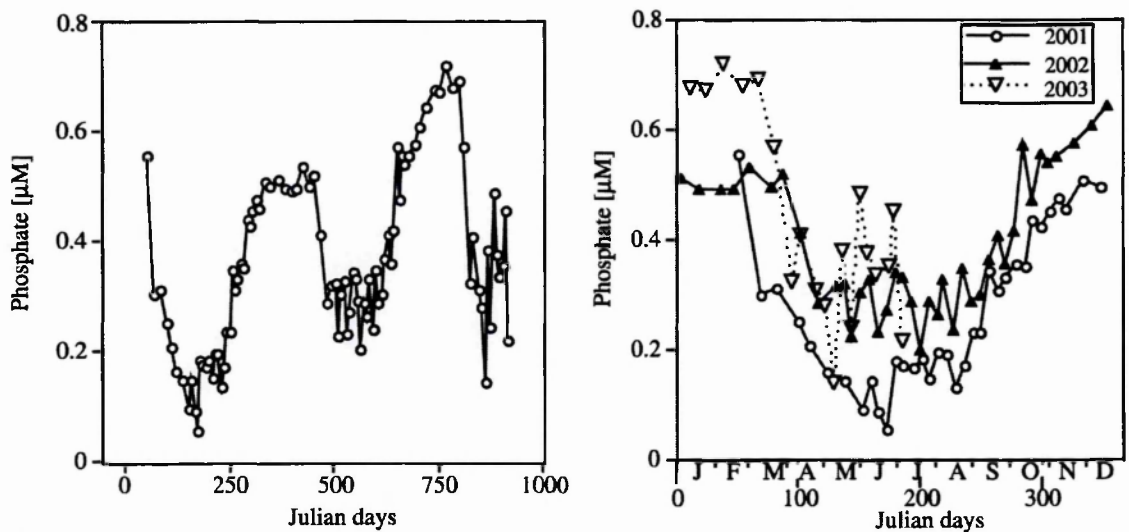


Fig. 2.10 Dissolved inorganic phosphate concentration in the water column at 10 m depth at LY1 from January 2001 until July 2003.

2.3.1.2.2 Silicate

The dissolved inorganic silicate (DSi) concentration in 10 m water (Fig. 2.11) followed a similar seasonal pattern as in the DIP concentrations, with highest values in February

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(2001 and 2003) or March (2002), which rapidly decreased until April (in 2002 and 2003) or May (in 2001) and thereafter showed an oscillating increase.

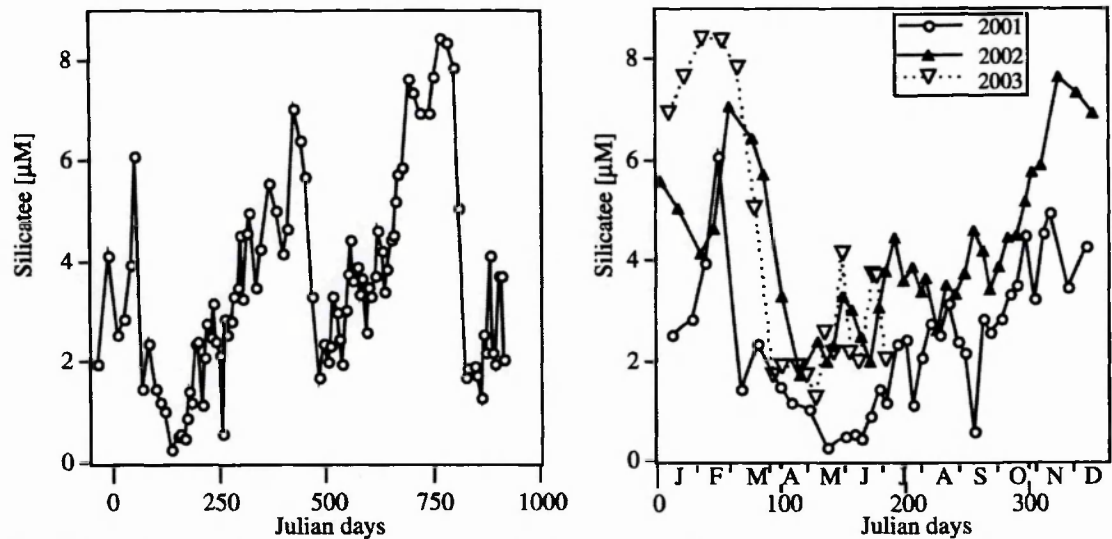


Fig. 2.11 Dissolved inorganic silicate (DSi) concentration in the water column at 10 m depth at LY1 from January 2001 until July 2003.

Interannual variability was expressed in generally higher concentration in February 2003 and during 2002 compared to 2001. The DSi concentration was negatively correlated with salinity at 10 m depth (Spearman rank, $p < 0.0001$).

2.3.1.2.3 Nitrate

The dissolved inorganic nitrate (DIN) concentration (Fig. 2.12) showed a clear sinusoidal, seasonal pattern with lowest concentrations during the summer months (June to August) and highest concentrations during late winter (end of February). Nitrate concentrations were generally lower during the spring and summer decrease in 2001, apart from this period interannual variability was low. A negative correlation between salinity and the DIN concentration was observed (Spearman rank, $p < 0.0001$).

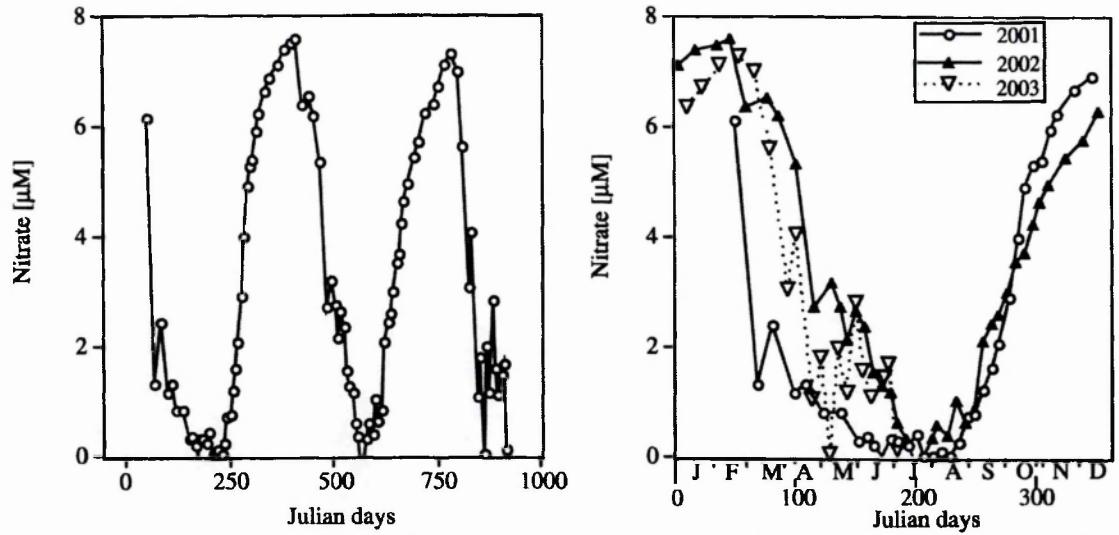


Fig. 2.12 Dissolved inorganic nitrate (DIN) concentration in the water column at 10 m depth at LY1 from January 2001 until July 2003.

2.3.1.2.4 Ammonium

The concentration of ammonium in 10 m water showed considerable variability (Fig. 2.13). The number of samples taken was not sufficient to make conclusions about interannual trends. In general, elevated ammonium concentrations were observed from May to September, with sudden decreases during July, and low values during autumn and winter. The ammonium concentration was not significantly correlated with temperature or salinity (Spearman rank, $p = 0.48$, $p = 0.22$, respectively).

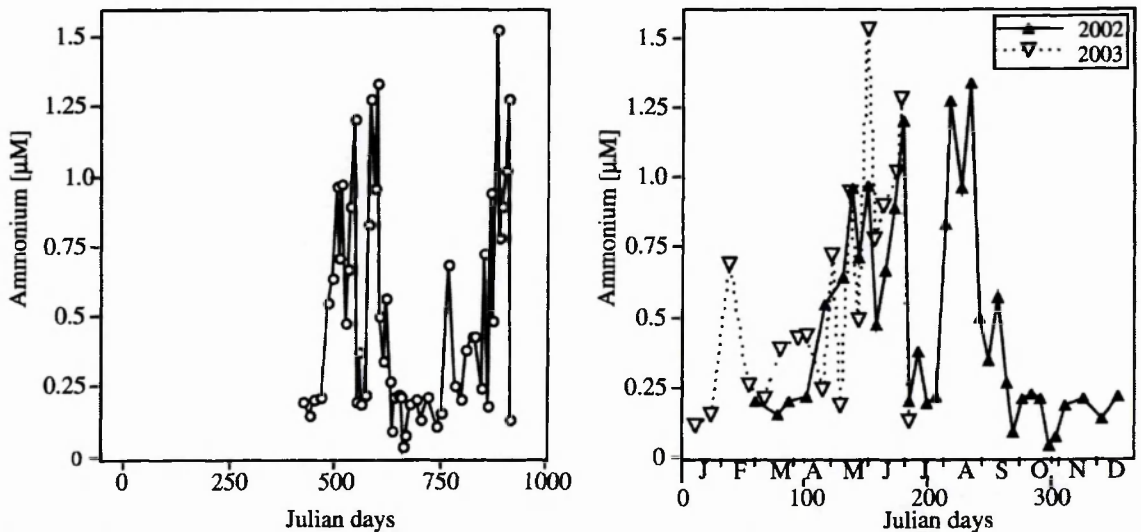


Fig. 2.13 Ammonium concentration in the water column at 10 m depth at LY1 from end of February 2002 until July 2003.

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2.3.1.3 Phytoplankton

During the phytoplankton monitoring at station LY1, between November 2000 and end of July 2003, 53 taxa were identified (see species list overleaf). They included 35 diatom taxa belonging to 20 genera, 16 dinoflagellate taxa comprising ten genera, which were mainly enumerated in two size classes, the silicoflagellate *Dictyocha speculum* and the ciliate *Mesodinium rubrum*. Based on the revision of Krainer & Foissner (1990), this ciliate is also called *Myrionecta rubra*. However, as in the literature it is mostly referred to as *Mesodinium rubrum*, this name is also used in this study. Other ciliates were present in low numbers, but were not identified or enumerated.

To obtain cumulative species densities, cell numbers from each sample were summed. Numerically the most abundant taxon was *Chaetoceros* spp. cells $< 10 \mu\text{m}$ (Tab. 2.4), reaching the highest cumulative value with about $27 \times 10^6 \text{ cells} \cdot \text{L}^{-1}$ and also the highest observed maximal number of cells in a single sample (about $4 \times 10^6 \text{ cells} \cdot \text{L}^{-1}$). The second most numerous species was *Skeletonema costatum*, a species which was highly abundant during the spring bloom in all three years and also during most of the rest of the year, occurring in 95% of the samples.

Pseudo-nitzschia species belonging to the *delicatissima* and *seriata* groups were the fourth and fifth most abundant species on a cumulative basis, with maximum cell numbers in a single sample of 1.6×10^5 and $1.1 \times 10^5 \text{ cells} \cdot \text{L}^{-1}$, respectively. They occurred in 95% (*P. delicatissima* group) and 83% (*P. seriata* group) of all samples. *P. americana* was usually found as an epiphyte on other diatoms such as *Chaetoceros* spp. and was not recognised prior to December 2002. Hence it was only identified in 7 of 23 samples.

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Species list:

Bacillariaceae

- P. seriata* group (including species with a width > 3 µm, such as: *P. australis* Frenguelli, *P. seriata* (P. T. Cleve) H. Peragallo, *P. fraudulenta* (P. T. Cleve) Hasle, *P. pungens* (Grunow ex P. T. Cleve) Hasle, *P. multiseriata* (Hasle) Hasle, *P. cf. subpacificata* (Hasle) Hasle)
- P. delicatissima* group (including species < 3 µm wide, such as: *P. delicatissima* (P. T. Cleve) Heiden, *P. pseudodelicatissima* (Hasle ex Hasle) Lundholm, Hasle et Moestrup, *P. calliantha* Lundholm, Moestrup et Hasle)
- P. americana* (Hasle) Fryxell
- Asterionellopsis glacialis* (Castracane) Round
- Chaetoceros* spp. [genus: Ehrenberg] (> 10 µm)
- Chaetoceros* spp. [genus: Ehrenberg] (< 10 µm)
- Corethron* sp. [genus: Castracane]
- Coscinodiscus* spp. [genus: Ehrenberg]
- Cylindrotheca closterium* (Ehrenberg) Lewin & Reimann
- Dactyliosolen fragilissimus* (Bergon) Hasle
- Ditylum brightwellii* (West) Grunow
- Eucampia zodiacus* Ehrenberg
- Guinardia delicatula* (P. T. Cleve) Hasle
- Lauderia annulata* P. T. Cleve
- Leptocylindrus danicus* P. T. Cleve
- Leptocylindrus minimus* Gran
- Meuniera membranacea* (P. T. Cleve) P. C. Silva
- Odontella mobiliensis* (Bailey) Grunow
- Paralia sulcata* (Ehrenberg) Cleve
- Pleurosigma* sp. [genus: W. Smith]
- Rhizosolenia setigera* Brightwell
- Rhizosolenia styliformis* Brightwell
- Skeletonema costatum* (Greville) P. T. Cleve
- Stephanopyxis turris* (Arnott in Greville) Ralfs
- Thalassionema nitzschioides* (Grunow) Grunow
- Thalassiosira* spp. [genus: P. T. Cleve] (including *T. anguste-lineata* (A. Schmidt) Fryxell & Hasle, *T. nordenskiöldii* P. T. Cleve, *T. rotula* Meunier, and other unidentified species)

Dinophyceae

Dinoflagellates > 20 µm

- (including *Protooperidinium* spp. [genus: Bergh], *Gonyaulax* spp. [genus: Diesing], *Lingulodinium polyedrum* (Stein) Dodge, *Gymnodinium* spp. [genus: Stein], *Gyrodinium* spp. [genus: Kofoid & Swezy], *Katodinium* spp [genus: Fott], *Alexandrium* spp. [genus: Halim])

Dinoflagellates < 20 µm

- (including *Gonyaulax* spp. [genus: Diesing], *Gymnodinium* spp. [genus: Stein], *Gyrodinium* spp. [genus: Kofoid & Swezy])
- Ceratium fusus* (Ehrenberg) Dujardin
- Ceratium furca* (Ehrenberg) Claparède & Lachmann
- Ceratium lineatum* (Ehrenberg) P. T. Cleve
- Ceratium longipes* (Bailey) Gran
- Ceratium tripos* (O.F. Müller) Nitzsch
- Dinophysis norvegica* Claparède & Lachmann
- Dinophysis acuta* Ehrenberg
- Dinophysis acuminata* Claparède & Lachmann
- Prorocentrum micans* Ehrenberg

Dictyochophyceae

- Dictyocha speculum* Ehrenberg

Ciliata

- Mesodinium rubrum* Lohmann

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Table 2.4 List of the enumerated taxa at LY1 in rank order of total density over the sampling period, with cumulative cell · L⁻¹ values, the maximum cell number found in a single sample, frequency of occurrence in 100 samples and the average number per sample in which a species was present. The latter was calculated by dividing the cumulative number by the frequency, this was used as the threshold cell number in Figure 2.6.

Taxon	Cumulative number [cells · L ⁻¹]	Max. number [cells · L ⁻¹]	Frequency	Average [cells · L ⁻¹]
<i>Chaetoceros</i> spp. < 10 µm	26,948,763	3,892,666	82	328,643
<i>Skeletonema costatum</i>	22,045,027	3,146,285	95	232,053
<i>Chaetoceros</i> spp. > 10 µm	4,416,369	478,176	81	54,523
<i>Leptocylindrus minimus</i>	4,293,640	1,676,487	55	78,066
<i>P. delicatissima</i> group	935,205	161,120	95	9,844
<i>P. seriata</i> group	824,240	105,840	83	9,931
<i>Thalassiosira</i> spp.	660,004	125,600	90	7,333
<i>Leptocylindrus danicus</i>	487,951	80,380	60	8,133
<i>Guinardia delicatula</i>	445,171	14,1894	71	6,270
Dinoflagellates < 20 µm	371,760	23,000	95	3,913
<i>Mesodinium rubrum</i>	356,741	150,096	21	16,988
<i>Asterionellopsis glacialis</i>	222,720	40,400	44	5,062
<i>Cylindrotheca closterium</i>	214,280	10,260	100	2,143
Dinoflagellates > 20 µm	163,320	13,120	92	1,775
<i>Dactyliosolen fragilissimus</i>	134,780	27,240	37	3,643
<i>Eucampia zodiacus</i>	121,020	39,860	47	2,575
<i>Lauderia annulata</i>	97,540	26,880	42	2,322
<i>Thalassionema nitzschioides</i>	37,740	6,000	52	726
<i>Dictyocha speculum</i>	21,300	2,520	58	367
<i>Paralia sulcata</i>	18,660	1,580	53	352
<i>Rhizosolenia setigera</i>	15,120	2,540	35	432
<i>Ditylum brightwellii</i>	12,500	1,760	63	198
<i>Prorocentrum micans</i>	11,800	2,200	49	241
<i>Rhizosolenia styliformis</i>	8,080	1,920	25	323
<i>Pleurosigma</i> sp.	5,420	720	62	87
<i>Meuniera membranacea</i>	4,600	1,120	16	288
<i>Ceratium fusus</i>	3,100	500	32	97
<i>Dinophysis norvegica</i>	2,400	340	37	65
<i>Stephanopyxis turris</i>	1,240	240	9	138
<i>Ceratium lineatum</i>	1,180	300	12	98
<i>Ceratium furca</i>	940	180	15	63
<i>Dinophysis acuta</i>	740	120	18	41
<i>Dinophysis acuminata</i>	560	220	10	56
<i>Coscinodiscus</i> spp.	460	180	10	46
<i>P. americana</i>	180	40	7 (out of 23)	26
<i>Ceratium longipes</i>	60	40	2	30
<i>Odontella mobiliensis</i>	40	40	1	40
<i>Corethron</i>	40	20	2	20
<i>Ceratium tripos</i>	40	20	2	20

Cylindrotheca closterium was the only diatom that was present in all samples, even during winter (between late November and early February), when only a few taxa were

present. Despite that it never reached a cell density higher than 10^4 cells \cdot L⁻¹. Some species occurred only rarely (in 2% of all samples or less) and in very low numbers (60 or less cells \cdot L⁻¹, representing 3 cells or less in a whole counting chamber), e.g. *Ceratium longipes*, *C. tripos*, *Odontella mobiliensis*, *Corethron* sp. and *Coscinodiscus* sp.. Those species were excluded from the analysis. Cell numbers at each sampling occasion for each taxon (including *Pseudo-nitzschia* species) are plotted in appendix 1. While some of the species were found at a particular season of the year, others were more variable (see appendix 1). *Chaetoceros* species and *Dactyliosolen fragilissimus*, for example, occurred mainly from April until the end of July, while *Asterionellopsis glacialis* and *Leptocylindrus danicus* were most abundant from June until October. *Paralia sulcata* showed an unpredictable abundance pattern with highest numbers (up to 1600 cells \cdot L⁻¹) during December. *Stephanopyxis turris*, although only observed in low cell numbers (< 250 cells \cdot L⁻¹), was only found in late August and September.

2.3.1.4 Chlorophyll *a* concentration and bloom events

Figure 2.5 presents chlorophyll *a* (chl *a*) concentrations from January 2001 until end of July 2003. The start of the "spring increase", defined by Tett & Wallis (1978) as the date on which chl *a* concentrations first exceed 1 μ g \cdot L⁻¹, was regular in its timing and occurred in 2001 between 9 March and 23 March, in 2002 between 18 March and 28 March and in 2003 between 7 March and 21 March. Due to the sampling interval of two weeks during that time of the year, the exact date was not recorded.

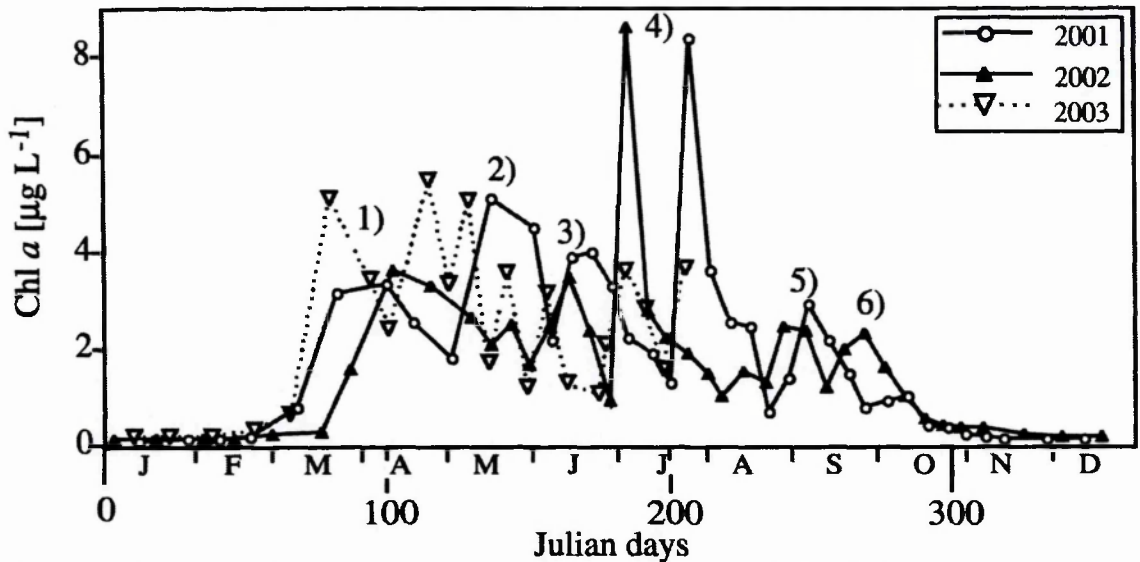


Fig. 2.14 Chlorophyll concentrations in 10m water at LY1 from 2001 to the end of July 2003. Numbers within the plot indicate chl *a* maxima, interpreted as reoccurring bloom events.

Chl *a* maxima were interpreted as events of high phytoplankton biomass (blooms) and are plotted in Figure 2.14. Taking into account chl *a* data from January 2001 until July 2003, six events of high chl *a* concentrations were identified in each year. The first maximum of the chl *a* concentration (1) (see Tab. 2.5 and Fig. 2.14) was observed in early April (2001 and 2002) and March (2003). At this time, *Skeletonema costatum* was the most abundant taxon during spring in all years, second and third highest numbers were found in diatoms belonging to the *P. delicatissima* group and *Thalassiosira* spp., respectively (in 2003 *Thalassiosira* spp. reached higher numbers than cells of the *P. delicatissima* group).

The second bloom, with a higher chl *a* maximum in 2002 and 2003 and a lower concentration than the first bloom in 2001, occurred in April (2003) or May (2001 and 2002) and was dominated by species belonging to the genus *Chaetoceros*. In 2003 this "second" bloom event was represented by two peaks. A high chl *a* concentration on April 25, was followed two weeks later, on 9 May, by a second peak in the chl *a* concentration at which time high numbers of *Chaetoceros* spp. were observed.

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The next bloom (3) was observed in late June (in 2003 in late May and early June) and was again dominated by *Chaetoceros* spp.. In 2002 and 2003 the *Chaetoceros* spp. bloom was accompanied by *Skeletonema costatum* and in 2002 also by diatoms belonging to the *P. delicatissima* group. This third bloom again occurred in two events at the end of May and beginning of June, where high chl *a* concentrations were observed.

Table 2.5 Events of high chlorophyll concentrations ("blooms") and taxa that were most abundant at that time.

Bloom/ Month	Date	Julian day	Most abundant taxa
1) March, April	10.4.2001	100	<i>Skeletonema costatum</i> , <i>Pseudo-nitzschia delicatissima</i> group, <i>Thalassiosira</i> spp.
	12.4.2002	102	<i>Skeletonema costatum</i> , <i>P. delicatissima</i> group, <i>Thalassiosira</i> spp.
	21.3.2003	80	<i>Skeletonema costatum</i> , <i>Thalassiosira</i> spp., <i>P. delicatissima</i> group
2) April, May	17.5.2001	137	<i>Chaetoceros</i> spp. < 10 µm, <i>Thalassiosira</i> spp., <i>Skeletonema costatum</i> , <i>Leptocylindrus minimu</i> .
	24.5.2002	144	<i>Chaetoceros</i> spp. (all sizes), <i>Eucampia zodiacus</i>
	25.4.2003, 9.5.2003	115 129	<i>Chaetoceros</i> spp. (all sizes). <i>Chaetoceros</i> spp. (all sizes), <i>Skeletonema costatum</i> .
3) May, June	22.6.2001	173	<i>Chaetoceros</i> spp. (all sizes)
	14.6.2002	165	<i>Chaetoceros</i> spp. (all sizes), <i>Skeletonema costatum</i> , <i>P. delicatissima</i> group
	23.5.2003, 6.6.2003	143 157	<i>Chaetoceros</i> spp. < 10 µm, <i>S. costatum</i> <i>Chaetoceros</i> spp. < 10 µm, <i>S. costatum</i>
4) July	27.7.2001	208	<i>Mesodinium rubrum</i> , <i>Leptocylindrus danicus</i> , <i>P.</i> group
	5.7.2002	186	<i>Mesodinium rubrum</i> , <i>P. seriata</i> group, dinoflagellates (all sizes)
	4.7.2003, 25.7.2003	185 206	<i>P. seriata</i> group, <i>L. minimus</i> <i>P. seriata</i> group, <i>Mesodinium rubrum</i>
5) August, September	7.9.2001	250	<i>Skeletonema costatum</i> , <i>Asterionellopsis glacialis</i> , <i>Chaetoceros</i> spp. > 10 µm
	29.8.2002	241	<i>Leptocylindrus danicus</i> , <i>Guinardia delicatula</i> <i>Chaetoceros</i> spp. (< 10 µm), <i>P. seriata</i> group
6) September, October	12.10.2001	285	<i>Skeletonema costatum</i> , <i>Chaetoceros</i> spp. (all sizes), <i>P. seriata</i> group
	27.9.2002	270	<i>Skeletonema costatum</i> , <i>Chaetoceros</i> spp. (< 10 µm), <i>P. seriata</i> group, <i>Thalassiosira</i> spp.

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2.1.1.5 Temperature and species succession

Figure 2.15 describes the range of the water temperature over which different taxa occurred. It also indicates the temperature at which each taxon was found in maximal cell density.

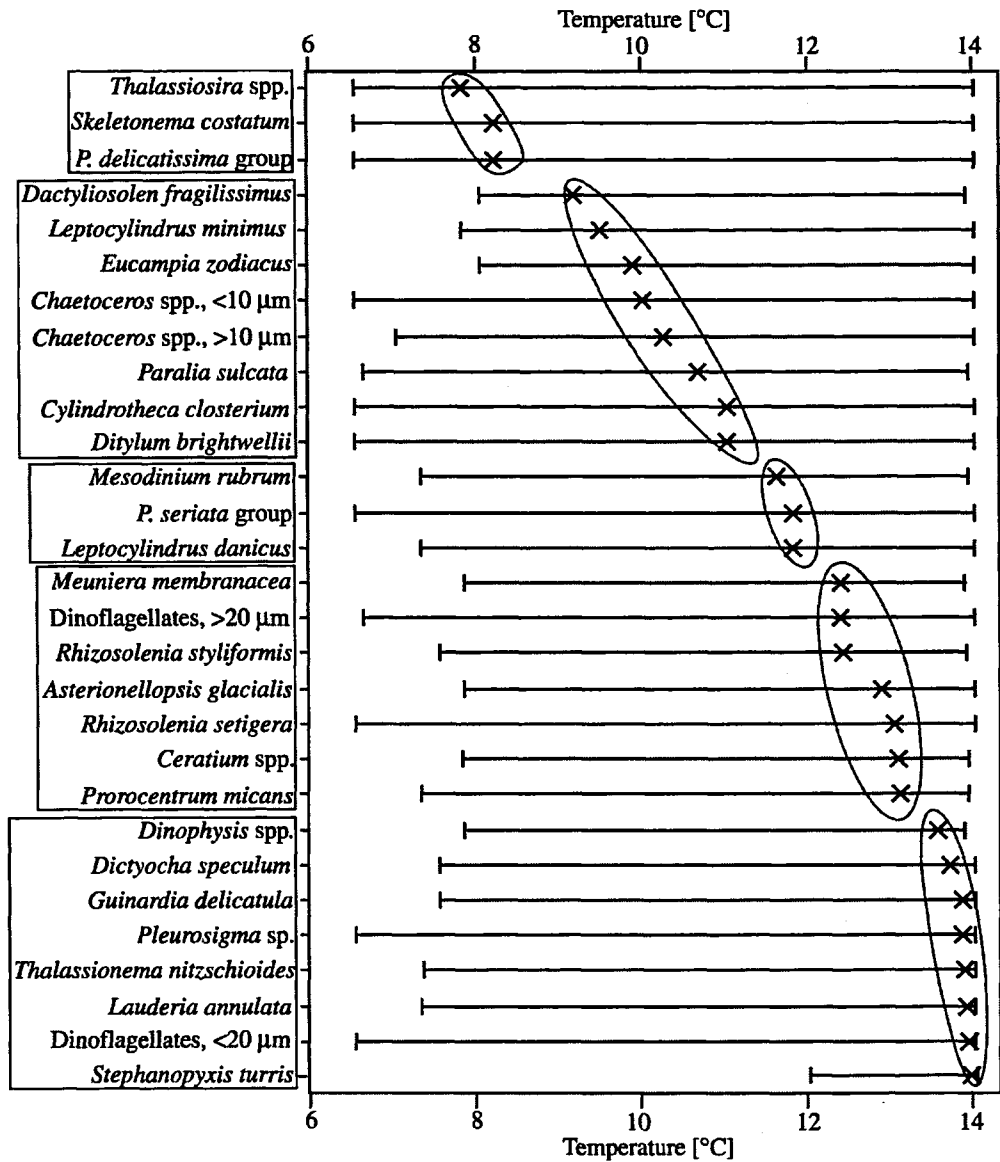


Fig. 2.15 Range of temperatures (|—|) and temperature (x) at which the maximum abundance of the individual taxa was observed.

The seasonal succession of taxa, as observed at LY1, is shown in Figure 2.16. For each species only a cell density above an individual threshold of cells · L⁻¹ (as indicated in parentheses) was taken into account. This threshold represents the average abundance of each taxon per sample in which it was present. It was calculated by dividing the total

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number of cells for each taxon by the number of samples in which that taxon was observed (see Tab. 2.4).

The water temperature observed at LY1 in 10 m depth between January 2001 and July 2003 ranged from 6.55 to 14.0 °C. Most of the species occurred over this entire temperature range, and apart from *Stephanopyxis turris* all taxa were found between 8 and 13.8°C. However, differences were observed in the temperature of their maximum cell density (Fig. 2.15), suggesting a preferred temperature. For easier description of the observations, taxa were divided into five groups related to their temperature preferences.

The first group consisted of *Thalassiosira* species, *Skeletonema costatum* and species belonging to the *P. delicatissima* group. They all occurred over the entire temperature range, but were most abundant in spring, when the water temperature was around 8°C (Fig. 2.15), regularly forming the spring bloom (Fig. 2.16). *Skeletonema costatum* and species of the *P. delicatissima* group were also regularly found between mid-March and late September, with occasional high cell densities during summer. More details about the occurrence of the *P. delicatissima* group are described below (see also Tab. 2.6). *Thalassiosira* species showed a maximal abundance in March, high numbers in May or June as well as later in July and September (Fig. 2.16).

The second group of species exhibited a temperature optimum of 9 to 11°C (*Dactyliosolen fragilissimus*, *Leptocylindrus minimus*, *Eucampia zodiacus*, *Chaetoceros* species, *Paralia sulcata*, *Cylindrotheca closterium* and *Ditylum brightwellii*). Together with *Thalassiosira* spp., *Skeletonema costatum* and *P. delicatissima* group, the above species represented the continuation of the spring bloom, also recognised in the second chlorophyll maximum (Fig. 2.14, peak 2). *Chaetoceros* species persisted mainly from April to early July, while *Dactyliosolen fragilissimus* cell numbers exceeded threshold for only about two month (Fig. 2.16). *Cylindrotheca closterium* was the only species

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present in all samples, and reached a cell density above the average mainly from May to early October and also occasionally showed high abundance in February or March. *Eucampia zodiacus* bloomed during the summer, from late May to early August. *Ditylum brighwellii* was found in relatively high cell densities over a period of seven months (from late March until mid-October).

Paralia sulcata was the only species that was present above its threshold throughout winter (Fig. 2.16). It was the earliest species to appear in each year (January or early February) and its highest density was found in December.

Leptocylindrus minimus reached greatest cell densities for a relatively short period from mid-May until early July, contributing to the spring and summer blooms.

The next group of taxa represents those responsible for the yearly observed summer bloom (Fig. 2.14, peak 4). *Mesodinium rubrum*, diatoms belonging to the *P. seriata* group and *Leptocylindrus danicus* were present in maximal cell numbers at temperatures between of 11 to 12°C (Fig. 2.15). The ciliate was regularly found in high numbers from July to mid-August, while *L. danicus* bloomed from late June until September. Species belonging to the *P. seriata* group were found in high numbers between mid-June and early October, and are listed in more detail below (see Tab. 2.7).

Group four included *Meuniera membranacea*, *Rhizosolenia styliformis* and *R. setigera*, *Asterionellopsis glacialis*, dinoflagellates > 20 µm, *Ceratium* species and *Prorocentrum micans*, and showed highest cell densities at a water temperature between 12.4. and 13.1°C. Those species might be typical summer/ early autumn species. While *Meuniera membranacea* was the second earliest species to be found at LY1 (end of January, Fig. 2.16) it reached its maximum cell density in late summer. *Asterionellopsis glacialis* was abundant between the end of June and mid-October (Fig. 2.16). *Rhizosolenia* species were found in higher cell densities between July and mid-October.

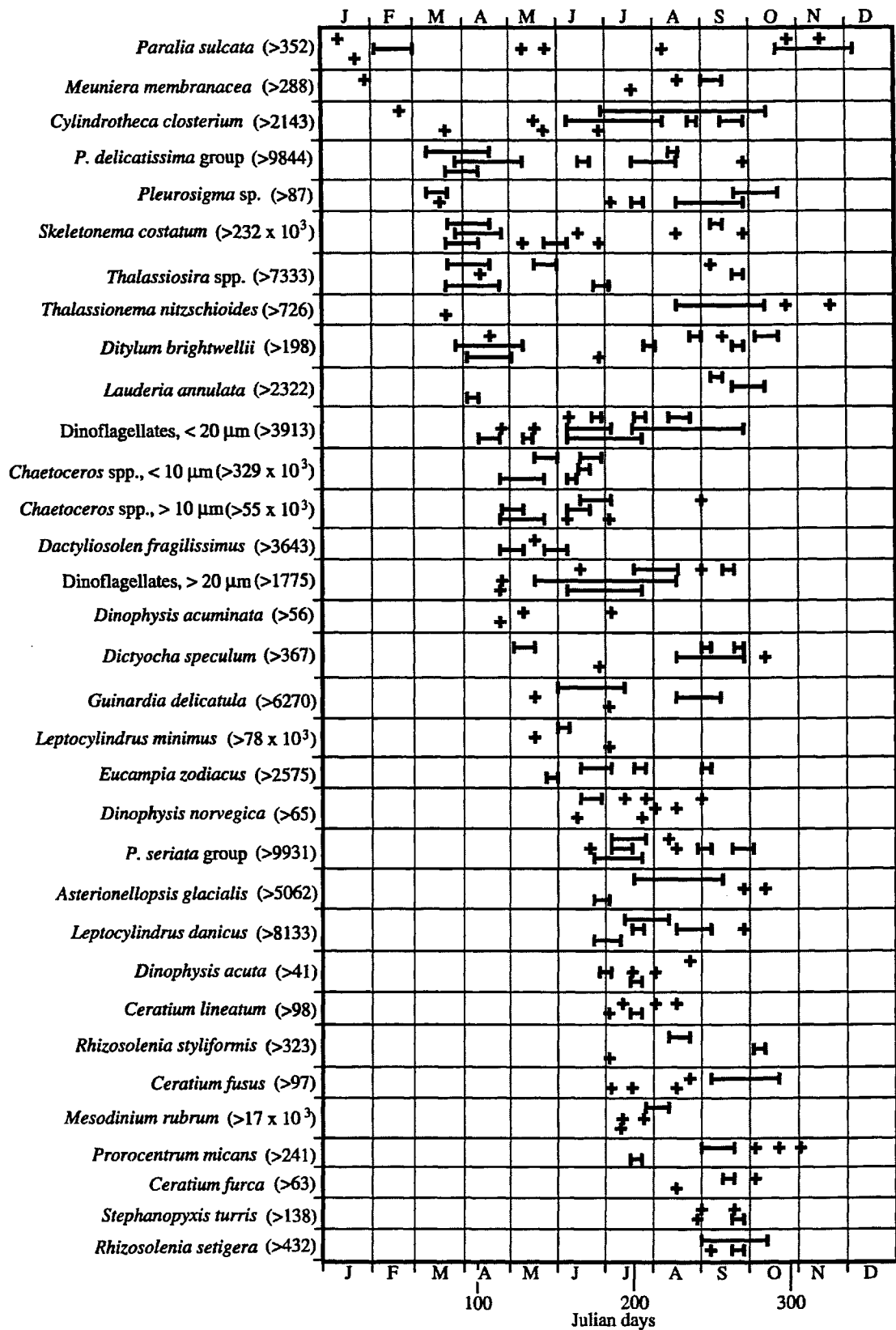


Fig. 2.16: Species succession at LY1, in 10 m water depth. For each species the period of its occurrence at a cell density · L⁻¹ above an individual threshold (total number of cells / frequency [cells · L⁻¹], given in parentheses behind each species name). Periods of high abundance are presented for each species from January 2001-July 2003 (for each species top bar = 2001, middle bar = 2002, lowest bar = 2003). In some years certain species did not occur in numbers above the threshold. A bar (|-|) indicates a period of time when cells · L⁻¹ were above the threshold, while + indicates a single observation of high cell density at that sampling occasion. Time is indicated in months and Julian days.

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Most of the dinoflagellates showed greatest abundance during summer and early autumn. The high numbers of dinoflagellates $> 20 \mu\text{m}$ in July reflects the high abundance of *Lingulodinium polyedrum* at that time. *Ceratium* species occurred in numbers exceeding the threshold cell number per litre from July until the end of October, *Prorocentrum micans* was present during July and again from September until early November (Fig. 2.16). The last group comprised taxa with maximum cell densities at the highest measured temperature (13.5-14°C): the diatoms *Guinardia delicatula*, *Pleurosigma* sp., *Thalassionema nitzschioides*, *Lauderia annulata* and *Stephanopyxis turris*, the silicoflagellate *Dictyocha speculum*, dinoflagellates $< 20 \mu\text{m}$ and those belonging to the genus *Dinophysis* (Fig. 2.15). *Thalassionema nitzschioides*, *Lauderia annulata*, *Guinardia delicatula*, *Pleurosigma* sp. and *Dictyocha speculum* were present in high cell numbers during spring and late summer/autumn (Fig. 2.16), but most abundant in late summer/autumn, demonstrating a preference for water temperatures representing late summer conditions.

Stephanopyxis turris had the smallest temperature range of all taxa. It was found at temperatures between 12 and 14°C (Fig. 2.15), reaching highest cell numbers between the end of August and end of September (Fig. 2.16). Dinoflagellates $< 20 \mu\text{m}$ showed maximal abundance from April to the end of September. Similarly, *Dinophysis* species were abundant from April to the end of August, with high cell numbers of *D. acuminata*, *D. norvegica* and *D. acuta* appearing at the end of April, in early and late June, respectively.

2.3.1.6 *Pseudo-nitzschia* spp. blooms

A major objective of this study was to investigate the occurrence and ecology of *Pseudo-nitzschia* species. Tables 2.6. and 2.7 show the occurrence of diatoms belonging to the *P. delicatissima* and *P. seriata* group in densities of approximately their average

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(= 10^4 cells · L⁻¹) and half average (= 5000 cells · L⁻¹) observed abundance (see appendix 1 for time series graphs).

Table 2.6 Occurrence of diatoms belonging to the *P. delicatissima* group from January 2001 to July 2003 in threshold or half threshold cell densities.

Year	Cells · L ⁻¹	Time period	Approx. number of weeks
2001	> 5000	9.3.-19.4.	6
	> 10000	9.3.-19.4.	6
	> 5000	10.8.-17.8.	1
	> 10000	10.8.-17.8.	
	> 5000	31.8.	1
2002	> 5000	28.3.-17.5.	7
	> 10000	28.3.-10.5.	6
	> 5000	7.6.-21.6.	2
	> 10000	14.6.-21.6.	1
	> 5000	18.7.-15.8.	4
	> 10000	18.7.-15.8.	4
	> 5000	27.9.-4.10	1
	> 10000	27.9.	1
2003	> 5000	21.3.-11.4.	4
	> 10000	21.3.-11.4.	4
	> 5000	23.5.	1
	> 5000	6.6.	1

Cell counts showed re-occurring seasonality in species belonging to the *P. delicatissima* (Tab. 2.6) and *P. seriata* (Tab. 2.7) groups. Diatoms belonging to the *P. delicatissima* group, in Scottish waters potentially including *P. delicatissima*, *P. pseudodelicatissima* and *P. calliantha* (see chapter 3), showed greatest abundance in spring and during the summer. Blooms with numbers > 10^4 cells · L⁻¹ persisted for up to six weeks in spring (March, April, also May) smaller and shorter blooms were present in June, July and August (Tab. 2.6, appendix).

Diatoms belonging to the *P. seriata* group, which included the toxic species *P. australis* and *P. seriata* and non-toxic strains of *P. fraudulenta*, *P. pungens* and *P. cf. subpacific* (see chapter 3) reached highest densities between June and October (Tab. 2.7). The *P. seriata* group regularly occurred in highest cell density in July. Bloom events with cell

numbers $> 10^4$ cells \cdot L⁻¹ would persist sometimes for just a short period of less than a weeks time (Tab. 2.7, appendix 1).

Table 2.7 Occurrence of diatoms belonging to the *P. seriata* group from January 2001 to July 2003, in threshold or half threshold cell densities.

Year	cells \cdot L ⁻¹	Time period	Approx. number of weeks
2001	> 5000	29.6.-17.8.	7
	> 10000	5.7.-27.7.	5
	> 5000	14.9.-5.10	3
	> 10000	10.8.	1
2002	> 5000	7.6.-26.7.	7
	> 10000	21.6., 5.7.-18.7.	1; 2
	> 5000	15.8.-11.10	8
	> 10000	15.8., 29.8.-6.9., 20.9.-4.10.	1; 1; 2
2003	> 5000	24.6.-25.7	> 4
	> 10000	24.6.-25.7.	> 4

2.3.1.7 Statistical analysis of samples from LY1

2.3.1.7.1 Multidimensional Scaling

The MDS plot in Figure 2.17 illustrates the seasonality observed in phytoplankton samples collected from station LY1. The analysis arranged the month, representing species composition and abundance at that time of the year, in an approximate circle. This configuration reflects species succession and changes in cell density over the year. Months representing winter (Nov, Dec, Jan, Feb) were configured closely together, March seemed to represent a month of transition between winter and spring. April, May and June continued the circle, representing typical spring and early summer samples. Species composition and abundance in summer were represented by July, September and August. September and July samples were more similar than July and August samples. October samples, as a transition between summer and winter samples, represented typical autumn samples. The relatively low stress factor of 0.04 indicates

that the degree of similarities between months is well represented in the 2-dimensional diagram.

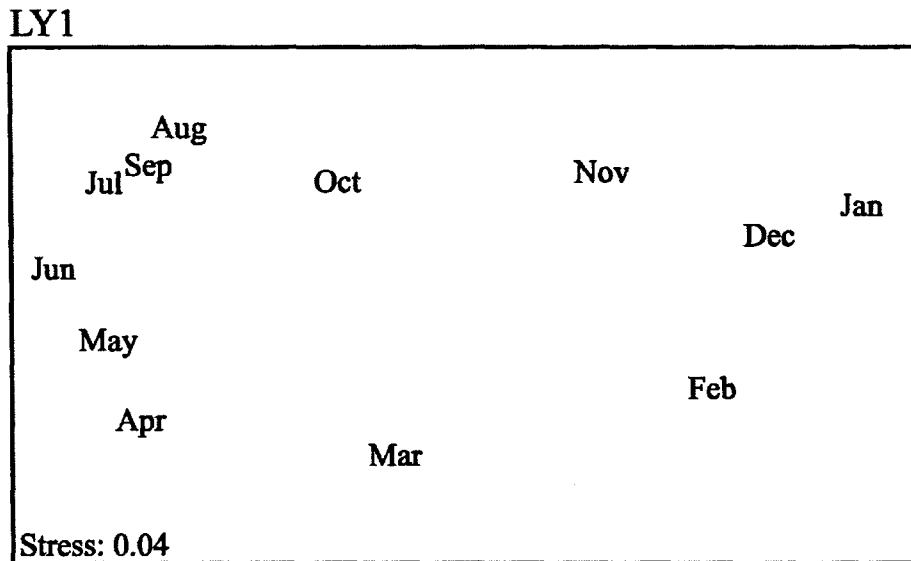


Fig. 2.17 MDS plot representing the assemblage similarities of samples taken at LY1 between November 2000 and July 2003, averaged for every month of the year (see Materials and Methods section 2.1.1.1 for details). The lesser the distance between two month, the greater was the similarity of the species composition and species abundance in those two month. Data were fourth root transformed.

2.3.1.7.2 Redundancy Analysis

The Monte Carlo test showed that dissolved inorganic silicate (DSi, $p = 0.002$), inorganic phosphate and nitrate (DIP and DIN, $p = 0.04$), temperature ($p = 0.04$) and salinity ($p = 0.064$) had significant impact on the phytoplankton assemblage.

Figure 2.18 illustrates the results of the RDA. Axes represent a multiple linear regression of the environmental parameters accounting for the species ordination. The length of an arrow reflects the relative importance of the environmental factor. Arrows pointing in the same direction indicate a positive correlation of factors and/or species, arrows pointing in opposite directions can be interpreted as negative correlations. In Figure 2.18, 25% of the species distribution was accounted for by the constrained axes. The diagram shows that of all environmental factors salinity (shortest arrow) had the lowest impact on the species assemblage. Arrows for DIN and DIP were positioned close to each other and show the same orientation, indicating that those two factors were

highly correlated. Opposed to DIN, DIP and DSi were typical summer species, such as *Eucampia zodiacus*, *Dinophysis norvegica*, *D. acuminata* and *Leptocylindrus minimus*. Those species were associated with low nutrient concentrations in the water column. *Paralia sulcata* was positively correlated with DSi.

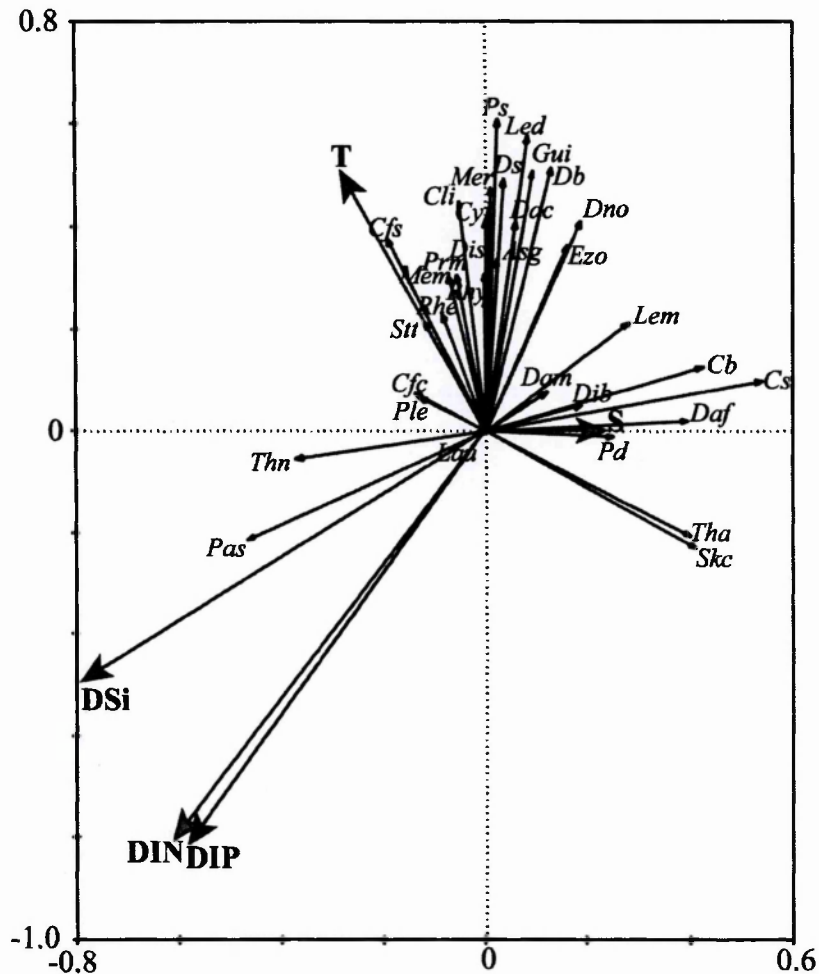


Fig. 2.18 RDA Correlation biplot of all samples taken at LY1 based on the absolute phytoplankton abundance and environmental parameters that significantly (Monte-Carlo test, $p < 0.1$) influenced the species ordination. All species were included, cell numbers were square root transformed prior to analysis. Species abbreviations: *Asg*, *Asterionellopsis glacialis*; *Cb*, *Chaetoceros* spp. $> 10 \mu\text{m}$; *Cfc*, *Ceratium furca*; *Cfs*, *Ceratium fusus*; *Cli*, *Ceratium lineatum*; *Cs*, *Chaetoceros* spp. $< 10 \mu\text{m}$; *Cyl*, *Cylindrotheca closterium*; *Dac*, *Dinophysis acuta*; *Daf*, *Dactyliosolen fragilissimus*; *Dam*, *Dinophysis acuminata*; *Db*, Dinoflagellates $> 20 \mu\text{m}$; *Dib*, *Ditylum brightwellii*; *Dis*, *Dictyocha speculum*; *Dno*, *Dinophysis norvegica*; *Ds*, Dinoflagellates $< 20 \mu\text{m}$; *Ezo*, *Eucampia zodiacus*; *Gui*, *Guinardia delicatula*; *Lau*, *Lauderia borealis*; *Led*, *Leptocylindrus danicus*; *Lem*, *Leptocylindrus minimus*; *Mem*, *Meuniera membranacea*; *Mer*, *Mesodinium rubrum*; *Pas*, *Paralia sulcata*; *Pd*, *P. delicatissima* group; *Ple*, *Pleurosigma* sp.; *Prm*, *Prorocentrum micans*; *Ps*, *P. seriata* group; *Rhs*, *Rhizosolenia setigera*; *Rhy*, *Rhizosolenia styliformis*; *Skc*, *Skeletonema costatum*; *Stt*, *Stephanopyxis turris*; *Tha*, *Thalassiosira* sp.; *Thn*, *Thalassionema nitzschioides*. Environmental parameters: DIN, dissolved inorganic nitrate; DIP, dissolved inorganic phosphate; DSi, dissolved inorganic silicate; T, temperature; S, salinity.

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Summer species as *Stephanopyxis turris*, *Ceratium fusus* and *Rhizosolenia setigera*, were positively correlated with temperature. *Thalassiosira* sp. and *Skeletonema costatum*, species associated with the spring bloom and colder water, were negatively correlated with temperature, but showed no association with nutrients. They were also potentially influenced by other factors, not measured in this study. The same applied for some other species (e.g. *P. seriata* group, *Leptocylindrus danicus*, *Asterionellopsis glacialis*), which were not directly correlated with any of the measured environmental factors.

2.3.2 Transect from Loch Spelve to Loch Creran

2.3.2.1 Hydrography

Along the Loch Spelve-Creran transect, temperature, salinity and density profiles of the upper 19 m water column were taken. In addition to the 19 m depth profiles, the physical data from the biological sampling depth of 5 m are shown.

2.3.2.1.1 Temperature

Temperature profiles of the 19 m water column along the Loch Spelve-Creran transect (Fig. 2.19) show in general increasing temperatures between station 700 and Loch Creran, and a decrease in temperature with depth. The water column at station 700 and LY3 was well mixed at all three sampling occasions. In September a slight temperature stratification of the upper 6 and 8 m of the water column was observed at LY2 and LY1, respectively. At LY1 a plume of colder water was found below 10 m depth, which stretched out towards C2, rising to about 8 m depth. The water column down to 6 m in Loch Creran reached temperatures above 13 °C and was only about half a degree colder below that depth.

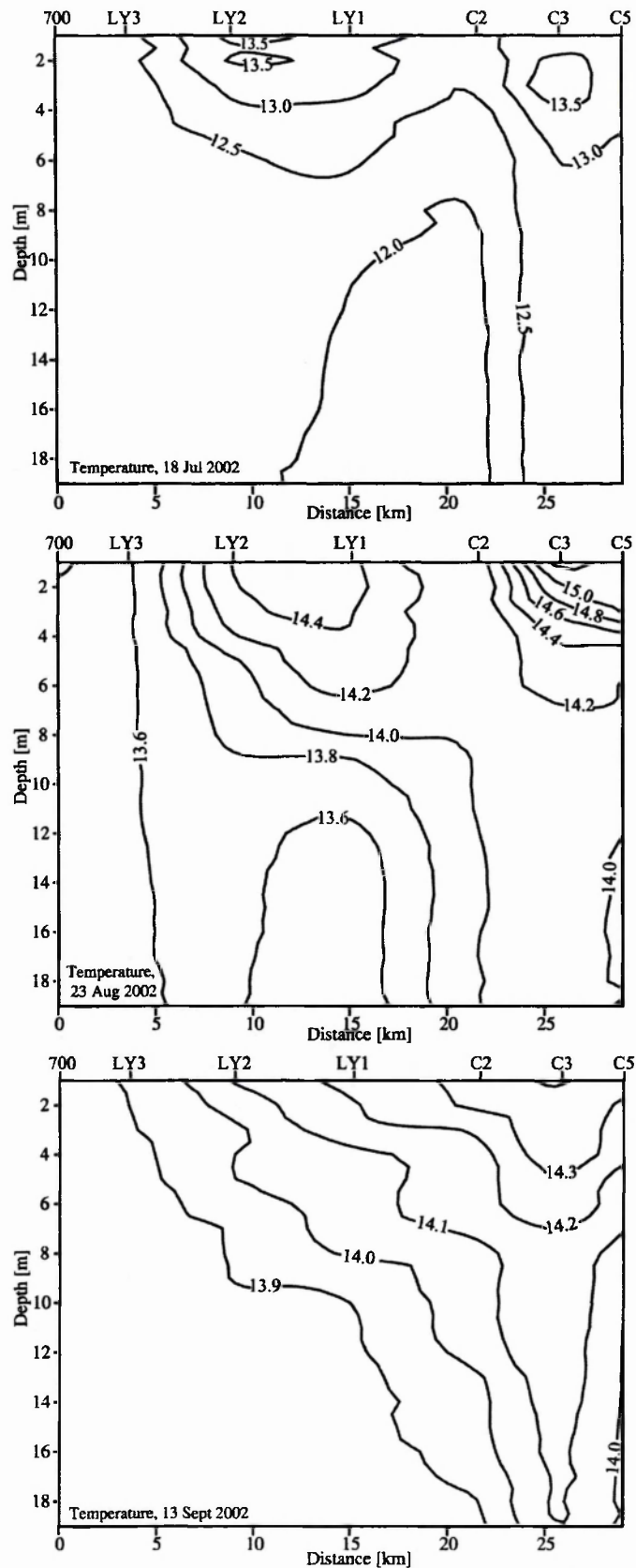


Fig. 2.19 Temperature profiles of the upper 19 m of the water column along the transect from Loch Spelve to Loch Creran, taken at three sampling occasions, on 18 July , 23 August and 13 September 2002. Recording of profiles started at 1m.

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In August the water column had warmed up by about a degree. The thermocline at LY1 was found in about 9 m depth, while the water column at C2 was well mixed. A temperature gradient with the thermocline at about 7 m was observed at C3 and C5.

In September the water was less stratified by temperature. The warmest surface temperature was found at C3 and decreased towards station 700. In general, from the temperature profiles the water column seemed to be well mixed at all stations, with temperature differences below 0.5 °C between the surface and 9 m at all stations.

At 5 m, a general increase from station 700 towards C3 was found in all months with a slight temperature drop at C5 (Fig. 2.20). The median temperatures were found at LY2 (in July), C2 (in August) and LY1 (in September). In general, the temperature increased between LY3 and C3, but the lowest temperature was found at C2. Temperatures were lowest in July and similar during August and September.

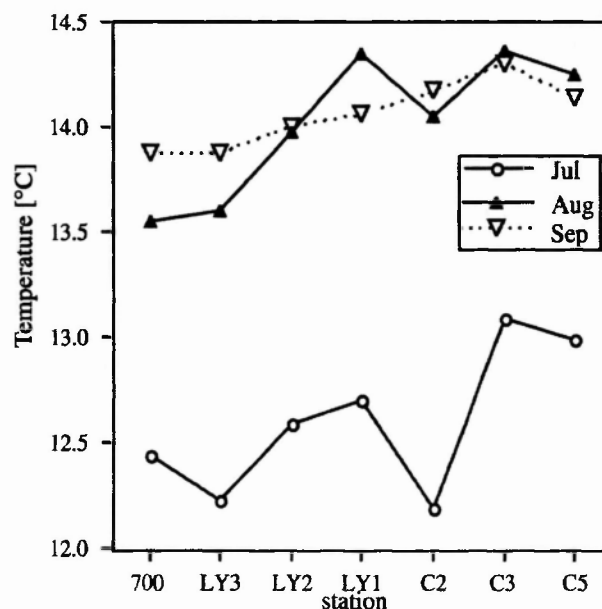


Fig. 2.20 Water temperature along the Spelve-Creran transect in 5 m on 18 July, 23 August and 13 September 2002.

2.3.2.1.2 Salinity

Similar to the temperature profiles, the salinity profile for the upper 19 m (Fig. 2.21) shows that there was little salinity invoked stratification at stations 700 and LY3. The most saline water was found at those two stations, due to their seawards location.

In July 2002 salinity stratification influenced the upper 6 or 10 m at LY2 and LY1, respectively. At C2 salinity gradients were not as strong as found at LY1 and LY2 and some stratification was observed at C3, with less at C5, affecting the upper 7 m of the water column. Interestingly, the lowest salinity was found in the upper 3 m at LY1. Close to the surface the water at LY2 was very similar in its salinity to that at C2 and C3.

In August 2002 the upper 10 m of the water column at LY2 and LY1 were stratified by a salinity gradient, the water column at C2 was again well mixed. Within Loch Creran water the surface layer (upper 5 m) was stratified.

In September, at LY2 and LY1 a salinity gradient had established in about 6 to 8 m. The water column at C2 and in Loch Creran showed only little stratification, Salinity in the upper 2 m was lower at LY1 and LY2 than at C3 and the Loch Creran stations.

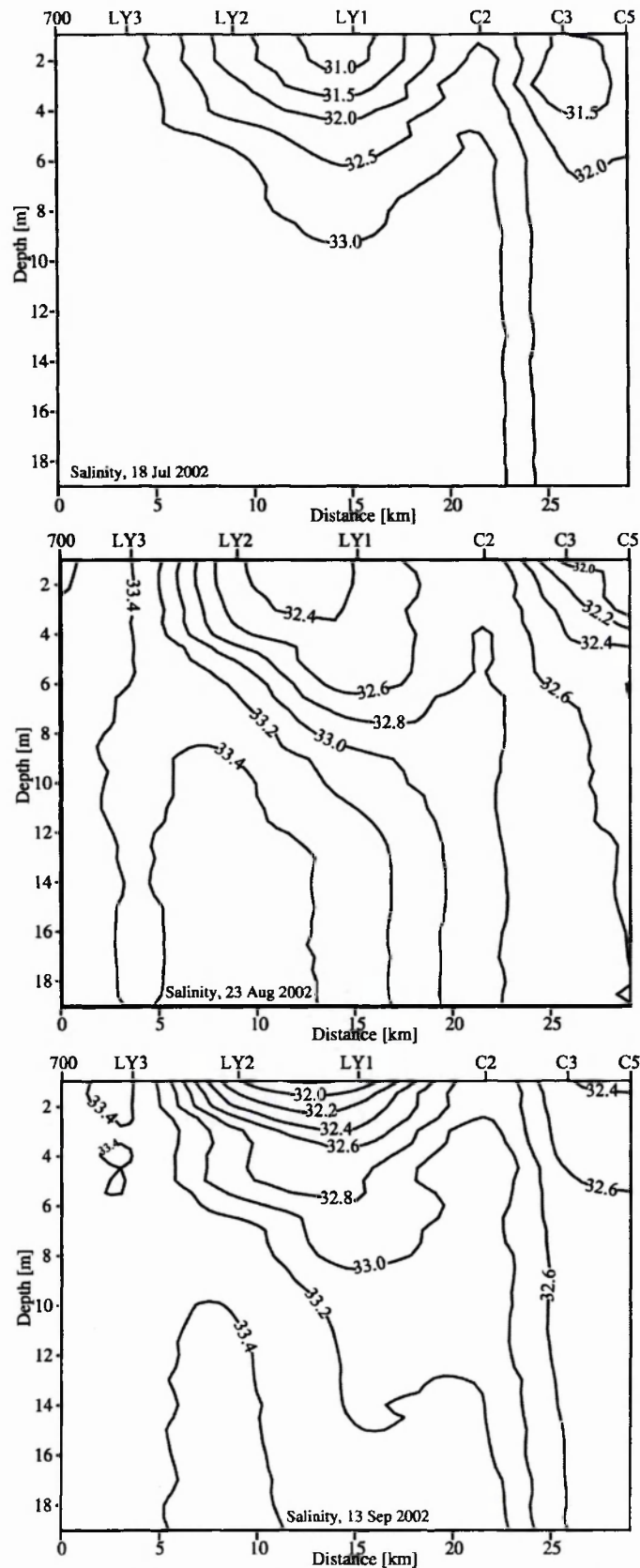


Fig. 2.21 Salinity profiles of the upper 19 m of the water column along the transect from Loch Spelve to Loch Creran, taken at three sampling occasions, on 18 July , 23 August and 13 September 2002. Recording of profiles started at 1m.

In general, salinity values in 5 m water depth showed a reverse trend compared to temperature, with highest values usually found near Loch Spelve and lowest in Loch Creran (Fig. 2.22). Salinity was higher at C2 than at LY2 and LY1. In all months relatively low values were found at LY1, in August they were even lower than in Loch Creran, indicating influx fresh water at stations LY2, LY1, C3 and C5. The median value was measured at LY2 (July and September) and C2 (August).

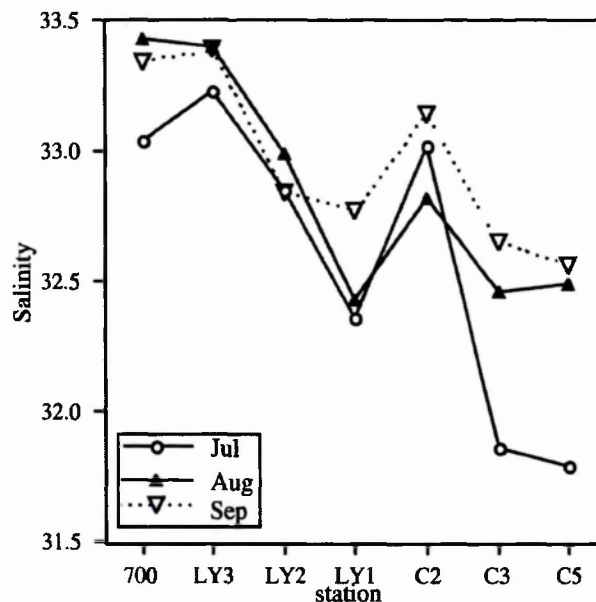


Fig. 2.22 Salinity along the Spelve-Creran transect in 5 m on 18 July, 23 August and 13 September 2002.

2.3.2.1.3 Density

The density profiles of the 19 m water column on the transect between Loch Spelve and Loch Creran (Fig. 2.23) mirrored the salinity profile.

The water column was, in general, well mixed at stations 700 and LY3. At stations LY2 and LY1 the water column down to about 10 m was stratified in July, a similar structure was found at C2. The lowest densities down to 23 were found in Loch Creran at C3 in 2 to 4 m. At C3 and C5 the surface of the water column down to about 6 m showed a slight stratification, but in general the water was well mixed.

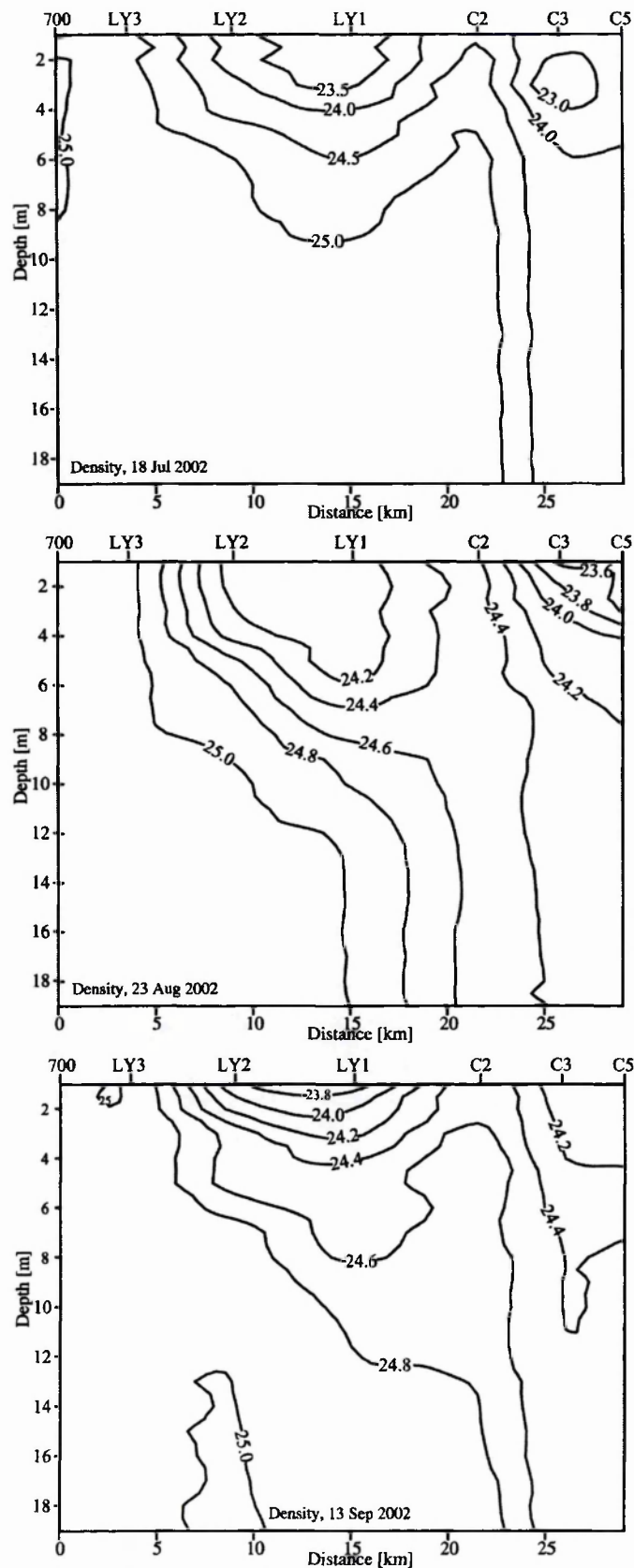


Fig. 2.23 Density profiles of the upper 19 m of the water column along the transect from Loch Spelve to Loch Creran, taken at three sampling occasions, on July 18, August 23 and September 13 2002. Recording of profiles started at 1m.

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In August profiles at LY2 to C5 showed the continuation of the stratification trends that were observed in July. LY2 and LY1 showed stratification down to 10 m and were well mixed below that depth. No density stratification was observed at C2, but a clear density gradient was found at C3 and C5 down to about 6 and 8 m respectively.

In September the stratification at LY2 and LY1 had stabilised and was found at LY2 to about 7 m depth and to about 10 m at LY1. The stratification was not so established at C2, but a slight gradient was observed down to about 12 m depth. At C3 and C5 the stratification observed in August had weakened but now reached deeper, down to about 11 m at C3 and 8 m at C5.

The density values in 5 m depth found along the transect mirror the salinity data (Fig. 2.24). In each month density decreased from Loch Spelve towards Loch Creran with low values at LY2 and LY1. The median was represented by LY2 (July, September) and C2 (August).

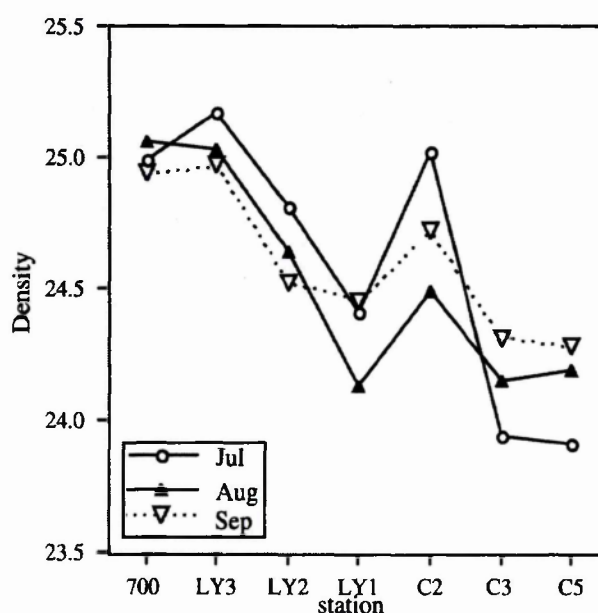


Fig. 2.24 Density [σ_t] along the Spelve-Creran transect in 5 m on 18 July, 23 August and 13 September 2002.

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2.3.2.2 Nutrients

Dissolved inorganic nutrient concentration did not show clear trends along the transect. However, for each station nutrient concentrations generally increased between July and September (except for silicate, which decreased from July to August at some stations; ammonium also was an exception).

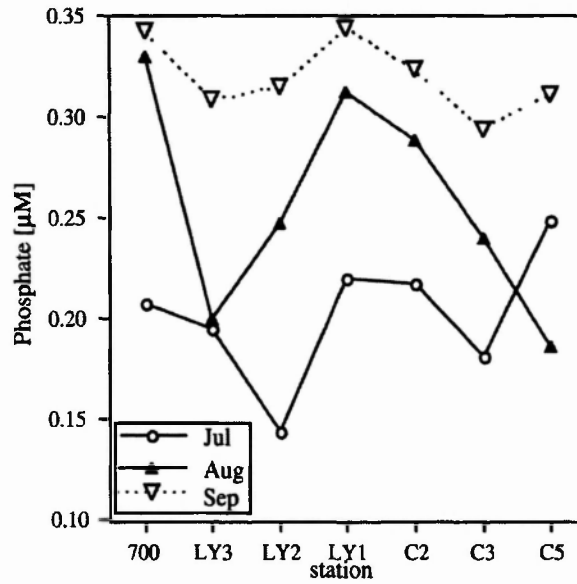


Fig. 2.25 Dissolved inorganic phosphate (DIP) concentration [μM] along the Spelve-Creran transect in 5 m on 18 July, 23 August and 13 September 2002.

The dissolved inorganic phosphate (DIP) concentrations showed a pattern of elevated values at stations 700, LY1 and C5 (Fig. 2.25). In July and September DIP concentrations were relatively constant, with some fluctuation, but more variability in August.

The patterns of the dissolved inorganic silicate (DSi) concentration differed between the three months (Fig. 2.26). Marked changes were observed within Loch Creran, where the DSi concentration was increased in July and September, but decreased in August.

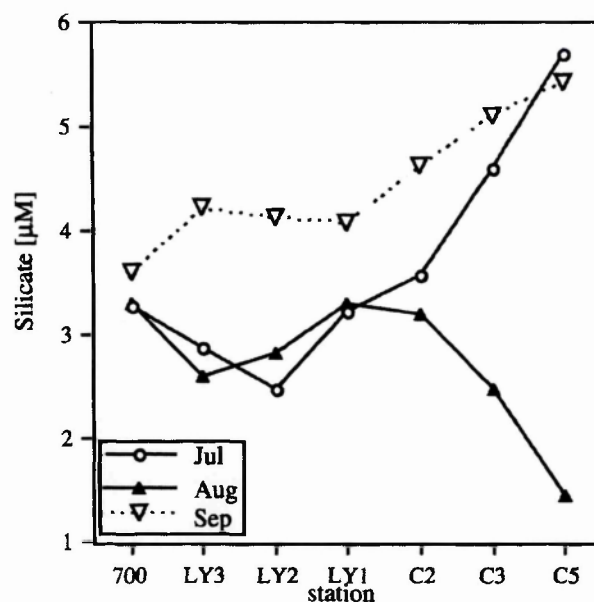


Fig. 2.26 Dissolved inorganic silicate (DSi) concentration [μM] along the Spelve-Creran transect in 5 m on 18 July, 23 August and 13 September 2002.

Similar to the pattern observed in the DIP concentrations, dissolved inorganic nitrogen (DIN) concentrations showed local maxima at LY1 (Fig. 2.27). In July 2002 this pattern led to DIN being depleted at all stations except stations 700 and LY1. In August and September increases at all stations were observed. In September DIN concentrations were generally higher at each station than in the previous months. The pattern along the transect was the same as in August with decreasing concentrations between station 700 and LY2, followed by an increase towards LY1, a steep decline towards C3 with a raised concentration at C5. The maximum concentration was measured at LY1 ($2.34 \mu\text{M}$) and the lowest at C3 ($0.88 \mu\text{M}$).

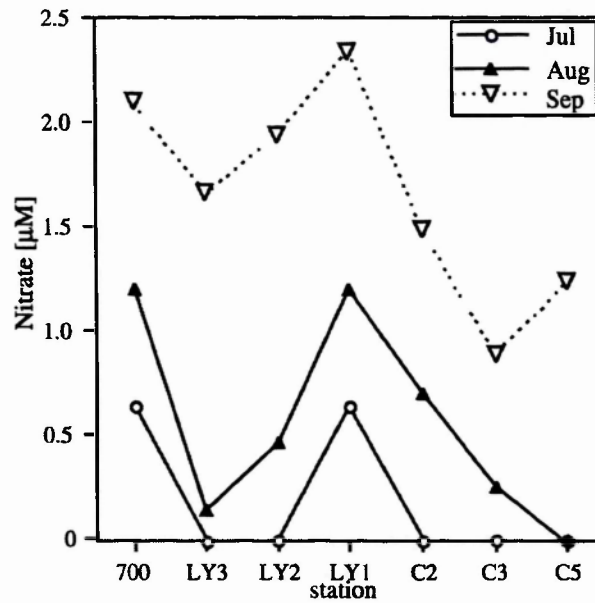


Fig. 2.27 Dissolved inorganic nitrate (DIN) concentration [μM] along the Spelve-Creran transect in 5 m on 18 July, 23 August and 13 September 2002.

Similar to the DIP and DIN concentrations, local maxima of the ammonium concentration were found at LY1 in all months (Fig. 2.28).

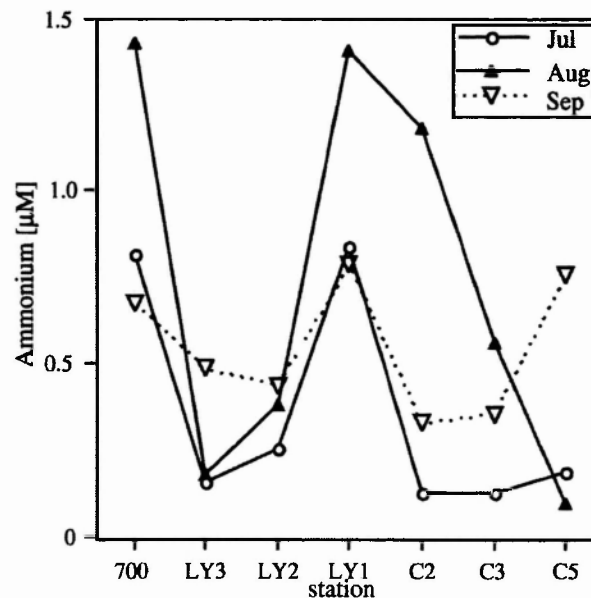


Fig. 2.28 Ammonium concentration [μM] along the Spelve-Creran transect in 5 m on 18 July, 23 August and 13 September 2002.

In each month the concentration decreased between station 700 and LY3 (LY2 in September), increased towards LY1, then was followed by a decrease towards C2.

2.3.2.3 Biological parameters

All species that were found at any of the other stations were also found at LY1. The dinoflagellate *Lingulodinium polyedrum*, that was included in the dinoflagellate group > 20 µm, when analysing samples from the monitoring programme at LY1, was counted separately, although it was only abundant in Loch Creran (stations C3 and C5 in August and September). For each species abundance in cells · L⁻¹ was plotted and figures are presented in appendix 2. Table 2.8 shows the total abundance for each taxon that was found at all stations along the Spelve-Creran transect, the highest total density of a taxon that was observed at any station, its percentage of the total abundance, and the station at which a taxon occurred in the highest abundance. In section 2.3.1.3 it was noted that *Chaetoceros* spp. < 10 µm was the most abundant taxon at LY1, when all seasons were included. In summer 2002 the taxa with highest cell densities along the Spelve-Creran transect were *Leptocylindrus danicus* and *L. minimus*, followed by *Chaetoceros* spp. < 10 µm (Tab. 2.8). Highest numbers of the former two were observed at LY1 (26.1 and 28.4% of total abundance) and the latter at C5 (30.8%). *Pseudo-nitzschia* species of the *P. seriata* group were, with a total of > 350 × 10³ cells · L⁻¹, the sixth most abundant taxon, about 27% of the cells were found at LY2. The highest percentage of *P. delicatissima* species was again found at LY2 (26.2%).

LY1 was the station where in total the maximal abundance of *Chaetoceros* spp. (all sizes), *Guinardia delicatula*, *Leptocylindrus danicus* and *Ditylum brightwellii* were found. More than 45% of all *Guinardia delicatula* cells were found at LY1, but abundance of other species at LY1 did not exceed 30% of the total abundance (of all stations).

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Table 2.8 Species found along the Spelve-Creran transect in summer 2002 in rank order of total density.

Taxon	Cumulative abundance [cells · L ⁻¹] at all stations	Max. abundance at one station [cells · L ⁻¹]	Max. abundance at one station [%] as percentage of total abundance at all stations	Station at which max. abundance was found
<i>Leptocylindrus danicus</i>	1,369,896	358,005	26.1	LY1
<i>Leptocylindrus minimus</i>	865,863	266,644	30.8	C5
<i>Chaetoceros</i> spp. < 10 µm	629,429	178,799	28.4	LY1
<i>Mesodinium rubrum</i>	484,292	178,803	36.9	C5
<i>Chaetoceros</i> spp. > 10 µm	406,912	115,287	28.3	LY1
<i>P. seriata</i> group	350,640	93,420	26.6	LY2
<i>Skeletonema costatum</i>	295,431	88,458	29.9	LY2
<i>Guinardia delicatula</i>	221,981	101,022	45.5	LY1
Dinoflagellates < 20 µm	96,560	24,140	25.0	C5
<i>Thalassiosira</i> spp.	84,160	33,680	40.0	C3
Dinoflagellates > 20 µm	84,020	19,340	23.0	C3
<i>P. delicatissima</i> group	73,220	19,160	26.2	LY2
<i>Cylindrotheca closterium</i>	54,480	11,720	21.5	C3
<i>Thalassionema nitzschioides</i>	45,580	9,820	21.5	LY2
<i>Asterionellopsis glacialis</i>	36,520	14,960	41.0	C3
<i>Lingulodinium polyedrum</i>	35,880	34,700	96.7	C5
<i>Lauderia annulata</i>	28,800	6,720	23.3	C2
<i>P. americana</i>	28,720	8,420	29.3	C3
<i>Ceratium furca</i>	26,020	25,500	98.0	C5
<i>Dictyocha speculum</i>	25,180	11,500	45.7	C5
<i>Eucampia zodiacus</i>	11,260	3,600	32.0	LY3
<i>Prorocentrum micans</i>	7,660	3,280	42.8	C5
<i>Rhizosolenia styliformis</i>	4,240	1,660	39.2	C3
<i>Pleurosigma</i> sp.	3,320	700	21.1	C2
<i>Ceratium lineatum</i>	2,680	1,360	50.7	C5
<i>Paralia sulcata</i>	2,240	780	34.8	LY3
<i>Meuniera membranacea</i>	2,140	460	21.5	C5
<i>Ditylum brightwellii</i>	2,040	560	27.5	LY1
<i>Stephanopyxis turris</i>	960	340	35.4	LY3
<i>Ceratium fusus</i>	940	380	40.4	C5
<i>Dinophysis acuta</i>	740	180	24.3	C5
<i>Dinophysis norvegica</i>	540	200	37.0	C5
<i>Rhizosolenia setigera</i>	200	60	30.0	C2, C3
<i>Dinophysis acuminata</i>	200	80	40.0	C5

The highest total abundance of all dinoflagellate taxa was found in Loch Creran. 23% of all dinoflagellates > 20 µm were observed at C3 and all other dinoflagellates occurred in highest percentages at C5. *Lingulodinium polyedrum* and *Ceratium furca* were with percentages of 96.7% and 98.0% were almost solely found at C5. More than half of all observed cells of *Ceratium lineatum* (50.7%) were present at C5. All other dinoflagellates (dinoflagellates < 20 µm, *Prorocentrum micans*, *Ceratium fusus*, and the

Dinophysis species) were observed at C5 in percentages between 23 to 40.4. Station 700 was the only station at which none of the taxa was found in higher abundance than at the others.

2.3.2.3.1 Pseudo-nitzschia spp. along the Spelve-Creran transect

The cell density of the *P. delicatissima* group was highest at all stations along the transect in July and decreased towards August and September (Fig. 2.29). In July *P. delicatissima* group abundance decreased from station 700 (10.5×10^3 cells · L⁻¹) towards Loch Creran (2.6×10^3 cells · L⁻¹ at C3), an increased cell density was observed at LY2, where numbers exceeded 15.8×10^3 cells · L⁻¹. In August cell numbers of the *P. delicatissima* group had decreased to less than 2.5×10^3 cells · L⁻¹ at all stations. Numbers increased from station 700 towards Loch Creran with highest densities at intermediate stations LY2 and LY1 (2.4 and 2.3×10^3 cells · L⁻¹, respectively). A further decrease of cell density was observed in September at all stations.

Abundance of the *P. seriata* group followed a more variable pattern than the *P. delicatissima* group (Fig. 2.29). In July and August, again the highest cell density of species assigned to the *P. seriata* group was found at LY2 and the lowest at station 700. Yet, at the same station (700), the highest cell density of that group was observed in September.

Cell densities of the *P. seriata* group were, in general, highest in August at each station, and in July and September decreased from LY2 or LY1 towards Loch Creran. In August, numbers in Loch Creran were elevated.

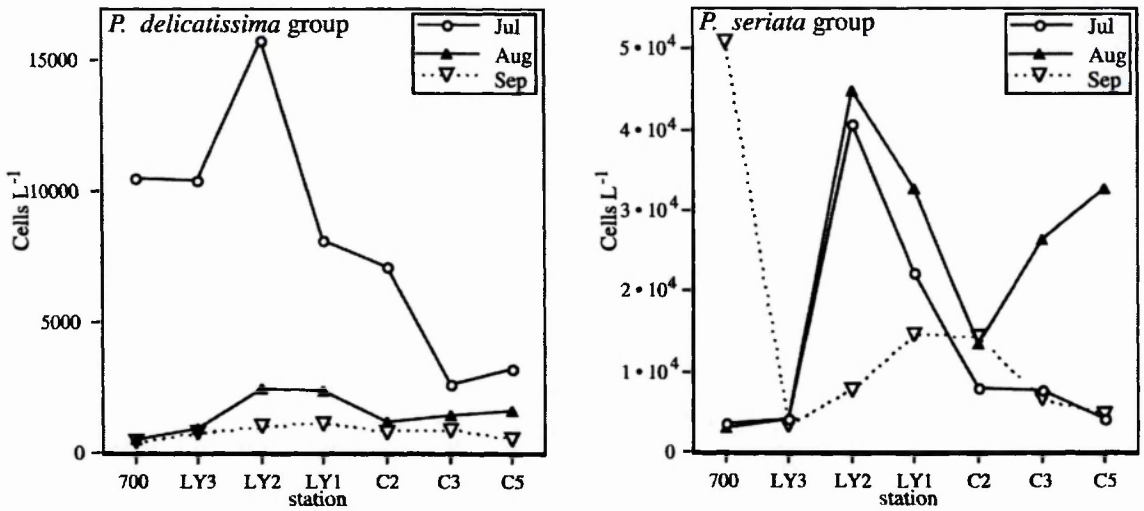


Fig. 2.29 Abundance [cells · L⁻¹] of species belonging to the *P. delicatissima* and *P. seriata* group along the transect from Loch Spelve to Loch Creran in summer 2002.

2.3.2.4 Chlorophyll *a* concentration along the Spelve-Creran transect

Chlorophyll *a* (chl *a*) concentrations generally increased along the Spelve-Creran transect towards Loch Creran, with elevated concentrations in July at LY1 and C2 and to a lesser extent in August at LY2 and LY1 (Fig. 2.30).

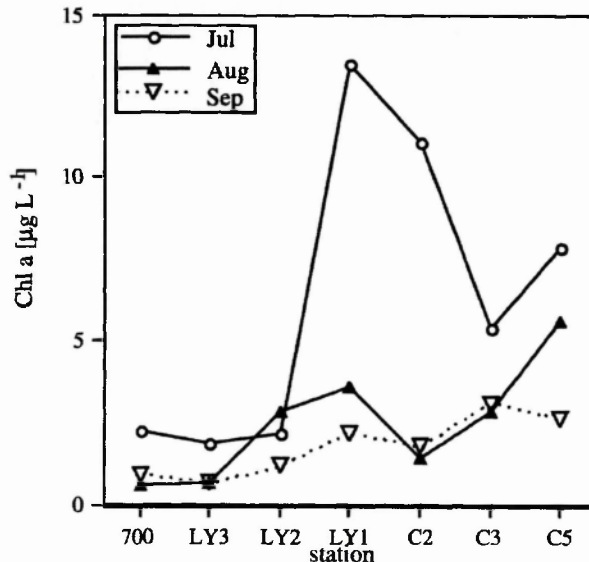


Fig. 2.30 Chl *a* concentration [µg · L⁻¹] along the Spelve-Creran transect at 5 m on 18 July, 23 August and 13 September 2002.

2.3.2.5 Statistical analysis of samples along the Spelve-Creran transect

2.3.2.5.1 *Multidimensional Scaling*

The MDS ordination in Figure 2.31A, based on phytoplankton abundance and species composition, shows that samples taken along the transect in July, August and September 2002 were configured in three groups, depending on the time of sampling. Samples taken in July clustered together on the left side, samples taken in August occupied the middle section and samples taken in September were found on the right side of the plot.

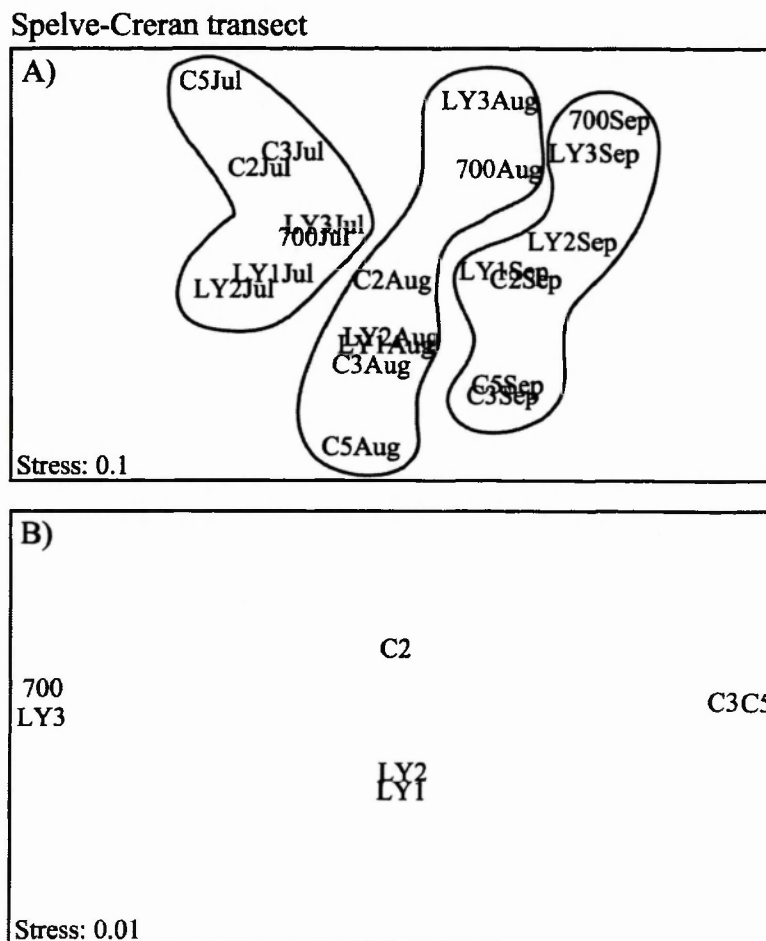


Fig. 2.31 MDS ordination of samples taken along the Spelve-Creran transect. In A) each sample, containing species abundances, is named after the station and month when it was taken. In B) for each station (700 to C5) the mean of species abundances for each station was taken. Data were fourth root transformed.

For each of the three months, stations 700 and LY3 were placed close together, indicating great similarity of those samples. In July LY1 and LY2, and C2 and C3 were

very similar in their species assemblage. In August samples from LY1, LY2 and C3 resembled each other well, while in September LY1, LY2 and C2 showed great similarity and C3 was clustered with C5. Samples at station 700 and LY3 from August and September had a high similarity in species abundance. The stress factor of 0.1 indicates that the 2-dimensional illustration is an accurate representation of similarities between stations.

The MDS ordination in Figure 2.31B indicates considerable similarity in species composition at stations 700 and LY3, LY1 and LY2 and C3 and C5. Station C2 is most similar to stations LY2 and LY1. This configuration closely reflects the geographical location of the stations: stations 700 and LY3 represent the seaward stations, stations LY2, LY1 and C2 are located in the Lynn of Lorne and C3 and C5 situated in Loch Creran. The MDS ordination, with LY1 in the middle, also shows that for summer 2002, LY1 represented a "median" of other stations along the transect. In the configuration LY1 was placed between stations 700, LY3, C3 and C5, which as monitoring sites would rather represent seawards conditions (700 and LY3) or sea-loch conditions (C3 and C5).

2.3.3 Discovery cruise D257

2.3.3.1 Hydrography

2.3.3.1.1 Open ocean stations

The water temperature at stations M and F ranged from ~ 13.5 to ~ 3.5°C at both ocean stations (Fig. 2.32). Highest values at both stations were found in the upper 50 m, with the lowest temperature in the greatest sampled depth. The thermocline was situated at around 150 m depth. For both stations salinity values were homogeneous in the top 70 m with values around 35.33 (M) and 35.34 (F). They increased rapidly to 35.41 (M) and

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35.39 (F) at about 100 m water depth, indicating the halocline. Lowest salinity values of 34.92 were found at ~ 1900 m. Derived from temperature and salinity, the density profile (expressed as σ_t) ranged at both stations from 26.53 and 26.57 in the top metres to 27.8 at the bottom of the water column, with the pycnocline situated in about the same depth as the thermocline (around 150 m).

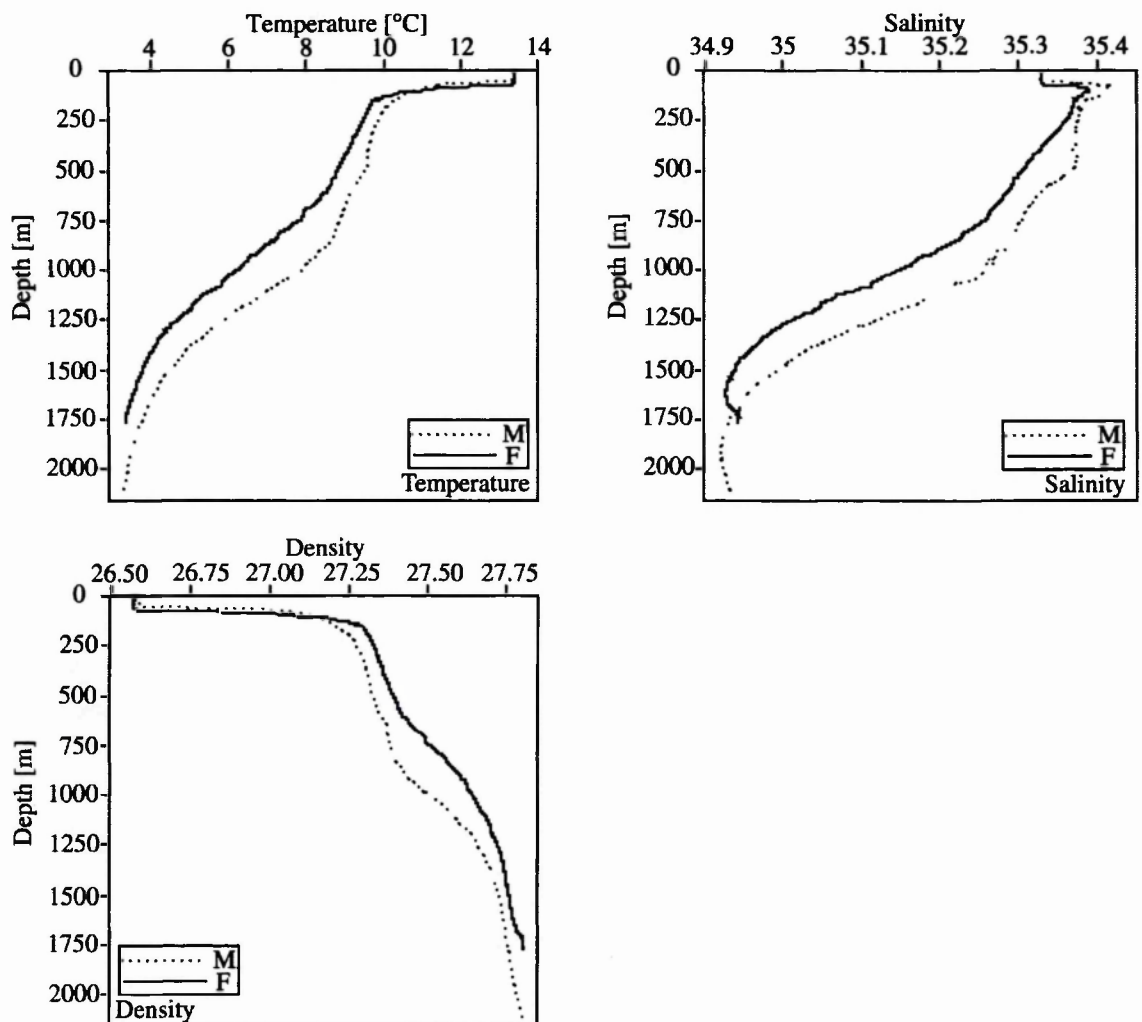


Fig. 2.32 Temperature, salinity and density profiles at stations M and F.

2.1.1.1.2 Shelf stations

2.1.1.1.2.1 Temperature

Figure 2.33 shows the temperature distribution within the water column at the shelf stations 10G to 1G. At stations 7G to 1G the water column temperature was relatively

homogenous. At station 9G the water temperature of the upper 50 m was half a degree warmer than at the other stations, a thermocline was found at about 75 m. 10G surface water was half a degree colder than the other shelf stations and temperature declined from about 25 m to reach 10.5°C in 100 m.

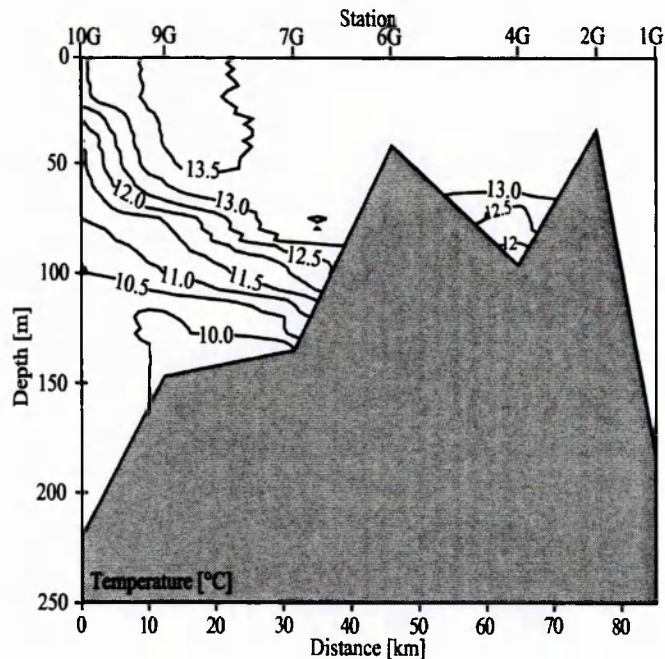


Fig. 2.33 Contour plot of water temperature [°C] along the Ellett Line shelf stations 10G to 1G.

2.1.1.1.2.2 Salinity

The salinity distribution in the water column is shown in Figure 2.34. Salinity in surface waters increased from stations 1G and 10G, as one moves offshore. At 1G and 2G slightly fresher water, with salinities between 34.2 and 33.8, lay on top of the otherwise well mixed water column. 4G and 6G were well mixed. At 7G water with higher salinity (above 35), was found at about 90 m. The water column at 9G was well mixed down to about 80 m and showed a salinity again higher than 35. At 10G salinity increased slowly with depth, no strong gradients were observed.

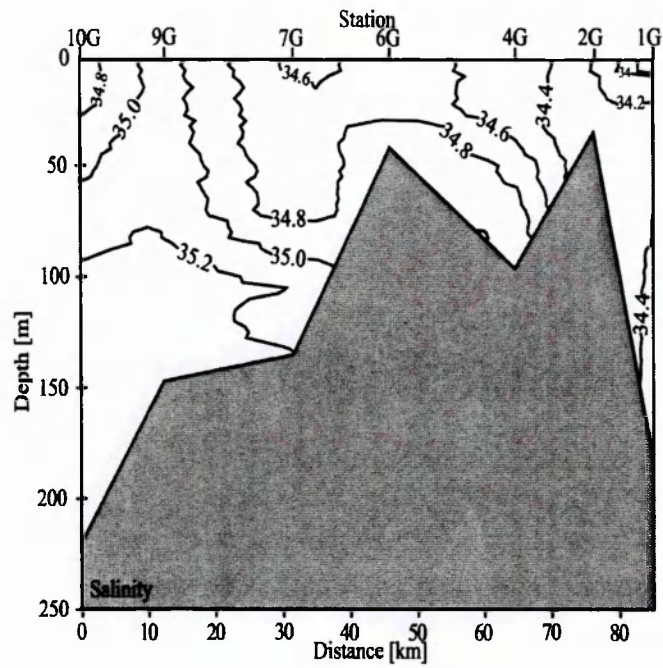


Fig. 2.34 Contour plot of salinity along the Ellett Line shelf stations 10G to 1G.

2.1.1.1.2.3 Density

Figure 2.35 indicates that the water column at all shelf stations was quite well mixed, with no strong density gradients. Stations 1G to 4G were influenced by the Scottish coastal current, with a relatively lower density in the upper water column, compared to the other shelf stations.

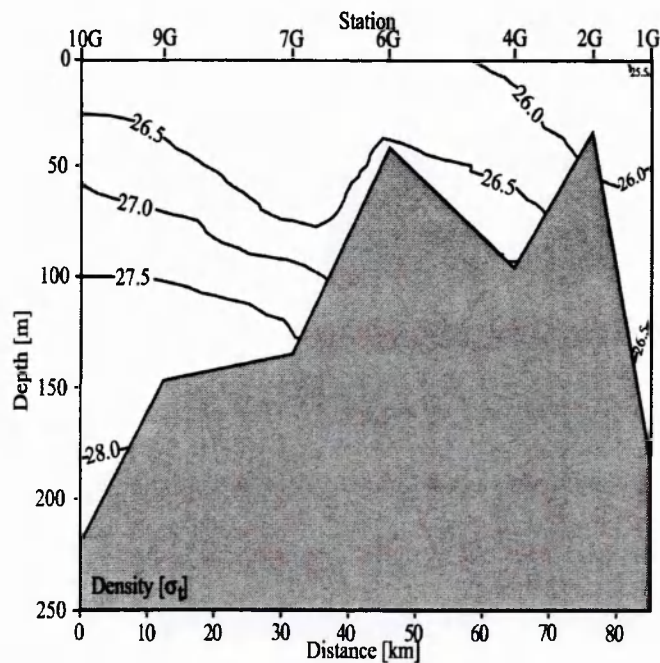


Fig. 2.35 Contour plot of density [σ_t] along the Ellett Line shelf stations 10G to 1G.

The density of the surface water at 1G was reduced by freshwater land runoff. Water below 50 m at 4G showed a density above 26.5, indicating it belonged to the same water mass that was found at 10G, 9G and 7G at approximately 30 m, 40 m and 70 m, respectively. Below those depths, stations 10G, 9G and 7G were clearly influenced by high salinity water. This is further demonstrated in the T-S diagram (Fig. 2.36).

2.1.1.1.3 T-S diagram

T-S diagrams are used to identify the origin of water masses and to distinguish between them (Dietrich et al. 1992), water belonging to one water mass having the same T/S relationships. Surface waters are influenced by the seasons, wind- and tidal mixing. Hence, their density changes with the seasons, due to alterations in surface temperature and salinity, and therefore the T/S relationship is not useful in distinguishing surface water masses. However, should water at a depth which is not strongly influenced by the seasons, have the same T/S relationship throughout the year, the TS-curve would be displayed by one value. T/S values for one station plotted from different depths are depicted in a single line (e.g. Fig. 2.36). T/S lines that are close to each other, or overlap, indicate that the same characteristic water mass was present at those stations.

Figure 2.36 presents the T/S diagram for the Ellett line stations.

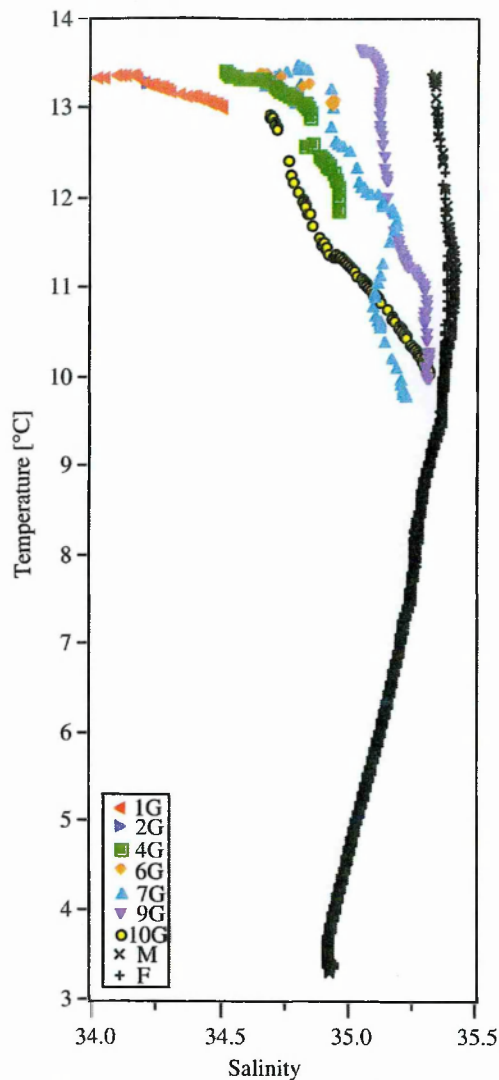


Fig. 2.36 Temperature-salinity diagram of stations along Ellett Line transect (D257 in autumn 2002).

In the T-S diagram (Fig. 2.36) surface values (those with the highest temperature) differed between the stations. As explained above, the T/S values of surface water should not be taken into account when making conclusions about similarities between water masses at different stations. In deeper waters, however, definite trends were present. T/S values for 2G appeared as a single data point (hidden by points for 1G). This indicated that the water column at 2G was homogeneous, well mixed, which was expected as the water depth at 2G was only 26 m, a depth that can still be affected by wind or tidal mixing especially in autumn. The co-incidence between the data of 2G and 1G, indicated that those two stations were located within the same water mass. Data points and lines for stations 4G, 6G and 7G all lie very close together. 6G, with only 36

m depth a relatively shallow station, was represented by the same water mass as that at station 7G from 34 to 80 m. 6G surface water, to a depth of about 24.5 m, also resembled that of 4G between 33 and 43 m. This water mass was the same as found at 7G from 8 m to 67 m. The deeper water at station 4G was an intermediate form of 10G and 7G water.

The upper water mass at 10G seemed to resemble a mixed form of water at stations 1G in 170 m and 4G from about 45 m depth. This is consistent with a current (Ellett 1979; McKay et al. 1986), which flows in a gyre, connecting station 10G with the area between 1G and 4G, as indicated in Fig. 2.2. At depth less than 68 m station 9G was relatively distinct from the other stations, but in its properties closest to 7G, M and F. The T-S diagram showed that deeper water at stations 7G, 9G and 10G had probably originated from the same water mass source. From approximately 97 to 100 m, 7G water was identical to that at 9G in approximately 68 m depth. 7G water at 107 m resembled 10G water at 75 m. 9G and 10G water were identical at 106 m and 190 m, respectively.

Stations M and F clearly represented one water body, indicated by their overlapping T/S lines. The closeness of T/S lines of 10G and 9G evidenced that the bottom water of stations 9G (124 m) and 10G (190 m) shared the same origin as water at approximately 228 and 146 m depth at M and F, respectively.

2.1.1.2 Inorganic nutrients along the Ellett Line transect

2.1.1.2.1 Open ocean stations

Figures 2.37, 2.38 and 2.39 show profiles of inorganic nutrient concentrations at the open ocean stations M and F. For all nutrients these generally increased with depth. DIP concentrations $< 0.1 \mu\text{M}$ were found between the surface and 80 m depth. Thereafter

DIP rapidly increased until about 150 m. The DIP concentration then increased again, but not as rapidly, between ~ 150 and ~ 1000 m at both stations. In greater depth DIP concentrations stayed relatively constant with maximum concentration 10 times that at the surface, reaching ~ 1 μM in the deepest samples.

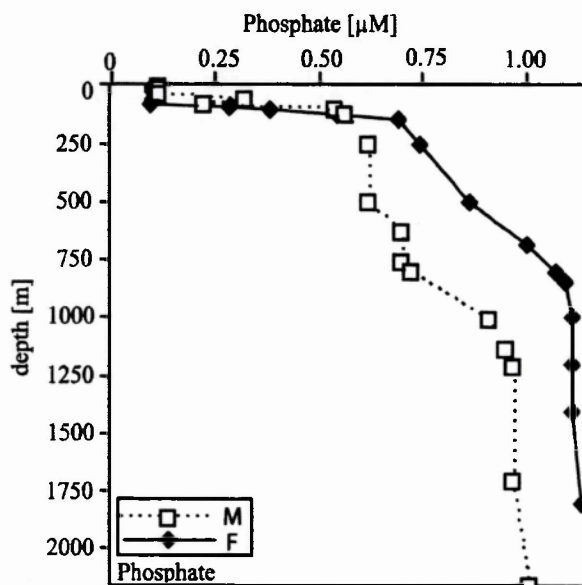


Fig. 2.37 Depth profiles of dissolved inorganic phosphate (DIP) concentration in μM at station M and F.

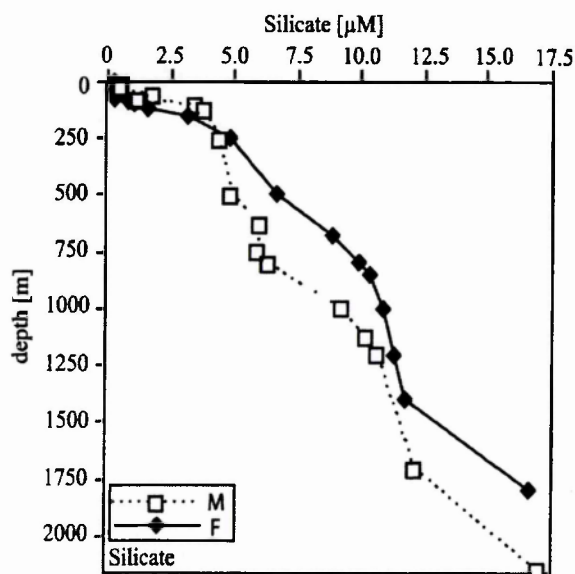


Fig. 2.38 Depth profiles of dissolved inorganic silicate (DSi) concentration in μM at station M and F.

At both open ocean stations dissolved inorganic silicate (DSi) was almost depleted in the top 15 m of the water column (Fig. 2.38). The DSi concentration at stations M and F were below 1 μM in the upper 15 m water depth. This increased to a maximum of $\sim 17 \mu\text{M}$ in the greatest depth.

The profile of the dissolved inorganic nitrate (DIN) (Fig. 2.39) concentration reflected that of the DIP concentration at both stations. The concentrations were lowest in the upper 80 m of the water column, increasing rapidly below this depth until about 150 m. Below that depth the increase was less rapidly until about 1000 m (at M) and 800 m depth (at F). Thereafter the DIN concentration stayed relatively constant with maximum values of 14.54 (1200 m, M) and 15.13 μM (1800 m, F).

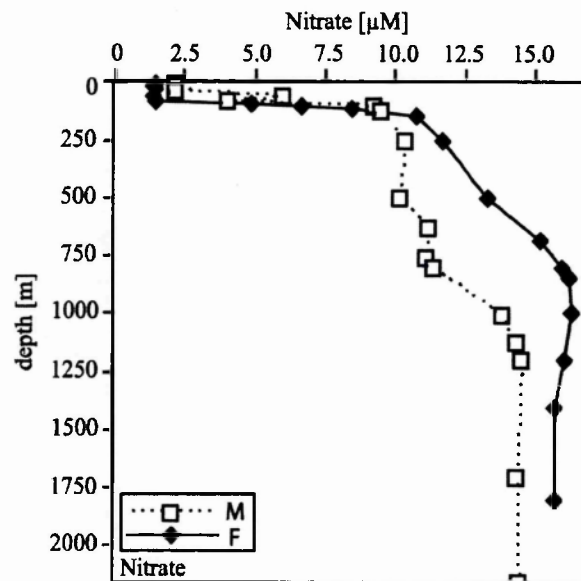


Fig. 2.39 Depth profiles of dissolved inorganic nitrate (DIN) concentration in μM at station M and F.

2.1.1.2.2 Shelf stations

Nutrient concentrations at the shelf stations 1G to 6G, were either homogeneous (mainly at station 2G) or concentrations increased only slowly with depth. A rapid

increase in the nutrient concentration was found at stations 7G (in about 60 to 80 m), 9G (in about 50 to 60 m) and in a weaker form at 10G (in about 20 to 30 m).

Figure 2.40 describes the DIP concentration in the water column along the shelf stations 10G to 1G. The DIP concentration at stations 1G to 6G was relative low throughout most of the water column, with weak increases observed at station 4G at about 75 m and at 1G about 100 m depth. In general, no steep gradients in the DIP concentrations were measured at those stations. However, at stations 10G, 9G and 7G zones of rapid DIP increase were observed in about 10 to 30 m, 50 to 60 m and 60 to 80 m depth, respectively. At those stations the DIP concentration in the deepest water had increased about six fold (at 10G), twelve fold (at 9G) to five fold (at 7G) compared to the surface water.

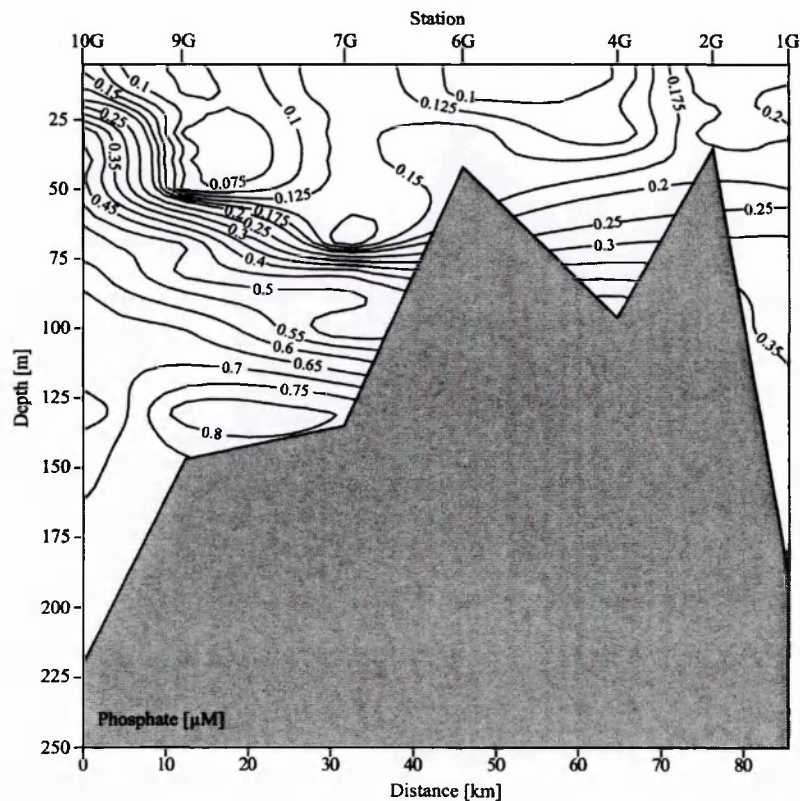


Fig. 2.40 Contour plot of dissolved inorganic phosphate [μM] of the water column along the Ellett Line shelf stations 10G to 1G.

The DSi concentrations in the water column at stations 10G to 1G are presented in Figure 2.41. DSi concentrations along the shelf transect ranged from 0.55 μM (4G, 30 m) to 6.93 μM (7G, 130 m). They were in general slightly higher in the surface water at stations 1G, 2G, 9G and 10G and increased with depth. As already observed in the DIP concentration, the DSi concentration at stations 1G, 2G and 6G did not vary rapidly with depth. Increases similar to those in DIP were observed at 10G, 9G and 7G.

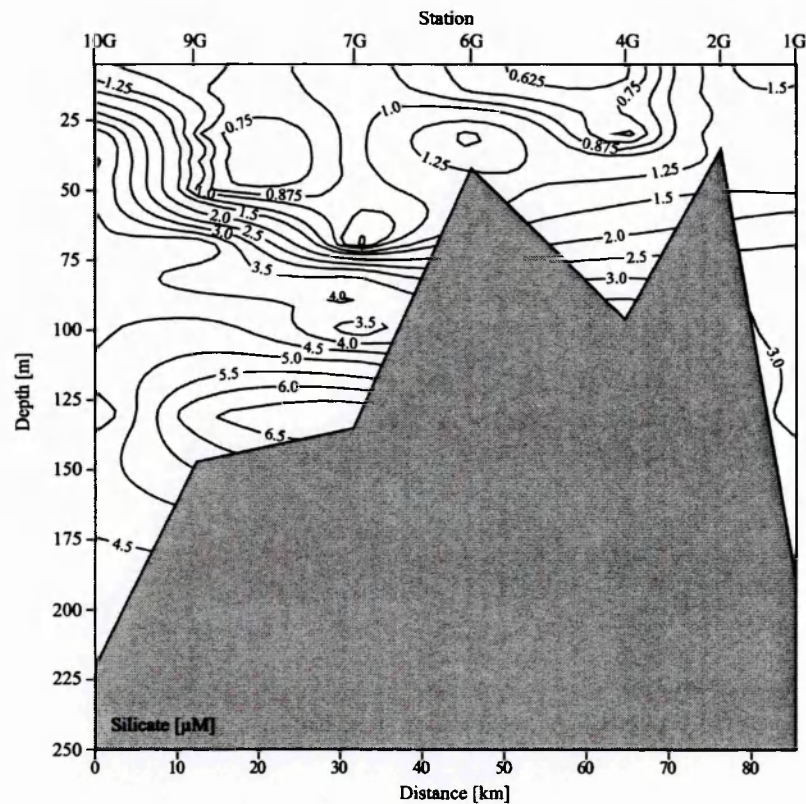


Fig. 2.41 Contour plot of dissolved inorganic silicate [μM] of the water column along the Ellett Line shelf stations 10G to 1G.

The DIN concentrations are illustrated in Figure 2.42. The lowest DIN concentration of 0.21 μM was observed at 5 m at 4G and the highest (11.53 μM) at 9G in 13 m. At 2G the DIN concentration was homogeneous within the whole water column at 2G and down to about 60 m at 1G. It increased more rapidly with depth at stations 6 G and 4G. The highest concentration in 4G bottom water was about 29 times higher than at the surface.

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As previously described for DIP and DSi, steep gradients were found at 10G, 9G and 7G in about 10 to 30 m (10G), 50 to 65 m (9G) and 65 to 80 m (7G). The DIN concentration was 6 times (10G), 20 times (9G) and 10 times (7G) higher than values in 5 m.

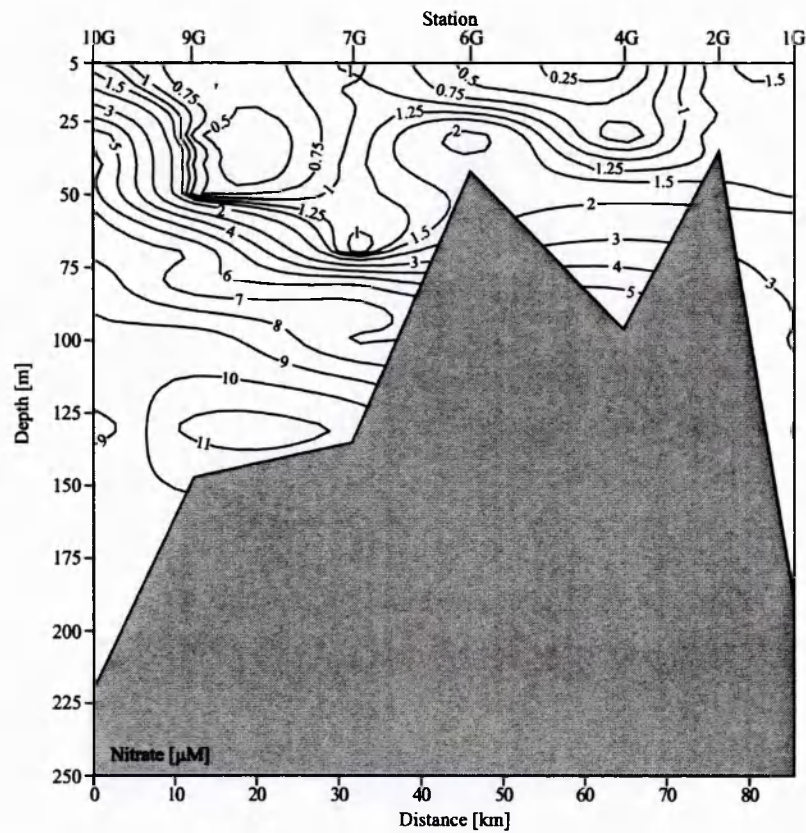


Fig. 2.42 Contour plot of dissolved inorganic nitrate [μM] of the water column along the Ellett Line shelf stations 10G to 1G.

In summary, nutrient concentrations in the surface waters of the shelf stations were in a similar range (~ 0.1 DIP, ~ 1.5 μM DIN and ~ 1.5 μM DSi) to those found at the open ocean stations. It was only at depths exceeding these of the shelf stations, that nutrient concentrations at M and F were further elevated.

2.1.1.3 Phytoplankton along the Ellett Line transect

Table 2.9 lists the species that were enumerated at shelf and open ocean stations along the Ellett Line. Most of the species found were also present at station LY1 between

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November 2000 and end of July 2003. The only species that were not previously enumerated at LY1 were the dinoflagellate *Ceratium horridum* (Cleve) Cleve, the silicoflagellate *Ebria tripartita* (Schumann) Lemmermann and the diatom *Guinardia striata* (Stolterfoth) Hasle. A diatom presumably belonging to the genus *Dactyliosolen*, which was not *D. fragilissimus*, as frequently observed in LY1 samples, was only found at stations M and F.

Species found at LY1, but not during cruise D257 were *Leptocylindrus minimus*, *Coscinodiscus* spp., *Odontella mobiliensis* and *Mesodinium rubrum*. These results were not surprising, as *L. minimus* was generally not found at LY1 after August and *Mesodinium rubrum* was only observed during July and August. *Coscinodiscus* spp. and *Odontella mobiliensis* were very rarely found in LY1 samples; *Coscinodiscus* spp. were found in low numbers mainly in July, and in total two cells of *O. mobiliensis* were enumerated in a February sample.

As stations differed in their depth and different numbers of samples were taken at each station, cell numbers for each species were averaged per station over the top 100 m of the water column. The top 100 m were chosen because most of the taxa were only abundant down to that depth.

The genus which was most abundant was *Chaetoceros*. *Chaetoceros* spp. > 10 µm were most prevalent at 4G (with more than 25.6×10^4 cells · L⁻¹ in the standardised sample at that station), while *Chaetoceros* spp. < 10 µm were most abundant at 2G, with about 71% of all enumerated *Chaetoceros* spp..

Diatoms belonging to the *P. seriata* group were the fifth most abundant group in the averaged samples, with mean maximal abundance of about 1.9×10^4 cells · L⁻¹ per sampled depth at station 2G. Most of the enumerated taxa were observed in highest average abundance at station 2G or 4G. Phytoplankton that was most abundant at one of the coastal stations (1G to 4G) was present at that station in a percentage of its total

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abundance below 50%, except at 6G, where 54% of all *Guinardia striata* cells were found. At 10G most of all *P. americana* and *Rhizosolenia styliformis* cells were observed. No species was found in maximal numbers at station 9G.

Table 2.9 List of the enumerated taxa along the Ellett Line transect. For each species the mean of cell abundance sampled in the top 100 m was taken. The maximal abundance of a species at one station is shown, the max. abundance observed at that station as percentage of the total mean abundance found in all samples, and the station at which the abundance of the taxon was highest (averaged per sample in the top 100 m depth at that station).

Species	Cumulative mean abundance [cells · L ⁻¹] at all stations	Max. abundance [cells · L ⁻¹] in one sample	Max. abundance at one station [%] as percentage of total abundance at all stations	Station at which max. abundance was found
<i>Chaetoceros</i> spp. > 10 µm	693,922	256,242	36.9	4G
<i>Chaetoceros</i> spp. < 10 µm	269,482	192,473	71.4	2G
<i>Lauderia annulata</i>	172,456	56,410	32.7	4G
Dinoflagellates < 20 µm	62,072	11,210	18.1	F
<i>P. seriata</i> group	56,407	18,780	33.3	2G
<i>Asterionellopsis glacialis</i>	22,407	7,413	33.1	2G
<i>Eucampia zodiacus</i>	21,332	5,853	27.4	2G
<i>Thalassiosira</i> spp.	19,881	6,393	32.2	4G
<i>P. delicatissima</i> group	19,660	4,340	22.1	F
Dinoflagellates > 20 µm	19,591	3,257	16.6	4G
<i>Leptocylindrus danicus</i>	17,380	4,603	26.5	4G
<i>P. americana</i>	9,898	2,625	26.5	10G
<i>Guinardia delicatula</i>	6,267	1,404	22.4	1G
<i>Ceratium lineatum</i>	4,600	1,520	33.0	2G
<i>Cylindrotheca closterium</i>	4,262	1,160	27.2	2G
<i>Rhizosolenia styliformis</i>	4,237	1,210	28.6	10G
<i>Thalassionema nitzschioides</i>	3,071	1,387	45.2	2G
<i>Ditylum brightwellii</i>	1,610	400	24.8	1G
<i>Rhizosolenia setigera</i>	1,475	920	62.4	4G
<i>Prorocentrum micans</i>	1,128	408	36.2	1G
<i>Paralia sulcata</i>	825	273	33.1	2G
<i>Dactyliosolen</i> sp.	758	545	71.9	M
<i>Ceratium fusus</i>	570	167	29.2	2G
<i>Ceratium furca</i>	519	247	47.5	2G
<i>Dictyocha speculum</i>	421	196	46.6	1G
<i>Pleurosigma</i> sp.	398	93	23.5	2G
<i>Guinardia striata</i>	394	213	54.1	6G
<i>Dinophysis acuta</i>	339	107	31.5	2G
<i>Corethron</i> sp.	299	127	42.4	2G
<i>Ceratium tripos</i>	198	55	27.7	F
<i>Ceratium horridum</i>	166	60	36.2	6G
<i>Dinophysis acuminata</i>	127	47	36.6	2G
<i>Skeletonema costatum</i>	123	57	46.2	4G
<i>Meuniera membranacea</i>	101	48	47.4	7G
<i>Dinophysis norvegica</i>	97	53	55.0	4G
<i>Ebria tripartita</i>	82	33	40.7	2G
<i>Stephanopyxis turris</i>	47	24	50.7	4G

At the open ocean stations M and F, diatoms belonging to the *P. delicatissima* group (station F), *Dactyliosolen* sp. (M), *Ceratium tripos* (F) and dinoflagellates < 20 µm (F) were most common. *Dactyliosolen* sp. was only present at M and F, while the *P. delicatissima* group and dinoflagellates were also found at the other stations. The average abundance of the *P. delicatissima* group at M represented ca. 33% of its average abundance at all stations. Of all species, only diatoms of the *P. delicatissima* group, *Dactyliosolen* sp., *P. seriata* (in very low numbers), *Chaetoceros* spp., *Cylindrotheca closterium*, *Guinardia striata* (only three cells), the two *Rhizosolenia* species, *Thalassiosira* spp., dinoflagellates enumerated in size classes, all *Ceratium* species, *Dinophysis norvegica*, *Dictyocha speculum* and *Ebria tripartita* occurred at the open ocean stations. In general densities were lower in the open ocean than at the shelf stations.

The abundance of each species averaged per sample for the top 100 m is shown in appendix 3. Cell density profiles for species that were abundant at three or more depths at the open ocean stations are shown below. Also presented are contour plots showing the distribution along the shelf stations of diatoms belonging to the *Pseudo-nitzschia* groups and other representative abundant diatom and dinoflagellate species.

2.1.1.3.1 Phytoplankton at M and F

Figure 2.43 shows diatoms that were found at stations M and F in three or more depths: *P. delicatissima* group, *Cylindrotheca closterium* and *Thalassiosira* spp.. All of these were also abundant at the shelf stations. Diatoms of the *P. delicatissima* group reached their highest concentrations at the open ocean stations (6.6×10^3 cells · L⁻¹ in 30 m at M), while *Cylindrotheca closterium* and *Thalassiosira* spp. were found in higher densities at the shelf stations. Cells were mainly found in the upper 100 m. For diatoms of the *P. delicatissima* group highest cell densities at M and F were found in 30 m

depth, only very few cells of the *P. delicatissima* group were observed below 100 m. *Cylindrotheca closterium* and *Thalassiosira* spp. were abundant in the top 30 m at both stations.

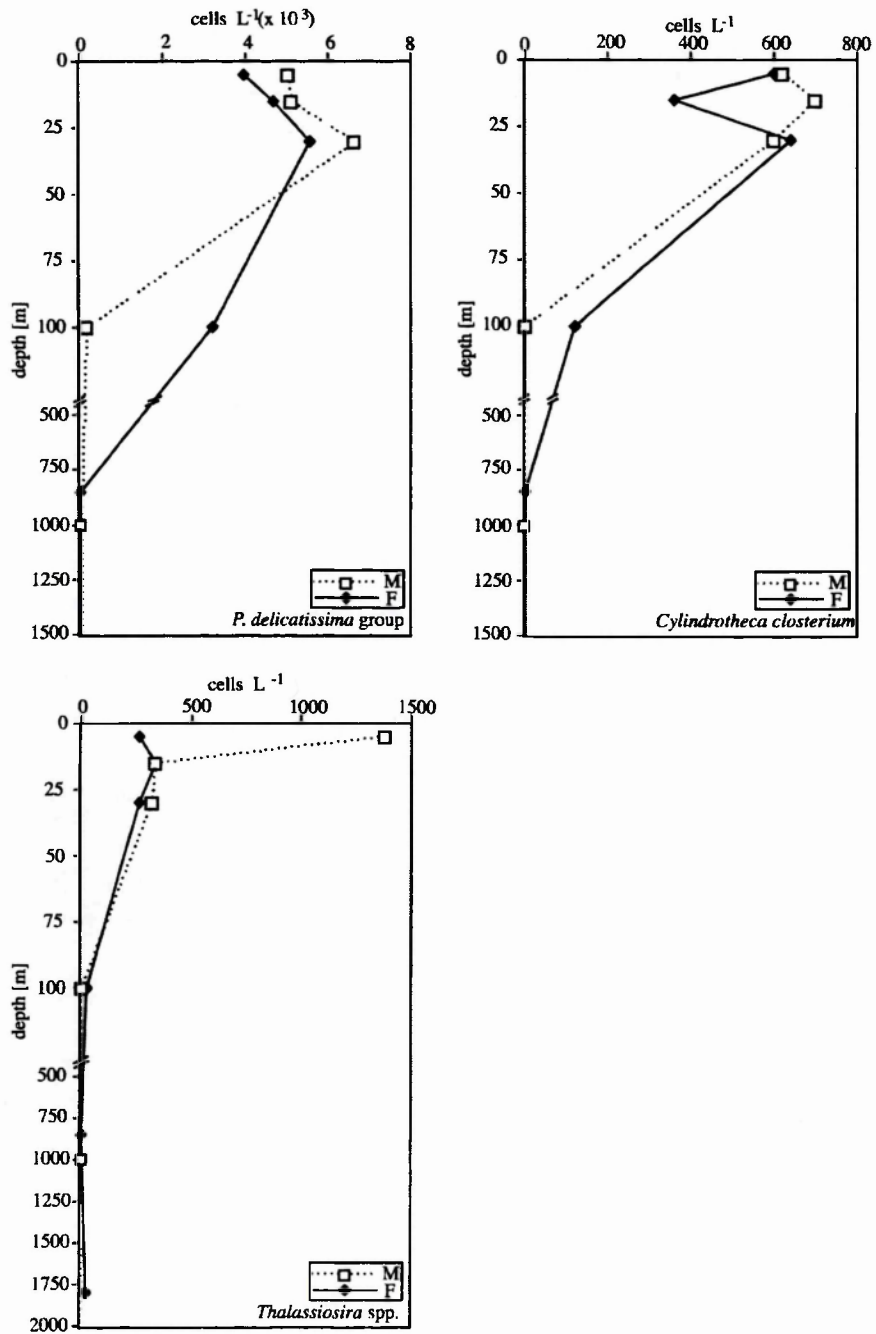


Fig. 2.43 Depth profiles of cell numbers of the most abundant diatoms at station M and F. Note the changing scale of the abscissa below 100 m depth.

Figure 2.44 illustrates the distribution from surface to bottom waters of dinoflagellates enumerated in size classes greater and smaller than 20 μm and *Ceratium* species.

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Similar to the diatoms, these organisms were also mainly found in the surface water. Dinoflagellates $> 20 \mu\text{m}$ were observed in greatest cell density within the top 30 m. About twice as many cells were found in the surface water at F than at M. No dinoflagellates of that size class were found below 100 m depth. Dinoflagellates $< 20 \mu\text{m}$ were observed in all depth at both stations, with maximum cell density at 30 m. Again, the dinoflagellates were more abundant at F than at M.

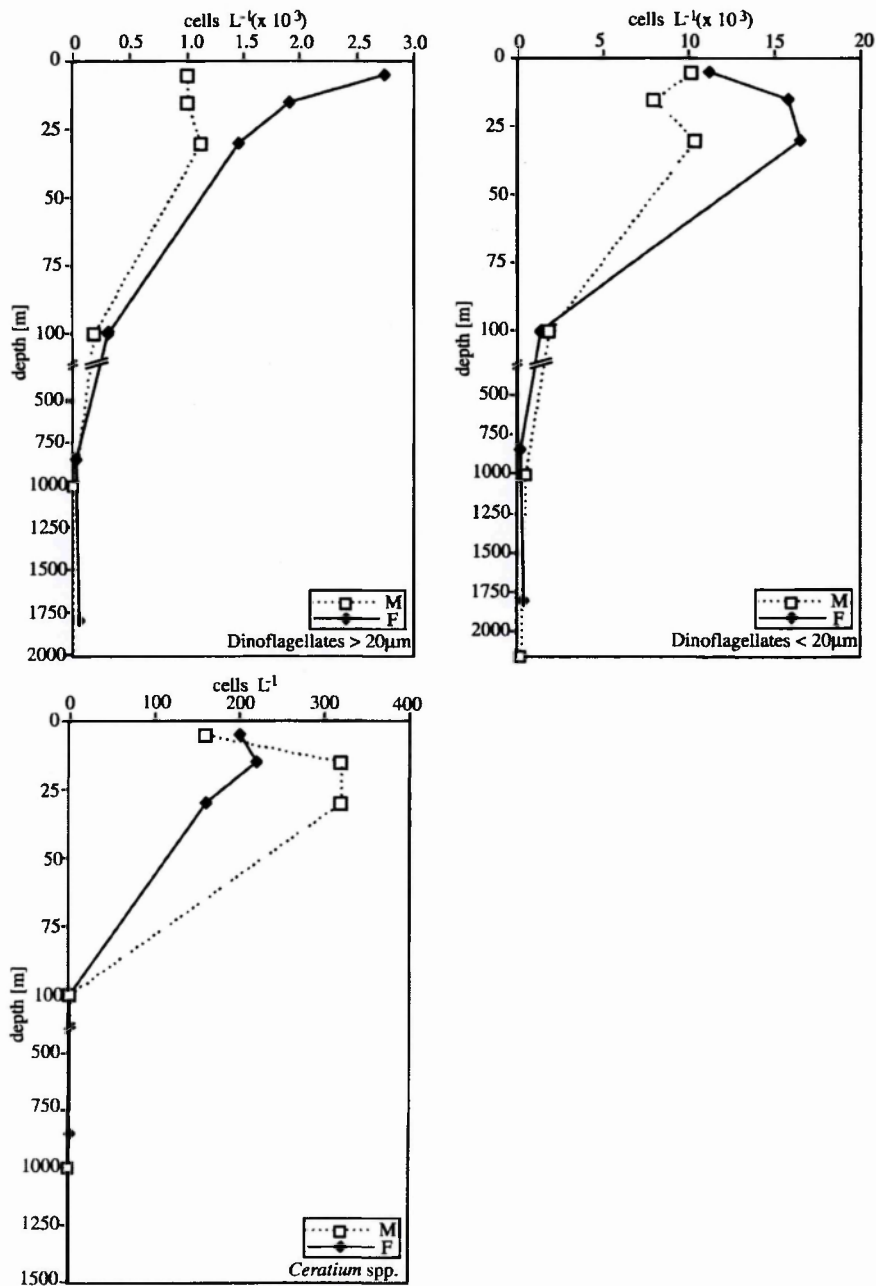


Fig. 2.44 Depth profiles of cell numbers of the most abundant dinoflagellates at station M and F. Note the changing scale of the abscissa below 100 m depth.

At both stations *Ceratium* species were found in maximal numbers in 15 m depth and no cells were found below 30 m. Although their cell numbers (with a maximum of 320 cells · L⁻¹, 15 m at M) were relatively low, as these cells were amongst the largest of all species mentioned in this study, their biomass may be significant.

2.1.1.3.2 Phytoplankton along the shelf stations

All taxa were most abundant in the top 100 m of the water column. For many taxa the maximum depth in which they were found decreased from 1G towards 10G. Lowest cell densities were found at 9G.

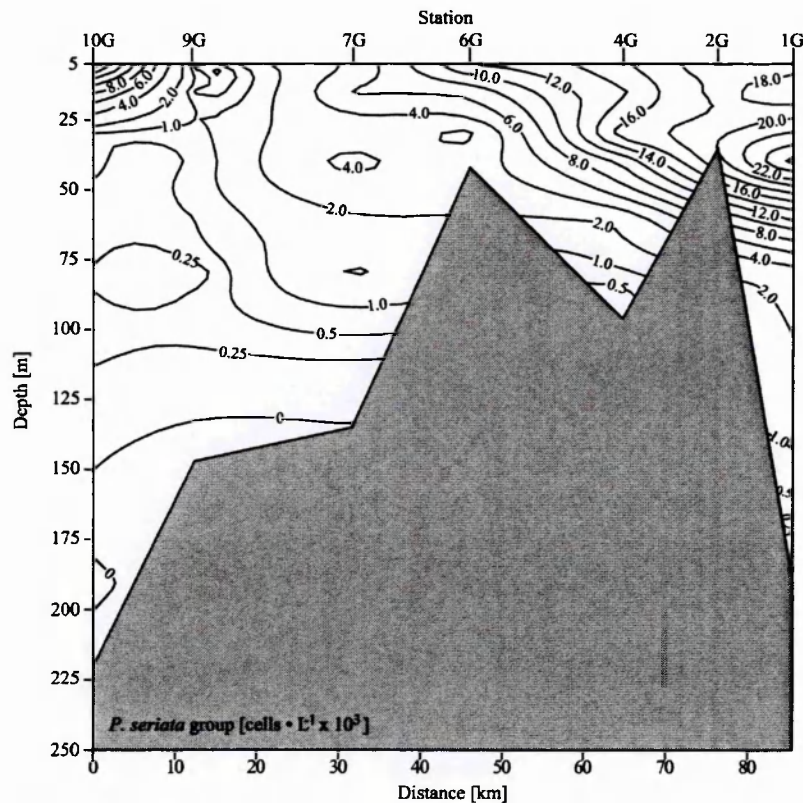


Fig. 2.45 Contour plot of vertical and horizontal distribution of *P. seriata* group abundance in [cells · L⁻¹ × 10³] along the shelf stations (10G to 1G).

Cells belonging to the *P. seriata* group (including *P. seriata*, *P. australis*, *P. cf. subpacificus*, *P. fraudulenta* and *P. pungens*, see chapter 3) were mainly found in the top

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100 m, with a local maximum (more than 26×10^3 cells \cdot L $^{-1}$) in 40 m depth at 1G, and a decrease in density from 1 G to 9G throughout the water column (Fig. 2.45).

Figure 2.46 shows abundance data for the *P. delicatissima* group (including *P. delicatissima* and *P. pseudodelicatissima*, see results chapter 3) obtained from stations 10G to 1G. Again highest cell densities were found above 100 m water depth.

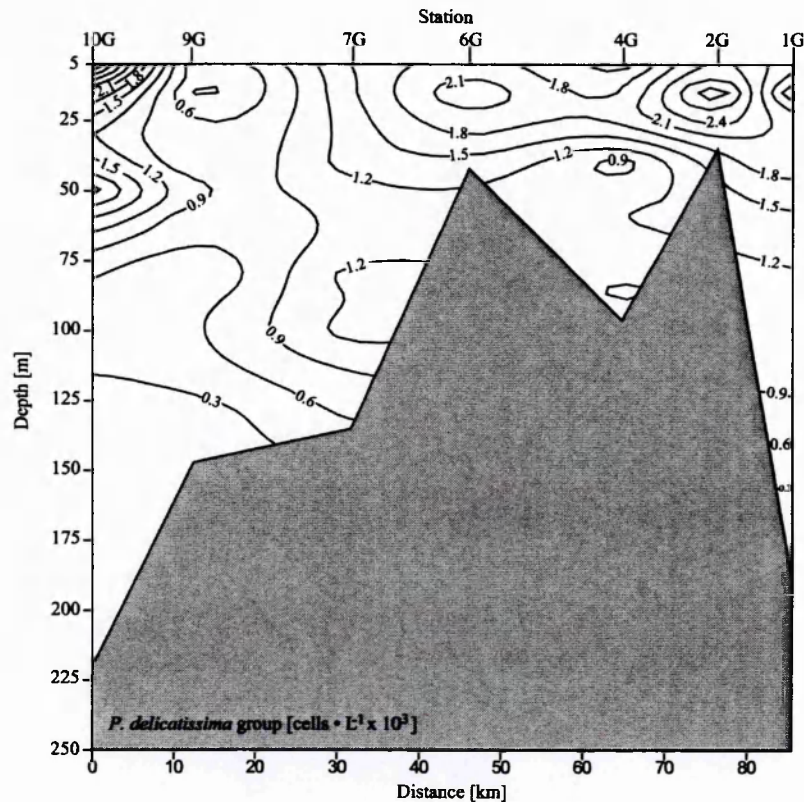


Fig. 2.46 Contour plot of vertical and horizontal distribution of *P. delicatissima* group abundance in [cells \cdot L $^{-1}$ \times 10 3] along the shelf stations (10G to 1G).

Similar patterns to the *P. seriata* group were observed, except at 6G, where a slightly elevated cell density was found in 15 m depth. However, cell densities were considerably lower, ranging from 300 cells \cdot L $^{-1}$ to 3.7×10^3 cells \cdot L $^{-1}$.

Cell densities of *Chaetoceros* species (both size classes) are presented in Figure 2.47. Below 100 m, cell density was low (at 1G), or zero. Highest *Chaetoceros* spp. cell numbers \cdot L $^{-1}$ were usually found between 5 and 30 m, and with maxima at stations 1G

($540 \times 10^3 \text{ cells} \cdot \text{L}^{-1}$, 5 m) and 4G ($364 \times 10^3 \text{ cells} \cdot \text{L}^{-1}$, 5 m). Lowest numbers were found at 9G. From 9G towards 10G *Chaetoceros* spp. cell density again increased in waters < 25 m, following the same pattern as the other species.

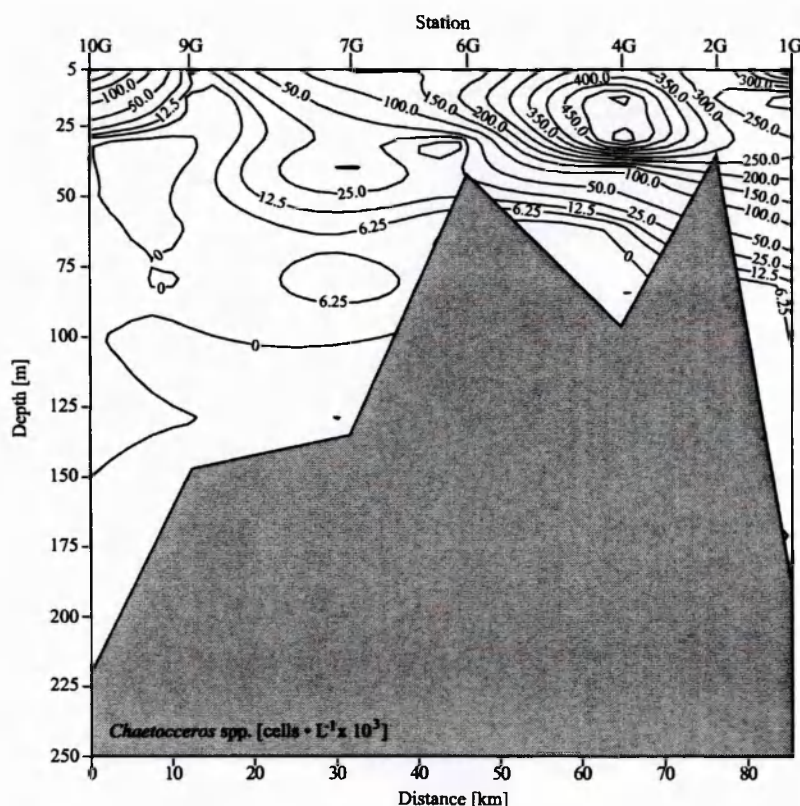


Fig. 2.47 Contour plot of vertical and horizontal distribution of *Chaetoceros* spp. (both size classes together) abundance in [$\text{cells} \cdot \text{L}^{-1} \times 10^3$] along the shelf stations (10G to 1G).

The vertical and horizontal distribution of abundance of other diatoms (e.g. *Thalassiosira* spp., *Lauderia annulata* and *Leptocylindrus danicus*) was very similar to that of *Chaetoceros* species, with no cells or very low densities below 100 m, maxima of cell densities at 4G and lowest numbers at 9G.

The distribution of the abundance of dinoflagellates enumerated in the size class > 20 μm is presented in Figure 2.48.

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They were found in maximal abundance ($6.6 \times 10^3 \text{ cells} \cdot \text{L}^{-1}$) in 5 m water depth at 4G. Cell numbers in that depth decreased towards 2G and 10G. At 9G most of the diatom taxa occurred in low numbers throughout the water column, this was different for the dinoflagellates. In 5 m at 9G $\sim 3.5 \times 10^3 \text{ cells} \cdot \text{L}^{-1}$ were found, their abundance decreased with depth, but still about 1000 cells were enumerated in 62 m depth. A similar concentration was found at 10G in 25 m depth, at 7 G in 90 m, 4G in 70 m and at 1G in about 60 m. At stations 1G, 6G and 7G local maximal concentrations of dinoflagellates $> 20 \mu\text{m}$ were observed in 15 m depth.

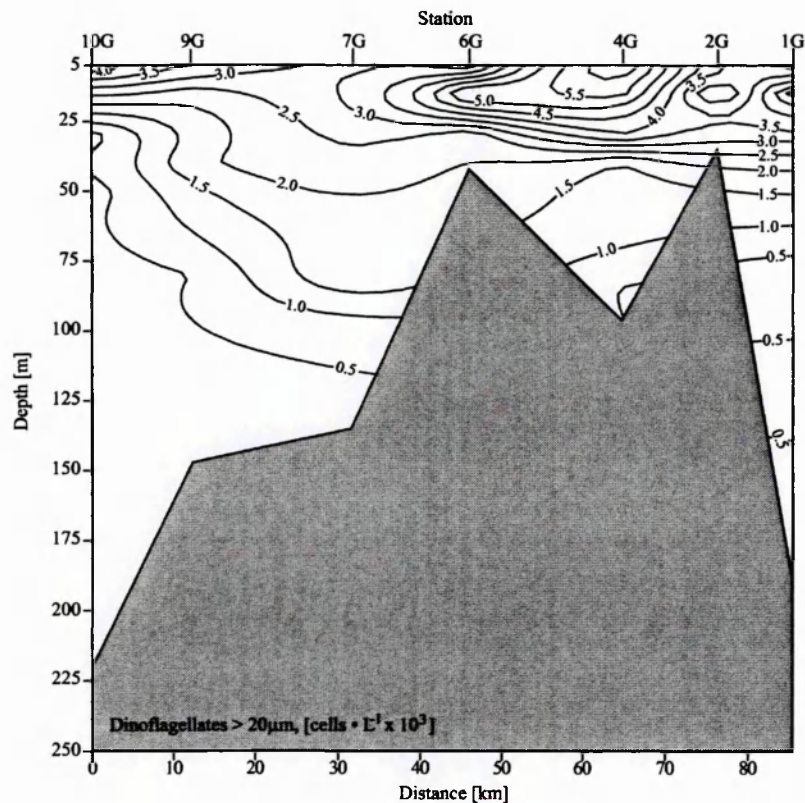


Fig. 2.48 Contour plot of vertical and horizontal distribution of dinoflagellates $> 20 \mu\text{m}$ abundance in $[\text{cells} \cdot \text{L}^{-1} \times 10^3]$ along the shelf stations (10G to 1G).

Dinoflagellates $< 20 \mu\text{m}$ showed a similar abundance pattern. However, the maximum cell density was observed at station 9G ($17.1 \times 10^3 \text{ cells} \cdot \text{L}^{-1}$). The distribution of *Ceratium* spp. was similar to that of *Chaetoceros* species, with highest cell numbers in about 15 m at 4G and a decreasing trend in abundance with depth and towards 9G.

2.1.1.4 Statistical analysis of samples along the Ellett Line transect

2.1.1.4.1 *Multidimensional Scaling*

Figure 2.49 illustrates similarity in species assemblage in samples taken along the Ellett Line transect. The MDS ordinations of samples divides open ocean stations (M and F) from the shelf stations, and indicates great similarity in species composition and abundance in samples taken at M and F and within the shelf stations.

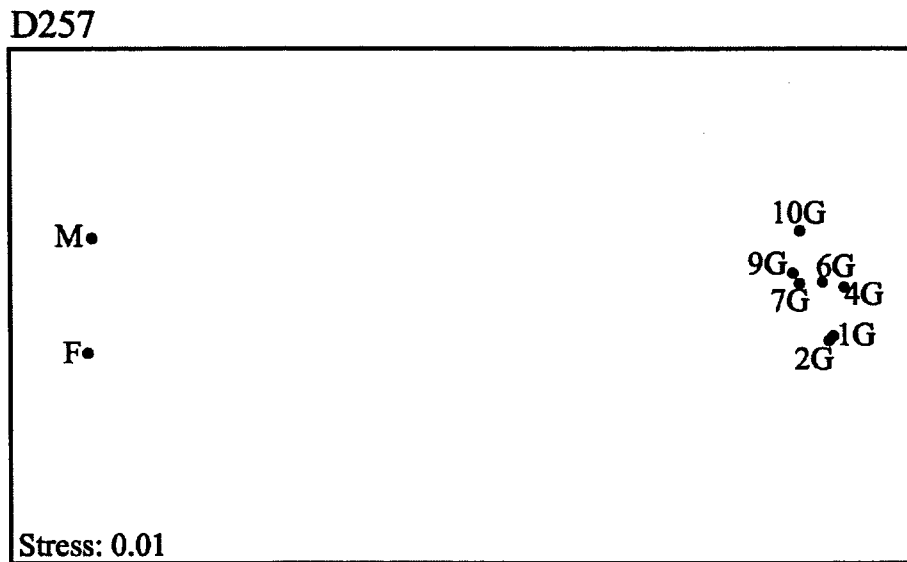


Fig. 2.49 MDS ordination of stations along the Ellett Line transect, sampled during D257. Samples above 100 m depth were included, data were fourth root transformed. Dots (•) symbolise the placement of station.

The ordination of the shelf stations broadly reflected their geographical location. The shelf stations 1G and 2G, 4G and 6 G and 7G and 9G were placed very closed to each other. The very low stress factor of 0.01 indicates the good 2-dimensional representation of the multidimensional species space.

2.1.1.4.2 *Principal component analysis*

The ordination of Ellett Line stations 1G to F according to their measured environmental parameters temperature, salinity, density, phosphate, silicate and nitrate using PCA is shown in Figure 2.50.

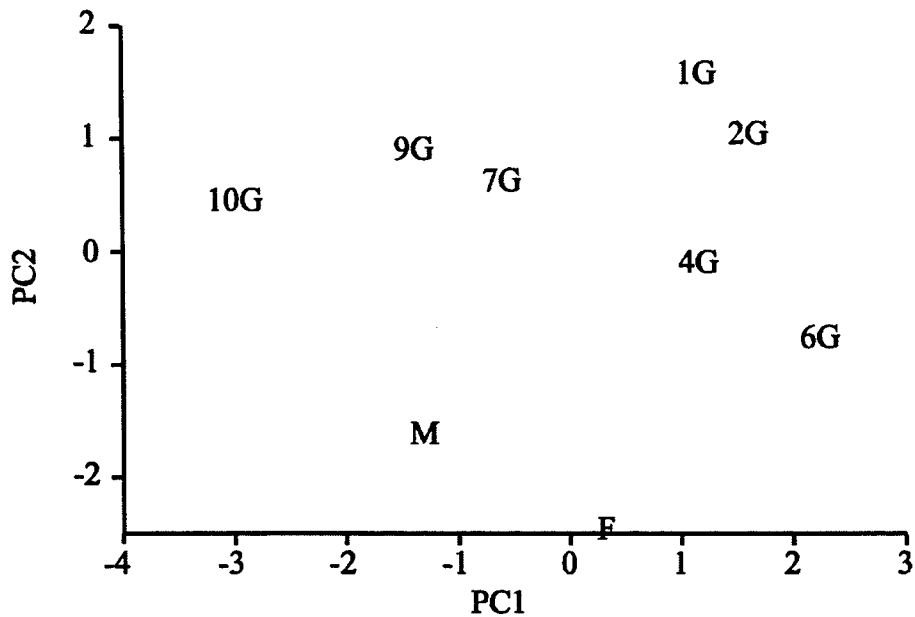


Fig. 2.50 Principal component analysis of stations along the Ellett Line transect, including normalised environmental data (temperature, salinity, density, phosphate, silicate and nitrate) of the upper 100 m of the water column. The ordinate represents the first component and the abscissa represents the second component.

The majority (58.4%) of the variance in the data was explained by the principal component 1 (PC1), while component 2 (PC2) accounted for 35% of the variation (total variation = 93.4%), making the 2-dimensional ordination a good representation of differences in environmental factors between stations. Within PC1 nitrate was the variable with the highest influence on the ordination, followed by temperature, phosphate, silicate and salinity (see Tab. 2.10). In PC2 salinity was most important for the placement of stations, followed by silicate, phosphate, temperature and nitrate. Similarly to the MDS plots, the PCA diagram placed stations 1G and 2G, 9G and 7G and M and F close to each other, indicating a high similarity in the effect of the above environmental factors at those stations. While PC1 separated the coastal stations 1G to 6G from the other shelf and the open ocean stations, PC2 divided all shelf stations (1G to 10G) from the open ocean stations (M and F).

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Table 2.10 Eigenvalues of the environmental variables (coefficients in the linear combinations of variables making up PC's).

Variable	PC 1	PC 2	PC 3	PC 4	PC 5
nitrate	-0.561	-0.12	0.524	0.014	0.629
phosphate	-0.46	0.436	0.205	-0.567	-0.485
salinity	-0.314	-0.62	0.215	0.358	-0.586
silicate	-0.314	0.582	-0.177	0.714	-0.062
temperature	0.509	0.27	0.778	0.203	-0.148

2.1.1.4.3 Redundancy analysis

RDA indicated that of all measured environmental parameters, only salinity (Monte Carlo test, $p = 0.044$) had significant impact on the composition and distribution of the phytoplankton assemblage. Species that were explained by up to 85% by the axes were included in Figure 2.51. The arrow representing salinity was placed along the x-axis, while the y-axis can be interpreted as standing for other, not measured, environmental factors. Open ocean, high salinity stations M and F are placed furthest left, in the same direction as salinity. Stations 10G to 4G were situated between x-axis positions -0.5 and +0.5, indicating intermediate salinity conditions, while 1G and 2G were explained by a negative correlation with salinity. All stations and species seemed to be influenced by other, not measured, factors. The analysis divided species that were present and relatively abundant at the open ocean stations M and F from those that mainly occurred at the shelf stations. Species, such as the *P. delicatissima* group and *Dactyliosolen* sp. were closely associated with stations M and F, where they were the most abundant species, together with dinoflagellates (both size classes), *Ceratium* species and the diatom *Cylindrotheca closterium*.

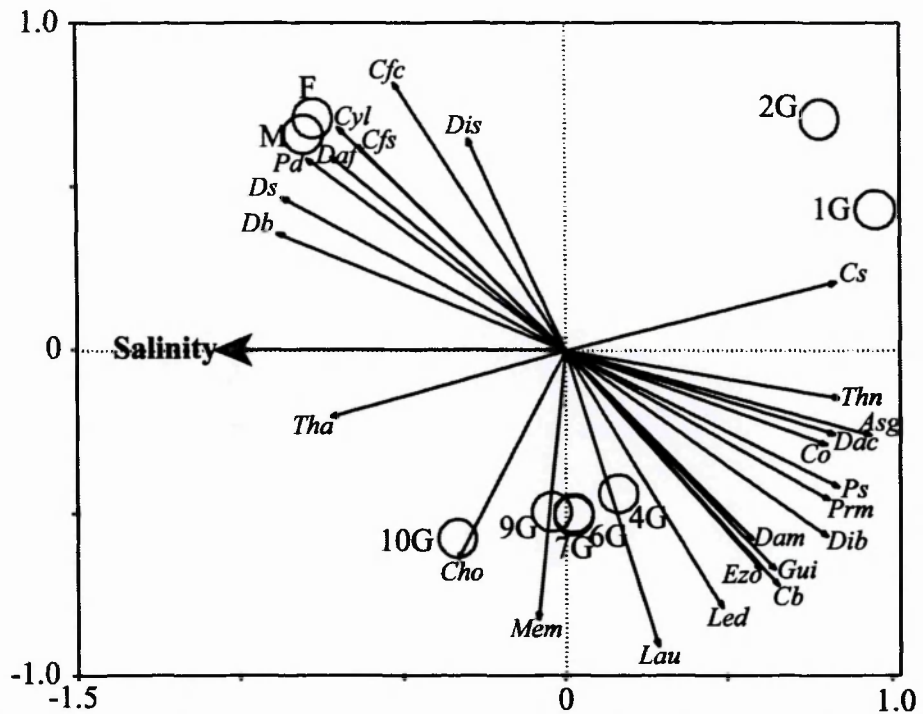


Fig. 2.51 RDA correlation triplot as result of an RDA of samples taken in the upper 100 m of the water column along the Ellett Line transect. Analysis was based on the relative phytoplankton abundance (standardised by norm) and environmental parameters significantly (Monte-Carlo test, $p < 0.1$) influencing the species ordination. Sampled stations are indicated by circles, phytoplankton species and salinity are represented by arrows. Species abbreviations: *Asg*, *Asterionellopsis glacialis*, *Cb*, *Chaetoceros* spp. $> 10 \mu\text{m}$; *Cfc*, *Ceratium furca*; *Cfs*, *Ceratium fusus*; *Cho*, *Ceratium horridum*; *Co*, *Corethron* sp.; *Cs*, *Chaetoceros* spp. $< 10 \mu\text{m}$; *Cyl*, *Cylindrotheca closterium*; *Dac*, *Dinophysis acuta*; *Daf*, *Dactyliosolen* sp.; *Dam*, *Dinophysis acuminata*; *Db*, *Dinoflagellates* $> 20 \mu\text{m}$; *Dib*, *Ditylum brightwellii*; *Dis*, *Dictyocha speculum*, *Ds*, *Dinoflagellates* $< 20 \mu\text{m}$; *Ezo*, *Eucampia zodiacus*; *Gui*, *Guinardia striata*; *Lau*, *Lauderia borealis*; *Led*, *Leptocylinndrus danicus*; *Mem*, *Meuniera membranacea*; *Pd*, *P. delicatissima* group; *Prm*, *Prorocentrum micans*; *Ps*, *P. seriata* group; *Tha*, *Thalassiosira* sp.; *Thn*, *Thalassionema nitzschioides*.

The other species shown in Figure 2.51, including the *P. seriata* group, *Asterionellopsis glacialis* and *Chaetoceros* species, were either not present, or occurred in very low numbers at the open ocean stations M and F. They were influenced by factors that were not measured and showed no direct correlation with salinity.

2.1.2 Main results

The main results from LY1, the Spelve-Creran transect and the Ellett Line are summarised here:

2.1.2.1 Temporal study at LY1

- The water column at LY1 was well mixed and occasionally influenced by freshwater influx; a seasonal pattern was reflected in the temperature profile.
- Elevated nutrient concentrations were found in winter and low nutrient concentrations in summer.
- DIP, DSi and DIN were negatively correlated with salinity.
- Fifty-three phytoplankton taxa were identified at LY1, *Chaetoceros* spp. and *Skeletonema costatum* were numerically the most abundant taxa.
- Six bloom events were observed in each year; while the *P. delicatissima* group was highly abundant during the spring blooms, the *P. seriata* group dominated the summer bloom.
- Most species occurred over a wide temperature range, although individual maximal cell densities were observed at certain temperatures (*P. delicatissima*: ~ 8 °C, *P. seriata* group: ~ 11.5 °C, *Stephanopyxis turris*: ~ 14°C).
- A significant positive correlation was found between the abundance of the *P. seriata* group and temperature.
- Several events of high cell density (exceeding the average density of all samples) of *P. delicatissima* and/or *P. seriata* occurred during the year, persisting from one week to several weeks.
- A statistically significant seasonal pattern in the floristic composition and abundance of phytoplankton was evident; temperature and nutrient concentrations were the main factors influencing the phytoplankton assemblage.

2.1.2.2 Spelve-Creran transect

- The seawards stations 700 and LY3 showed physical characteristics of the open sea (higher salinity, lower temperature), stations LY2, LY1 and C2 shared hydrographical features, although LY2 and LY1 were influenced by freshwater runoff, C3 and C5 represented sea-loch conditions.

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- The DSi concentration rapidly decreased in Loch Creran between July and August.
- The most abundant species in summer 2002 was *Leptocylindrus danicus*, dinoflagellates were more abundant in Loch Creran than at other stations.
- The similarity of LY1, LY2 and C2 in their physical, chemical and biological parameters, indicated that the monitoring site represented Scottish inshore waters of the Firth of Lorne - not sea-loch or "exposed, open sea" conditions.

2.1.2.3 Ellett Line transect

- Open ocean and shelf stations were well mixed in the upper ~ 100 m.
- Salinity increased from coastal waters towards the open ocean, while temperature decreased (apart from 9G, where temperature was elevated in the top 50 m).
- Phytoplankton was abundant in the top 100 m.
- Open ocean stations M and F consisted of the same water mass.
- The properties of the water mass at station 9G were more similar to 7G, M and F than 10G water.
- Stations 1G and 2G as well as 4G and 6G represented the same water masses.
- The inorganic nutrient concentrations were similar throughout the top 100 m at shelf stations.
- The concentration of phytoplankton was higher on the shelf than in the open ocean.
- Within the shelf the phytoplankton density was lowest at 9G, except for dinoflagellates < 20 µm.
- The *P. delicatissima* group, was more abundant at the open ocean stations than at the shelf stations.
- Open ocean stations M and F showed significant differences from the shelf stations in the phytoplankton composition and abundance.
- Salinity was the only factor measured that had a significant statistical influence on the phytoplankton assemblage; other, not measured, factors might have played an important role as well.

2.2 Discussion

2.2.1 Temporal monitoring at LY1

2.2.1.1 Long-term phytoplankton monitoring

Long-term monitoring of phytoplankton is necessary to detect any differences in the phytoplankton assemblage that have occurred due to climatic or hydrographic changes (e.g. as speculated in Lange et al. 1992). An intensive monitoring program can help detecting trends against a background of short-term, seasonal and interannual variability.

Although some sampling of the phytoplankton community and related environmental factors was conducted in the Firth of Lorne and Loch Creran in the seventies and the early eighties (e.g. Tett & Wallis 1978; Tett et al. 1981a, b; Grantham 1983a, b; Grantham et al. 1983), a time series was not maintained. A long term series in the Firth of Lorne could have provided information about occurrences of toxic blooms in the past before the establishment of fish farming and the shellfish industry, and hence given insight about potential anthropogenic impact on the ASP situation in Scottish waters. Therefore there was a requirement to start a new time series, and also to conduct laboratory experiments (see chapter 4), to investigate the impact of environmental factors on *Pseudo-nitzschia* species and domoic acid production.

The information from early studies is of limited use due to changes in taxonomy and/or grouping of phytoplankton species (e.g. Dortch et al. 1997; Vrieling et al. 1996). When studying the phytoplankton in Loch Creran in 1979, Tett et al. (1981b) summarised *N. delicatissima*, *N. seriata* and *Cylindrotheca closterium*. However, their drawings suggested the species *P. delicatissima* (then called *N. delicatissima*), *P. seriata*, *P. australis* and/or *P. fraudulenta* (all called *N. seriata* in that study) might have been present. Within this study, original phytoplankton samples that had been taken by P.

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Tett during the 1970's in local waters were obtained and re-examined. However, unfortunately all diatom frustules in the Lugol's Iodine preserved samples had dissolved and hence could not be re-examined for *Pseudo-nitzschia* species determination using modern taxonomy.

2.2.1.2 LY1 suitability as a monitoring site

Sampling the transect from Loch Spelve to Loch Creran in summer 2002 indicated that LY1 represented western Scottish coastal waters, that were not typical for a sea loch and also did not mirror exposed, seawards locations. The conditions in Loch Creran were characteristic of a typical Scottish sea loch, in its dimensions, freshwater input and tides (Tett & Wallis 1978) and temperature and salinity stratification down to about 7 m (in August 2002). In contrast to the Loch Creran stations, station 700 and LY3 were influenced by the open sea, showing higher salinities and well mixed water columns. Hence, not characterised by sea loch or open sea conditions, stations LY2, LY1 and C2 were represented the general properties of the Lynn of Lorne. The occasional fresh water influx observed at LY1 only rarely influenced 10 m depth, from which phytoplankton and nutrient samples were taken. Results of multidimensional scaling, showed that in the configuration LY1 was placed between the seawards stations and the sea loch stations, supporting its good representation of "average" western Scottish coastal waters.

2.2.1.3 Phytoplankton monitoring at LY1

The phytoplankton monitoring at LY1 showed a repeatable annual cycle of phytoplankton species succession. *Pseudo-nitzschia* species blooms also occurred in a regular pattern. A typical species succession pattern, related to availability of inorganic

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nutrients and their recycling, as observed in temperate waters (e.g. Drebes 1974) was found, with diatoms dominating the spring bloom and the occurrence of ciliates and dinoflagellates during summer and early autumn. However, in this study 6 chl *a* peaks were observed throughout the year (see table 2.5, section 2.3.1.4), which is different from the "classical" species succession model, consisting of one phytoplankton spring bloom, and a late summer/ early autumn bloom (e.g. Ott 1996). Those chl *a* peaks reoccurred with a similar timing and species composition, and were always composed of multiple diatom species.

The multiple chl *a* maxima also demonstrate the necessity of regular and temporally highly resolved sampling, when monitoring phytoplankton, and in particular harmful algae. Some blooms, including those of potential harmful *Pseudo-nitzschia* species would sometimes persist for just a short period of less than a weeks time. A monthly or fortnightly sampling would likely miss blooms that are potentially of only short duration, but could be highly toxic.

Some species were found at one time of the year (e.g. *Stephanopyxis turris*, *Rhizosolenia setigera*), while others were found during the whole year (e.g. *Paralia sulcata*, *Cylindrotheca closterium*). Species like *Stephanopyxis turris* and *Rhizosolenia setigera* might have a preference for higher temperatures as found in western Scottish waters in late summer/ early autumn. The occurrence of *Paralia sulcata*, which was the only species to be found regularly during winter, might be related to the fact that it is generally a benthic species, but might have been transported into the water column by tidal mixing and turbulence caused by storms, as observed during the winter. *Cylindrotheca closterium* was the only species that was present in every sample, despite its relatively low numbers, indicating its broad tolerance to many environmental factors.

With exception of the ciliate blooms in summer, the study confirmed the impression that the Firth of Lorne phytoplankton was diatom dominated, in contrast to the Firth of Clyde, where large dinoflagellates, including *Ceratium* spp. and *Protoperidinium* species, were abundant in summer (Tett 1992).

'Small flagellates' (< 5 µm) were not enumerated, but were present in the samples throughout the year. They might be an important component of the phytoplankton community in Scottish waters, as for example observed in late spring in Loch Striven by Wood et al. (1973).

2.2.1.3.1 Spring blooms

Two consecutive blooms were observed in spring, the first one lasting from March until April and the second one in April and May. This was consistent with results from Sakshaug & Mykkestad (1973) from the Trondheimsfjord in Norway, who also observed two spring blooms in 1970 and 1971.

In the present study, the first chl *a* peak of the year with a concentration above 1 µg · L⁻¹ occurred in March, which agrees with observations from Loch Creran in the 1970's (Tett & Wallis 1978). The dominant species in the first spring bloom in this study and past studies of local waters and the Trondheimsford (Sakshaug & Mykkestad 1973; Tett et al. 1981b; Tett et al. 1986; Tett & Edwards 2002) were *Skeletonema costatum*, *P. delicatissima* and *Thalassiosira* spp..

Similar to the first bloom of the year described in the Trondheimsfjord, the first spring bloom at LY1 started to develop in waters abundant in nutrients. Sakshaug & Mykkestad (1973) described those conditions for the first spring bloom as analog to a "batch culture". A store of nutrients was built up during winter and was likely to be the main source of nutrients for this bloom. The same build up of nutrients over the winter

months, and their rapid uptake by the first spring bloom was observed at LY1 from 2001 to 2003.

The second bloom in the present study was dominated by *Chaetoceros* species, which continued as a third bloom into June. As inorganic nutrients were largely used up during the first spring bloom, the second bloom and the following ones until July might have been governed by nutrient supply from runoff and outflow of nutrient rich water from the sea lochs. Additionally, the increasingly available light due to the increase in day length may have contributed to the following events of high phytoplankton density.

2.2.1.3.2 Summer blooms

In each year, the ciliate *Mesodinium rubrum*, diatoms of the *P. seriata* group and *Leptocylindrus danicus* (in 2001) dominated the summer bloom at LY1. High abundances of that ciliate are common in summer in Scotland (Tett et al. 1992) and world-wide (e.g. Bay of Fundy, Martin et al. 1990; Crawford 1989). *Mesodinium rubrum* contains cryptomonad endosymbionts, which possess a red phycobiliprotein pigment, giving blooms their characteristic red colour and enabling the ciliate to photosynthesise (for review see Crawford 1989). Water discoloration due to *M. rubrum* has previously been reported from Loch Spelve (Isle of Mull) (Tett et al. 1981b). Although there is no evidence of toxin production, blooms of *M. rubrum* have been associated with harmful effects on marine life (reviewed by Landsberg 2002). During summers of 2001 and 2002, when water discoloration due to *M. rubrum* was observed in the Lynn of Lorne, SAMS was contacted by local salmon farmers who inquired about the current phytoplankton situation. They reported that their fish were showing an unusual behaviour ("vigorously jumping in the cages"). One might speculate that this behaviour was caused by a *M. rubrum* bloom. The ciliates, which carry cirri that are arranged in a ring around the cell, might have clogged the fish gills.

The measured chl *a* concentrations at LY1 and along the Spelve-Creran transect in summer 2002 were related to the abundance of *Mesodinium rubrum*, the *P. seriata* group, *Chaetoceros* species and *Leptocylindrus danicus*. Again, those observations are consistent with results from Loch Creran from the late 1970's, where the summer bloom was then dominated by small *Chaetoceros* species, which reached numbers up to 10^6 cells · L⁻¹ and *Leptocylindrus danicus* (10^6 cells · L⁻¹ at beginning of September) (Tett et al. 1981b). Confirming observations of this study, in earlier studies *Leptocylindrus danicus* and '*Nitzschia seriata*' were often reported together as the dominant phytoplankton species in local waters (e.g. in Scottish sea lochs in August 1926, Marshall & Ott 1927; Loch Etive in August 1970 and 1971, Wood et al. 1973) and southern UK waters in the summer (Maddock et al. 1989).

In this study dinoflagellates were most abundant during the summer and early autumn, but they did not dominate the phytoplankton assemblage. Similarly, in Loch Creran in the late 1970's, Tett et al. (1981b) observed dinoflagellates in high numbers only in late summer and early autumn. Interestingly, along the Spelve-Creran transect highest cell numbers of dinoflagellates (all taxa) were observed in Loch Creran. One factor influencing the abundance of dinoflagellates might have been the low salinity that was measured in the surface layer in Loch Creran. From observations in Loch Etive in the early seventies, Wood et al. (1973) concluded that dinoflagellates, which were numerous only in the surface layer, seemed to be favoured by low salinities.

2.2.1.4 Temporal distribution patterns of *Pseudo-nitzschia* spp.

The *P. delicatissima* and *P. seriata* group had different temporal distributions (see appendix 1), with the former occurring in highest densities during spring and the latter in summer and early autumn. The main differences between spring and summer conditions in western Scottish waters were high inorganic nutrient conditions in spring

as opposed to low concentrations in summer, short day length in spring, long light availability in summer and cold water temperatures in spring contrasted by relatively warmer temperatures in summer. Hence the different time of maximal abundance suggests that both *Pseudo-nitzschia* groups have different tolerances of those factors.

For the *P. delicatissima* group the field observations suggested a tolerance for low temperature and short light conditions (the latter was confirmed in a laboratory experiment, see chapter 4, experiment C). The occurrence of *P. delicatissima* in summer and early autumn, although in a reduced cell density might indicate, that in spring and summer the group was composed of different species. Indeed, it is likely that the *P. delicatissima* group in spring was dominated by the non-toxic species *P. delicatissima*, as all cultures that were isolated during that time were identified as that species (chapter 3). On the other hand in summer and early autumn, potentially toxic species of that group, such as *P. calliantha*, *P. pseudodelicatissima* and *P. cf. delicatissima* were identified by TEM from net samples together with *P. delicatissima*. This was also observed by Hasle et al. (1996) in samples from the Skagerrak and Norwegian coast between 1980 and 1993, who found *P. delicatissima* in highest abundance in spring and maximal *P. pseudodelicatissima* densities mainly in summer. The maximal cell density of diatoms belonging to the *P. delicatissima* group (1.6×10^5 cells \cdot L⁻¹) in the present study was found in April. This cell density is comparable with other reported maximal abundances of the *P. delicatissima* group found in other parts of the world (Table 2.11). In contrast to the *P. delicatissima* group, the *P. seriata* group seemed to be adapted to low inorganic nutrient conditions and long day lengths. In western Scottish waters it contained at least two toxic species, *P. australis* and *P. seriata* (see chapter 3), which frequently occurred together with non-toxic *P. fraudulenta*, *P. pungens* and *P. cf. subpacificus*. The maximum number of diatoms belonging to the *P. seriata* group in western Scottish waters was $\sim 10^5$ cells \cdot L⁻¹. This is the trigger level of *Pseudo-*

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nitzschia density, which was noted by Bates et al. (Bates et al. 1998) to cause ASP. A cell density of the same magnitude had caused ASP in southern California in the 1990's, killing pelicans (Buck et al. 1992; Work et al. 1993) and sea lions (Scholin et al. 2000). Although the average number of cells belonging to the *P. seriata* group was a magnitude smaller (10^4 cells \cdot L⁻¹) than the ASP trigger density, blooms in that density persisted for some weeks (Tab. 2.7), and might have lead to accumulation of DA in the food chain. *Nitzschia seriata* counts from Loch Creran in 1979 and 1980 (Tett & Edwards 2002) showed that potentially toxic species have occurred in numbers $\sim 10^5$ cells \cdot L⁻¹ for a couple of weeks, suggesting that DA contamination of marine life might have been possible at that time. However, shellfish was then not tested for DA and reports of shellfish poisoning in humans or marine wildlife are not known.

Cell numbers of diatoms belonging to the *P. seriata* group were comparable with other observations of blooms of those taxa at world-wide locations (see Tab. 2.12).

The precise timing of *P. delicatissima* and *P. seriata* maximal cell density might help forecasting blooms. However, to exactly predict and model the *P. seriata* occurrence, a better understanding of their physiology and the influencing factors are needed. The experimental work conducted in this study (chapter 4) gave some insight into *P. seriata* growth dynamics and its toxin production, but to establish a model for ASP prediction, further experiments on the algae and also algae-shellfish interactions are needed.

Table 2.11 Literature information on species belonging to the *P. delicatissima* group regarding their approximate maximal cell densities [$\text{cell} \cdot \text{L}^{-1}$], time of the year, and location where it was observed in high abundance.

Species / Group	Time	Cell number [$\text{cells} \cdot \text{L}^{-1}$]	Location	Reference
<i>P. delicatissima</i> group	Apr ('02)	max. 1.6×10^5	LY1, western Scotland, UK	this study
<i>Nitzschia delicatissima</i>	Mar/April ('80) Aug/Sep ('79)	10^5	Loch Creran, western Scotland, UK	Fig. 3.2. in (Tett & Edwards, 2002)
<i>P. delicatissima</i>	May, Sep ('96)	max 2×10^6 (total P-n species)	Irish waters	(Cusack et al. 2000)
<i>Nitzschia delicatissima</i>	Apr ('70)	2.5×10^5	Trondheimsfjord, Norway	(Sakshaug & Mykkestad, 1973)
<i>P. delicatissima</i>	Aug ('00)	max. 1.8×10^6	Gulf of St. Lawrence, Canada	(Couture et al. 2001)
<i>P. calliantha</i>	Jul - Sep ('98)	max 1.2×10^6	Bay of Fundy, Canada	(Martin et al. 1990)
<i>P. pseudodelicatissima</i>	Sep ('98)	15.4×10^6	Washington state, USA	(Trainer et al. 2002)
<i>P. pseudodelicatissima</i>	Sep ('98)	max 9.5×10^6	Washington state, USA	calculated from tables (Stehr et al. 2002)
<i>P. pseudodelicatissima</i>	Jun ('98)	10^6	Monterey Bay, California	(Scholin et al. 2000)
<i>P. delicatissima</i>	Apr ('98)	max. 7×10^6	Louisiana, USA	(Parsons et al. 1999)
<i>P. pseudodelicatissima</i>		max. 1.4×10^7		

Table 2.12 Literature information on species belonging to the *P. seriata* group regarding their approximate maximal cell densities [cell · L⁻¹], time of the year, and location where it was observed in high abundance.

Species / Group	Time	Cell number [cells · L ⁻¹]	Location	Reference
<i>P. seriata</i> group	Jun to Aug, Sep/Oct ('01-'03)	10 ⁴ - 10 ⁵ 10 ⁴	LY1, Western Scotland, UK	this study
<i>Nitzschia seriata</i>	Jul ('79, '80) early May ('80) Sep to Nov ('79)	6 x 10 ⁴ 10 ⁵ 10 ⁴	Loch Creran, Western Scotland, UK	Fig. 3.2. in (Tett & Edwards, 2002)
<i>P. australis</i>	Sep ('99)	max 2 x 10 ⁶ (total P-n species)	Irish waters	(Cusack et al. 2000)
<i>P. australis</i>	Dec ('99)	6.8 x 10 ⁴	Galician coast, Spain	(Rodriguez et al. 2001)
<i>P. fraudulenta</i>	June/July ('99)	8 x 10 ⁵		
<i>P. multiseriata</i>	Dec- Feb Oct ('87/'88)	max 1.5 x 10 ⁷ max 4 x 10 ⁵	PEI, Canada	(Bates et al. 1989)
<i>P. seriata</i>	Jun/Aug ('97, '99, 2000)	> 10 ⁴ > 5 x 10 ⁴	Gulf of St. Lawrence Gulf of St. Lawrence	(Couture et al. 2001) J-Y Couture pers . com.
<i>P. seriata</i> group	Jun - Sep Jun / Jul ('99) Aug/Sep ('98)	max 7.9 x 10 ⁶ max 2.4 x 10 ⁶	Washington state, USA	calculated from tables (Stehr et al. 2002)
<i>P. seriata</i> group	Jul ('97)	max 1.4 x 10 ⁷	Washington state, USA	(Trainer et al. 1998)
<i>P. multiseriata</i> & <i>P. pungens</i>	May ('92)	> 10 ⁵	Washington state, USA	(Horner & Postel, 1993)
<i>P. australis</i>	Sep to Nov ('91)	max 6.7 x 10 ⁵	Monterey Bay, California, USA	(Buck et al. 1992) (Work et al. 1993)
<i>P. australis</i>	May ('98)	max 1.3 x 10 ⁵	Monterey Bay, California	(Scholin et al. 2000)
" <i>N. seriata</i> "	Feb/Mar, Apr/May, Jun, Jul/Aug ('28, '36, '40, '41)	10 ⁴ to 10 ⁶	SIO Pier, California, USA	(Lange et al. 1994)
<i>Nitzschia seriata</i> group	Mar ('83)	3 x 10 ³	SIO Pier, California, USA	(Reid et al. 1985)
<i>P. australis</i>	Mar ('91) Jul ('92)	10 ⁵ 10 ⁴	SIO Pier, California, USA	(Lange et al. 1994)
<i>P. multiseriata</i>	April ('98)	2.2 x 10 ⁵	Louisiana, USA	(Parsons et al. 1999)
<i>N. seriata</i> (<i>P. fraudulenta</i>)	Sep - Oct (austr. spring, '78/'79)	up to 2 x 10 ⁶	Sydney, Australia	(Hallegraeff, 1981)

2.4.1.5 Physical and chemical parameters at LY1

Physical and chemical parameters at LY1 obtained between 1978 and 1983 by Grantham (1983a, b), Grantham et al. (1983) and Tyler et al. (1983) were compared with this study (Fig. 2.52). Grantham (1983a, b) reported a relationship between low salinity surface water and high silicate concentration at LY1. This relationship was confirmed (Spearman rank $p < 0.0001$) and further negative correlations of DIP and DIN ($p < 0.0001$) at 10 m with salinity were identified. Salinity at LY1 showed a variable annual pattern in years 2001 to 2003. This may be interpreted as a reflection of precipitation and fresh water influx influencing LY1, at 10 m depth. Precipitation was not measured, but the density and salinity profiles from LY1 showed that, even at 10 m depth, LY1 was from time to time affected by fresh water influx. This fresh water, presumably originating from outflow from Loch Creran, Loch Etive, or from further up the Firth of Lorne (from Loch Linnhe), would have carried nutrients that contributed to the increase in the dissolved inorganic nutrient concentration at LY1 and hence the observed negative correlation with salinity. Observations along the Spelve-Creran transect suggested that LY1 was at times more strongly influenced by freshwater runoff from Loch Etive, than from Loch Creran. In July 2002, when lowest surface salinities were found at LY1 an elevated salinity was observed at the entrance to Loch Creran (C2), compared to surface water in the Loch (see Fig. 2.22). The greater influence of Loch Etive freshwater runoff was due to the location of LY1, close to the entrance of Loch Etive and the higher fresh water runoff from Loch Etive, which is with $\sim 3 \times 10^9 \cdot \text{m}^3 \cdot \text{year}^{-1}$ about a magnitude higher than that from Loch Creran ($\sim 2.9 \times 10^8 \cdot \text{m}^3 \cdot \text{year}^{-1}$) (Edwards & Sharples 1986).

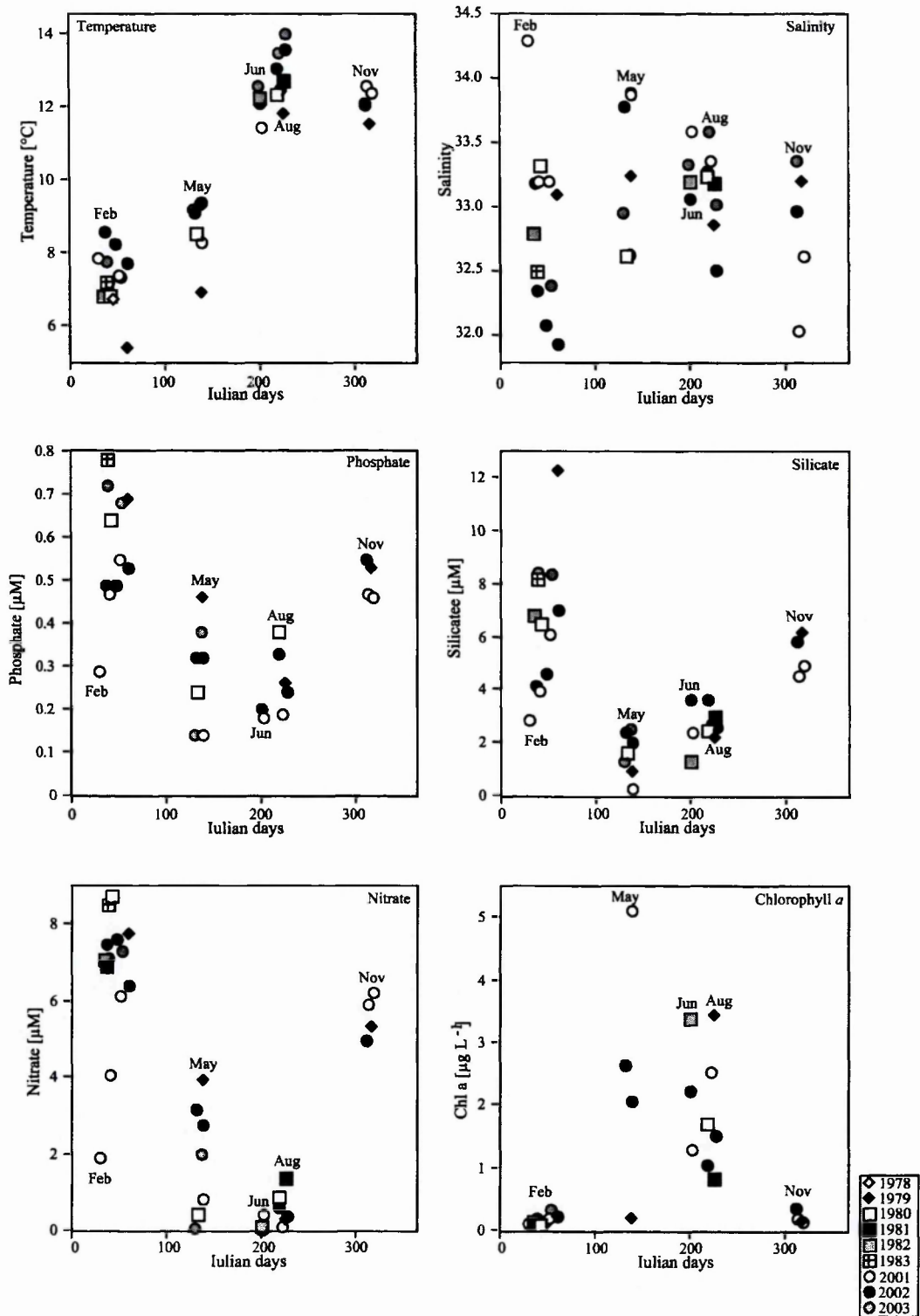


Fig. 2.52 Comparison of physical (T, S) and chemical (NO_3 , PO_4 , Si, Chl a) parameters measured at LY1 at 10m depth in 1978 - 1983 (diamond, squares, Grantham 1983a, b; Tyler et al. 1983) and in 2001-2003 (circles, this study).

Statistical analysis showed a significant difference in temperature (Mann-Whitney-U test, $p = 0.007$) in February between recent data (2001-2003) and those obtained between 1978 and 1983. While the average temperature in February between 1978 and

1983 was 6.58°C (average from 5 data points), it was more than 1°C warmer between 2001 and 2003 (7.74°C, average from 8 data points). This increase in temperature might be a result of warmer air temperature in winter, due to NAO (North Atlantic Oscillation), or possibly global warming, and could potentially cause an earlier start of the spring bloom. However, that would have resulted in an earlier peak of chl *a*, which was not observed. For other physical and chemical parameters and other times of the year (see Fig. 2.52) differences between recent and data from more than 20 years ago were not significant ($p > 0.05$). Most of the parameters, such as salinity, dissolved inorganic nutrient concentrations and chl *a* showed a high interannual variability.

2.4.1.6 Environmental factors and phytoplankton distribution

Redundancy analysis and Spearman rank correlation ($p < 0.0001$) showed that cell density of the *P. seriata* group was positively correlated with temperature, while abundance of the *P. delicatissima* group was not. In Monterey Bay, California during monitoring from 1989 to 1991, *P. australis* was also significantly correlated with temperature (maximum abundance during the warmer time of the year). However, many periods of high abundance were also associated with weak upwelling events that resulted in cooling at the sea surface (Buck et al. 1992).

While most of the species belonging to the *P. seriata* group that occur in Scottish waters are known as temperate species (Hasle & Syvertsen 1996), *P. seriata*, is thought to be a cold water species (Smith et al. 1994), with a restricted distribution to the North Atlantic (Hasle 2002). It has previously been described as part of the under-ice community (e.g. Smith et al. 1994; Quillfeldt von 1996; Fehling 2000), and can also occur in the infiltration assemblage at the snow-ice interface, when the snow on the ice surface is flooded with seawater (Horner 1990). However, cultured strains within this study (chapter 3) were not affected by an upper lethal temperature limit of $> 12^{\circ} \text{C}$, as

found by Smith et al. (1994); they were successfully grown and maintained at 15° C. Furthermore, the temporal monitoring at LY1 showed that diatoms of the *P. seriata* group were most abundant during summer and early autumn, when water temperatures reached maximal values. Similar observations were made in eastern Canada, the Gulf of St. Lawrence region, where the higher abundances of *P. seriata* ($> 5 \times 10^4$ cells · L⁻¹) are habitually found between June and September, when water temperatures range between 10° C and 16° C (J.-Y. Couture, personal communication). The Scottish and Canadian field observations suggest that although seen as a psychrophilic species, *P. seriata* can adapt to higher temperatures and may occur at water temperatures $> 12^\circ$ C.

Apart from the correlation of some species with temperature, redundancy analysis showed a negative relationship between taxa that were abundant in the summer (including *P. seriata* group) and dissolved inorganic nutrients. Lowest nutrient concentrations in the water were associated with highest phytoplankton abundance. Dortch et al. (1997) also observed a significant negative relationship between *Pseudo-nitzschia* spp. presence in Louisiana (USA) coastal waters and all nutrient concentrations. The low nutrient concentrations can be interpreted as a consequence of high phytoplankton cell density, rather than an indication of a preference of summer taxa for nutrient low water. The measured concentrations in the water are the remains of the nutrients that have been taken up and utilised by the phytoplankton, enhancing their biomass. As only dissolved inorganic nutrients were measured, it is not known what proportion of nutrients was steadily supplied, e.g. through efflux of nutrient enriched water from the sea lochs Etive, Creran, or water from further up the Lynn of Lorne, and what proportion was immediately taken up by phytoplankton. It is likely that some nutrients such as silicate, phosphate or nitrate were limited in their abundance during the summer and may have governed the composition of the phytoplankton community at that time. In competition experiments with 11 marine phytoplankton species,

including *Pseudo-nitzschia pungens*, Sommer (1994) showed that *P. pungens* (*P. seriata* group) was the dominant species at relatively low Si:N ratios. From this result one may conclude that in the field diatoms belonging to the *P. seriata* group might have still reached high cell numbers in the summer even at low DSi concentrations. Led by field observations from LY1, indicative of relatively high *P. seriata* group abundance during times of low DIP and DSi concentrations, the effect of phosphate and silicate limitation on *P. seriata* growth dynamics and domoic acid production was tested in laboratory experiments within this study (chapter 4, Fehling et al. 2004a). Results showed that *P. seriata* domoic acid production was enhanced by DIP or DSi nutrient limitation, and that the species was capable of utilising undetectable low concentration of silicate that had dissolved from empty frustules for renewed growth in late stationary phase. In the field, this ability to utilise even low amounts of silicate might hence explain the relatively high cell densities of the *P. seriata* group in summer at times of low DSi concentrations.

During summer 2002 a sharp decrease in the DSi concentration (see Fig. 2.26) was evident in Loch Creran from June to August. In the same time cell numbers of the *P. seriata* group increased rapidly (see appendix 2). Calculations from the silicate limitation experiment (chapter 4) showed that in stationary phase, before DSi was limited, cells had taken up approximately $0.6 \text{ nM DSi} \times \text{cell}^{-1} \cdot \text{day}^{-1}$. To diminish the DSi concentration, in Loch Creran, *Pseudo-nitzschia* species would have taken up about 2 to 4 $\text{nM DSi} \times \text{cell}^{-1} \cdot \text{day}^{-1}$, which is about an order of magnitude higher than the value obtained from the laboratory experiment. Diatoms belonging to the *P. seriata* group might have been only one of the diatom taxa that were causing the decrease in the DSi concentration in Loch Creran between July and August. At the same time rapid cell density increases were as well observed in *Asterionellopsis glacialis*, *Chaetoceros* species, *Leptocylindrus danicus* and *Thalassionema nitzschioides* (see appendix 2).

RDA showed for LY1 that apart from temperature and nutrient concentrations other factors, that were not measured in this study might have influenced the phytoplankton assemblage. Tett (1992) suggested for Scottish waters that apart from nutrients the phytoplankton floristic composition depends on light and mixing. One other factor might be grazing by zooplankton. It is known that zooplankton can influence phytoplankton dynamics by selective grazing and by differential excretion of limiting nutrients (e.g. Katechakis et al. 2002 and references therein). Data from continuous plankton records (CPR) (Colebrook 1982) showed a coupling between phytoplankton and zooplankton abundance, with highest numbers of zooplankton following those of phytoplankton. For the region between 48°N and 60°N it was suggested that zooplankton grazed the daily production of the phytoplankton (Colebrook 1982).

It has been hypothesized that DA might act as an antifeedant (summarized in Bates 1998), due to the finding that DA was toxic to small estuarine copepods, although it was not toxic to larger copepods. Other studies (summarized in Bargu et al. 2003; Turner & Tester, 1997) found no effect of DA on copepod survival, but suggested that copepods may act as vectors to transport DA to zooplanktivorous consumers. For *P. australis* Buck et al. (1992) showed that they were grazed in high amounts by the midwater polychaete *Poebius meseres*. Assuming that DA production doesn't negatively affect grazing on *Pseudo-nitzschia* species, it is likely that in Scottish waters one of the factors regulating *Pseudo-nitzschia* spp. and other phytoplankton blooms is grazing by zooplankton. However, in Loch Creran, Tett et al. (1985) observed relatively weak mesozooplankton grazing.

2.4.2 Ellett Line transect

2.4.2.1 Hydrographical features

Previous studies investigating the phytoplankton distribution and hydrographical parameters along (Savidge & Lennon 1987), or in the greater vicinity (Dodge 1993; Gowen et al. 1998; Yallop 2001) of the Ellett Line were conducted in spring and/or summer and hence make comparison with the present study difficult. However, some general hydrographical features, that influenced the phytoplankton distribution, were evident and may explain some of the observed results within the present study.

When sampling the Ellett Line, Savidge and Lennon (1987) distinguished between coastal waters (east of Barra Head), shelf waters (west of Barra Head) and open ocean (further west, beyond the shelf). Shelf waters were separated from the open ocean by a salinity boundary, this was also earlier reported by Ellett & Edwards (1983) and confirmed in the present study, where a strong difference in surface salinity was obvious between the shelf stations (mean surface salinity: ~ 35) and open ocean stations F (surface salinity > 35.3). However, because no additional shelf stations between 10G and M were sampled, the exact location of a salinity boundary could not be identified. Savidge and Lennon (1987) located a zone of active vertical mixing associated with strong tidal currents immediately adjacent to Barra Head (west of 10G). This spatially restricted zone is predominantly influenced by coastal water. Although in the present study this exact geographical region was not sampled, a change of hydrographical features was recognised at 10G, which was located close to that zone. Here the thermocline reached the surface and might have indicated the beginning transition to the hydrological features commonly observed in the Barra Head zone.

Physical, chemical and biological parameters at the shelf stations were affected by the general surface current patterns (see Fig. 2.2). The Scottish west coast water is mostly influenced by the Scottish coastal current, a northwards flowing stream of low-salinity

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water originating from the Irish Sea and Clyde Sea (McKay et al. 1986). The flow of this current was first shown by Ellett (1979) to diverge in the vicinity of Skye with part continuing northward through the Minch. A substantial fraction of this water was flowing in a narrow current southwards, close along the east coast of the Outer Hebrides. It would flow round Barra Head (southern tip of Outer Hebrides), continue as a northwards current west of the Outer Hebrides and mix with Northern Atlantic water (Fig. 2.2). As a result of the circular direction of part of the surface water current, surface water at 10G would have been composed of water that had flown northwards along the Scottish coast past the region of 1G, 2G and 4G. This was confirmed by the T-S diagram (Fig. 2.36), which placed 10G water between water at 1G, 2G and 4G, indicating that their water masses were similar. However, the surface temperature at 10G was slightly colder than that at 1G, 2G and 4G and PCA and the MDS ordination separated 10G from the other stations, indicating that the water might have been modified by physical processes after passing stations 1G, 2G and 4G. Temperature, salinity and nutrients in the top 30 m showed that surface water at 10G was represented by a similar water mass as found at 1G, 2G and 4G in greater depth. Hence, it might be speculated that the deeper portions of the Scottish coastal current, surfaced somewhere between the eastern stations (1G, 2G and 4G) and 10G. The distribution of phytoplankton further connected 10G with the eastern stations. Most of the taxa (e.g. *P. seriata* and *P. delicatissima* group, *Chaetoceros* spp., *Lauderia annulata*, *Asterionellopsis glacialis*, *Thalassiosira* spp.) occurred in maximal cell densities at 1G or 4G and again showed high numbers at 10G, in contrast to 9G and 7G.

At 9G the water column was well mixed in the upper 80 m and T and S values were elevated. Furthermore, the nutrient concentrations and phytoplankton density was lower in the top 50 m compared to the other shelf stations. This might have been due to the influence of Atlantic water which was evident in the increased salinity found at that

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station and towards 7G. Apart from the different origin of the water at 9G, the weather conditions during sampling might have affected the upper 50 m metres of the water column. 9G was sampled under severe gale conditions (Beaufort scale 9, 47-54 miles · h⁻¹), the storm might have mixed the water column.

The results from PCA separated coastal stations 1G to 6G from the other stations mainly based on their nitrate concentrations, but also on T, the other nutrients and S. Stations 1G to 6G were located closest to the coast and hence influenced by coastal runoff (evident in decreased surface salinity) which might have supplied nutrients. The separation of shelf stations from open ocean stations along PC2, with salinity as the main influencing factor, demonstrated that shelf and open ocean stations were subjected to different water masses and current systems. While the shelf stations were influenced by the Scottish coastal current and water that was modified by freshwater and Atlantic water influx, the open ocean stations were located in Atlantic water, with higher salinity.

The dissolved inorganic nutrient concentration increased with depth. This is in accordance with the general patterns of nutrient uptake by phytoplankton in the photic zone and typical regeneration processes (e.g. Parsons et al. 1984).

Savidge and Lennon (1987) found nitrogen concentrations in surface waters around 0.5 $\mu\text{M} \cdot \text{L}^{-1}$ in August 1983. Concentrations observed in this study were in a similar range (1.5 to 0.25 $\mu\text{M} \cdot \text{L}^{-1}$) at the shelf stations, but elevated (up to 2.2 $\mu\text{M} \cdot \text{L}^{-1}$) in surface water at the open ocean stations. This might be an indication of nitrate resupply in open ocean waters. The deepening of the surface mixed layer, as observed in autumn, would entrain water from the thermocline, where nutrient concentrations are higher (see Tett & Wilson 2000).

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2.4.2.2 Phytoplankton distribution

2.4.2.2.1 General phytoplankton

Most of the taxa that were found at LY1 during the year were also observed during the cruise in late September/early October. However, physical, chemical and biological parameters indicated the end of summer conditions with a weakening vertical stratification and the beginning of a transition to autumn. This was evident by the lack of stratification, consequently enhanced sinking rates and declining phytoplankton growth due to lower light irradiance:

At stations 1G to 6G, the difference between temperature in bottom and surface waters was less than 2°C. At stations 7G, 9G, M and F the upper 100 m and at 10G the top 80 m showed little temperature stratification and hence significant mixing. This reflected the typical autumn decrease of phytoplankton in Atlantic waters north of 48°N, which is associated with a reduction in the extent of thermal stratification to 2°C temperature difference in the top 200 m (Colebrook 1982). Mixing of the water column affected the vertical distribution of the phytoplankton. For some diatom species cell density maxima were found in about 40 m depth (e.g. *Lauderia annulata* and *Leptocylindrus danicus* at 7G, *Chaetoceros* spp. in 15 m at 4G). Savidge & Lennon (1987) made similar observations in August 1983, with some diatoms being distributed with maxima of cell density spread over a great depth range.

Bienfang (1981) observed sinking rates of 0.2-1.7 m · day⁻¹ for natural populations of temperate diatoms. The lack of stratification of the water column would have enhanced sinking rates. Furthermore, the phytoplankton decline would have been enhanced due to the lower irradiance levels in greater water depth and limited light availability as a consequence of shortening of the day length in these latitudes at that time of the year.

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2.4.2.2.2 Dinoflagellates

Dinoflagellates were present at all stations and distributed over a wide depth range. In the open ocean dinoflagellates < 20 µm exhibited very high cell numbers in the upper water column, while diatom numbers were insignificant. However, at the shelf stations dinoflagellates only played a minor role in terms of cell numbers, as diatoms dominated the phytoplankton. This was also observed by Savidge & Lennon (1987) in August 1983. One station that proved an exception was 9G, where numbers of dinoflagellates < 20 µm were elevated, although the water column was well mixed. This contrasted with results from other studies, which associated high dinoflagellate concentrations with vertical stability (intermediate to strong stratification) of the water column (Holligan et al. 1980; Lewis 1985; Dodge 1993). However, physical data indicated that 9G was strongly influenced by open ocean water, Hence, dinoflagellates might have been carried on the current to 9G.

2.4.2.2.3 Pseudo-nitzschia species

The phytoplankton monitoring had shown that the toxic *Pseudo-nitzschia* strains had appeared in late May and reached maximal abundance during July. However, this autumn cruise demonstrated the still high abundance of *Pseudo-nitzschia* species in late September, early October.

2.4.2.2.3.1 Horizontal distribution

The main difference between the *P. delicatissima* and *P. seriata* group was, that diatoms belonging to the *P. delicatissima* group were most abundant at the open ocean stations, while the *P. seriata* group was most abundant in shelf waters. In general, numbers of the *P. delicatissima* group were relatively low (~ 34% of *P. seriata* group cell numbers). Results from the temporal phytoplankton distribution at LY1 showed that diatoms of the *P. delicatissima* group occurred in highest densities during spring and

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had generally low concentrations in late summer/ early autumn, indicating that this group did not show preferences for autumn conditions. At the shelf stations species belonging to the *P. delicatissima* group were identified as *P. delicatissima*, *P. cf. delicatissima* and *P. pseudodelicatissima* (chapter 3). All species were present at the open ocean stations as well, although in higher numbers. This higher abundance in the open ocean might suggest a preference of the *P. delicatissima* for open ocean conditions, or might simply be related to the fact that the water mass in the open ocean was different to the water mass on the shelf and hence carried a different species composition.

In contrast to the *P. delicatissima* group, diatoms of the *P. seriata* group were a common and significant part of the phytoplankton community at LY1 at that time of the year. Species belonging to the *P. seriata* group identified from shelf station samples included *P. australis*, *P. seriata*, *P. pungens*, *P. fraudulenta* and *P. cf. subpacific*a (chapter 3). The only with TEM and genetic methods identified and isolated species belonging to the *P. seriata* group found in the open ocean was *P. cf. subpacific*a. Interestingly, none of the toxin producing species was confirmed amongst the few cells belonging to the *P. seriata* group found in the open ocean. However, the numbers at M and F were low and other *P. seriata* group species might have been overlooked, because of their low abundance. In the vicinity of 59°N, 20°W in June 1996, diatoms belonging to the *P. seriata* group (then enumerated as the '*Nitzschia seriata* complex') had been observed, confirming that they are present in open ocean waters, however, species were not identified (Yallop 2001). In the present study, the high density of potentially toxic *P. seriata* group species in coastal waters as opposed to the open ocean showed that at that time of the year the *P. seriata* group was mainly occurring in the Scottish coastal current. Conditions in the coastal water might have favoured the autumn bloom of that group.

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The difference of the spatial distribution between the *P. delicatissima* and *P. seriata* group might be explained by the different water masses found in coastal waters and the open ocean. Colebrook (1986) noted that results from the continuous plankton recorder studies in the northern Atlantic and North Sea showed the phytoplankton species composition tended to be closely related to the current system.

P. americana was found in relatively high densities at the shelf stations (see appendix 3). Cells were commonly found attached to *Chaetoceros* species and hence showed a similar distribution. This observation of *P. americana* as an epiphyte is common for the species (Lundholm et al. 2002b), but its occurrence had not previously been reported from north-western Atlantic waters (see also chapter 3).

2.4.2.2.3.2 Vertical distribution

In the present study both groups were distributed over the top 100 m with maximal cell densities in certain depths, indicating decreasing stratification due to mixing of the water column. The *P. delicatissima* group was observed in highest abundance at about 15 m (1G to 6G) and at the open ocean stations in 30 m depth. A similar depth distribution of this group was observed by Savidge & Lennon (1987) in the same waters, who found increased concentrations of *Nitzschia* species at 20-40 m. They did not specify the group of *Nitzschia* species, but as they made their observations in May (late spring) it can be assumed, that they had observed species belonging to the *P. delicatissima* group.

Diatoms of the *P. seriata* group were also distributed in high numbers in the upper 40 m of the water column, with maximal concentrations found at 40 m at 1G, 30 m at 4G and 15 m at 7G. Those *Pseudo-nitzschia* species might have a tolerance for low light intensities and could be adapted to a distribution in layers in depth exceeding 15 m. In coastal waters a layer of freshwater and enhanced mixing, might have displaced surface

blooms of the *P. seriata* group to greater depth (40 m). This had previously been observed in a fjord in Washington state, USA, coastal waters (Rines et al. 2002), where *P. pseudodelicatissima*, *P. fraudulenta* and *P. pungens* were observed with maximum concentrations in thin layers in about 30 m depth. The *Pseudo-nitzschia* species thrived at that depth for several weeks, indicating their low light intensity tolerance.

2.4.2.3 Factors influencing the phytoplankton distribution

Redundancy analysis of samples averaged over the top 100 m showed that salinity influenced the phytoplankton composition and distribution significantly. Species occurring at the open ocean stations were clearly separated from those occurring at the shelf stations. Salinity mainly characterises the water mass, hence the results from RDA might suggest that species were associated with a specific water mass (Atlantic water or Scottish coastal current) and distributed with it. The contribution of Atlantic water to the shelf water (especially at stations 9G and 7G) would have transported species found at M and F to shelf waters. However, cell numbers at M and F were considerably lower than at the shelf stations and it was likely that an increased mixing of the water column might have already decimated cell density of some of the species. While the measured inorganic nutrients did not show a statistically significant influence on the phytoplankton distribution in RDA and PCA, nutrients that were immediately taken up by phytoplankton (and hence not measured), might have played a role. Other, not measured factors such as grazing, mixing and light availability might have contributed to the separation of ocean and shelf stations. This was also demonstrated in previous studies of temperate waters, where differences in the species composition of phytoplankton have been explained in terms of the interaction amongst light, mixing and nutrient availability (Holligan et al. 1980; Jones & Gowen 1990).

3 Chapter 3: *Pseudo-nitzschia* cultivation, identification and domoic acid quantification

3.1 Introduction

P. multiseriis, *P. australis* and *P. calliantha*, are the three *Pseudo-nitzschia* species that have been identified to have caused ASP events or the closure of shellfish harvesting areas in Canada and North America. *P. multiseriis* and *P. australis* have also been found to occur in Scottish waters (Gallacher et al. 2001), where in 1999 high levels of DA in king scallops (*Pecten maximus*) prompted the largest fisheries closure to date, due to a harmful algal bloom (Campbell et al. 2003). Two strains of *P. australis* from Scottish waters (Campbell et al. 2001) and one from Irish waters (Cusack et al. 2002) from prior to, and following, this event were cultured and their DA production was confirmed. However, it remained unknown whether this species alone was responsible for ASP. Therefore, there remains a need to determine if other species are also DA producers and which of these are of most concern in Scottish waters.

With light microscopy, *Pseudo-nitzschia* species can only be separated into two main groups, the *P. delicatissima* and *P. seriata* group (see chapter 1; Hasle 1965; Hasle & Syvertsen 1996). The delineation to species level requires electron microscopy (EM) or molecular methods (see chapter 1). Prior to this study, *Pseudo-nitzschia* species from Scottish waters had been identified by TEM, but only a few preserved samples from inshore-areas had been analysed (Gallacher et al. 2001). Potential DA producers such as *P. multiseriis*, *P. seriata*, *P. fraudulenta*, *P. pungens* and *P. delicatissima* were named. However, those identifications have not been confirmed.

Even using EM, species identification based on the morphological fine structure can be ambiguous. *Pseudo-nitzschia seriata*, for example, has previously been mistaken for *P. australis* and vice versa (e.g. Villac et al. 1993b; Fryxell et al. 1997; Hasle 2002;

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Fehling et al. 2004b). However, molecular methods such as PCR (polymerase chain reaction) and sequencing can be applied for precise *Pseudo-nitzschia* species identification. By a pairwise sequence alignment search of databases (e.g. using the FASTA algorithm, Altschul et al. 1994; Pearson 1990) the obtained sequences can be matched with sequences belonging to identified species.

Previously (see chapter 1, section 1.7.2.2) the small subunit (SSU), large subunit (LSU) and internal transcribed spacer (ITS) regions of nuclear DNA, coding for ribosomal RNA have been sequenced for *Pseudo-nitzschia* species from round the world, but Scottish strains had not been studied. For DNA extraction either single, non-preserved cells (for example from net samples) can be used and directly amplified. Or preferably, a subsample of a clonal culture is taken to provide greater amounts of DNA.

In this study toxic and non-toxic *Pseudo-nitzschia* spp. from western Scottish waters were isolated, cultured and identified using both, classic morphological and molecular techniques. New cultures were obtained from western Scottish waters during 33 months of monitoring site LY1 and a cruise from the western Scottish coast across the shelf towards the open Atlantic (chapter 2). Cultures previously established by C. Bolch (in August 1999) were also examined. Most of the cultures were also tested for DA production.

Prior to this study, phylogenetic relationships among *Pseudo-nitzschia* species were established applying parsimony and maximum likelihood methods (for an introduction see Swofford et al. 1996) to partial LSU rDNA sequences (Lundholm et al. 2002a; Lundholm & Moestrup 2002; Orsini et al. 2002; Lundholm et al. 2002b) or ITS rDNA sequences (Lundholm et al. 2003). In this study ITS and partial LSU rDNA data were combined, giving larger sequence fragments and hence providing more data for the phylogenetic analysis. Maximum likelihood and Bayesian analysis were conducted on the Scottish strains, establishing their phylogenetic relationships.

3.2 Material and Methods

3.2.1 Isolation and cultivation of *Pseudo-nitzschia* spp.

Most of the Scottish *Pseudo-nitzschia* cultured strains established during this study were isolated from plankton net samples (20 µm mesh size) taken at the monitoring station LY1 (Lynn of Lorne, 56°28'.9 N, 5°30'.1W) in close vicinity to SAMS over a period of 33 months between 2000 and 2003, and during cruise D257 of *RV Discovery* D257 in autumn 2001 (chapter 2). Further *Pseudo-nitzschia* cultures were obtained by taking additional plankton net samples from the Dunstaffnage Marina pier.

Immediately after sampling, non-preserved fresh 20 µm plankton net samples were inspected under an inverted Zeiss Axiovert S100 microscope on 100 x magnification. To obtain clonal cultures, a single chain or cell of *Pseudo-nitzschia* spp. was isolated by micropipette, washed several times in sterile filtered seawater and transferred into 5.5 cm diameter petri-dishes containing 10 ml sterile F/2 medium (Guillard, 1975) plus 107 µM Si (as metasilicate), referred to as F/2 + Si. Dishes were sealed with Parafilm™ and incubated at 15° C under an irradiance of ca. 100 µmol · photons m⁻² · s⁻¹ (12:12 h L:D cycle) and grown for approximately two weeks. If cells had undergone several divisions, and reached a density of at least ten cells per 100 x magnification field of view, the content of the petri-dish including cells and medium, was transferred into 100 ml Erlenmeyer flasks containing 50 ml of sterile F/2 + Si medium and were incubated under the same temperature and light conditions as above. Two to three weeks later a subsample of about 10 ml was then transferred into 250 ml Erlenmeyer flasks containing 100 ml F/2 + Si medium and grown under the above conditions. To sustain the cultures, every three weeks approximately 10-20 ml of the parental culture were transferred under sterile conditions into a fresh 250 ml Erlenmeyer flask containing fresh, sterile F/2 + Si medium (autoclaved at 121°C for 15 min). All cultures were

maintained in the culturing facilities of CCAP (Culture Collection for Algae and Protozoa at SAMS).

Cultures were named after the site and sampling occasion, from which they were isolated (e.g. PLY1St.70 was isolated at LY1 from the 70th sample, D257F was isolated during cruise D257 from station F).

3.1.2 *Pseudo-nitzschia* identification in samples and cultures

After identification and enumeration of phytoplankton in water samples taken at the monitoring site LY1 and cruise D257 by light microscopy (chapter 2), plankton net samples from 15 sampling occasions at which high densities of *Pseudo-nitzschia* species were observed, were inspected by TEM (Tab. 3.1).

In total, 37 cultures were identified by TEM. From 26 of the 37 cultures, the fine structural frustule features of the cells were measured in detail (Tab. 3.2). Culture Paustralis3 (isolated by C. Bolch) was identified by scanning electron microscopy (SEM). The remaining 11 cultures were identified (together with N. Lundholm) by recognition of distinct structures (Tab. 3.5).

Field samples that contained high cell densities of diatoms belonging to the *P. delicatissima* or *P. seriata* group were additionally inspected by TEM and the species composition of the dominant *Pseudo-nitzschia* group present during that sampling event was analysed.

3.1.2.1 Acid cleaning of diatoms

Prior to electron microscopy, subsamples from cultures and preserved net samples were acid cleaned to remove organic material from the cells. This was done after the method of Lundholm et al. (2002a): 3-10 ml sample were poured into a 100 ml Erlenmeyer

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flask, 2 ml of 30% H₂SO₄ (sulfuric acid) and 10 ml saturated KMnO₄ (potassium permanganate) were added. The mixture was left for 24 h and shaken from time to time. 5-10 ml freshly made (COOH)₂ (oxalic acid) was added and the transparent sample was divided into centrifuge tubes and spun for 20 min at 3500 rpm. The supernatant was aspirated and the sample washed with distilled water five times. Cleaned diatom samples that were not immediately used for microscopy were stored in glass vials after the distilled water was exchanged with absolute ethanol.

3.1.2.2 Sample preparation for electron microscopy

For TEM a drop of acid cleaned diatom culture or field sample was mounted on a 300 µm mesh formvar film coated copper grid (3.05 mm, Agar Scientific). Pictures of the morphological fine structures of the frustules were taken with a JEOL-100SX TEM at different magnifications (ranging from 1000 to 50,000 x). The negatives were developed using standard dark room techniques.

For SEM acid cleaned diatom frustules were concentrated on 1 µm polycarbonate filters (Osmonics), water was exchanged in an acetone dilution series and filters were mounted on SEM stubs, which were then sputter coated with gold. The stubs were then examined with a JEOL-35C SEM and pictures were taken digitally for measurements of the morphological frustule fine structures.

3.1.2.3 Measurement of the morphological fine structure on TEM micrographs

TEM pictures taken at magnifications greater than 1000 x show just a part of the *Pseudo-nitzschia* frustule, because of their size and elongated shape. Ideally, per cell one picture was taken of the centre of the frustule, and another one of the tip, allowing recognition of its shape. If possible, a picture at 1000 x magnification showing the

whole length of a frustule was taken (unfortunately the exposure times are difficult to adjust with the microscope at SAMS at that magnification and most of the those pictures were overexposed). In some cases, mainly with species belonging to the *P. delicatissima* group, the poroids were pictured with a high magnification (max. 50,000 x) to visualise the structure of the hymena covering them.

For identification, the following fine structural features of the *Pseudo-nitzschia* valve were taken into account and measured on the negatives or prints (see also Fig. 1.4 chapter 1): the width was noted, if possible the length was measured and the middle of the frustule was inspected for the presence or absence of a central interspace. The rows of poroids per interstria were counted and the number of poroids in 1 μm measured. The number of fibulae and interstriae in 10 μm was noted. The fine structural features from up to 10 cells (~ 30 cells for *P. seriata* strains) were measured from cultured samples. Taking all the fine structure measurements into account, the cells were delineated to species level by comparing the measurements with keys from Hasle (1965), Hasle & Syvertsen (1996) and Skov et al. (1999).

As mixed field samples contained not only *Pseudo-nitzschia* cells, and grids were sometimes covered with other diatom species and detritus, only about 1-4 cells were measured per species at each station.

3.1.3 Genetic identification of *Pseudo-nitzschia* species

3.1.3.1 DNA extraction, amplification and sequencing.

For genetic analysis DNA was extracted from 10 ml culture subsamples of cells in exponential phase, using a phenol-chloroform extraction after Bolch et al. (1998).

The ITS1 (internal transcribed spacer), 5.8S and ITS2 rDNA genes (hereafter referred to as ITS region) were amplified and sequenced from isolates of Scottish *Pseudo-nitzschia*

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spp. listed in Table 3.1. Total DNA was used as a template to amplify the ITS region in a 50 µl reaction containing 75 mM Tris-HCl (pH 8.8), 20 mM (NH₄)₂SO₄, 0.01% [v/v] Tween 20, 2.5 mM MgCl₂, 0.2 mM dNTP, 0.5 µM or 1.0 µM of the ITS or LSU primer pairs, respectively, 1.0 U *Taq* polymerase (ABgene, UK) and 25 ng target DNA. The forward primer ITS-A (5' - CCAAGCTTCTAGATCGTAACAAGGTHTCGTTAGGT-3') and reverse primer ITS-B (5' - CCTGCAGTCGACAKATGCTTAARTTCAGCRGG-3') (Adachi et al. 1996) were used. Amplification was carried out using the following conditions: an initial denaturation of 94°C for 2 min, followed by 30 cycles each containing 30 sec at 55°C, 2 min at 72°C and 10 sec at 94°C, finally 30 sec at 60°C and 10 min at 72°C. PCR products were analysed by agarose gel electrophoresis and purified prior to DNA sequencing with the Amicon® Microcon®-PCR centrifugal filter devices (Millipore Corporation, UK), following the manufacturers instructions.

A fragment of the large subunit (LSU) rDNA gene was amplified after the same procedure as the ITS region. This time the LSU-specific primers D1R-F (5' - ACCCGCTGAATTTAAGCATA-3'; Scholin et al. (1994) and reverse primer D3B*-R (5' - ACTTCGGAGGGAACCAGCTAC-3'; modified from Lenaers et al. (1989) were used, the annealing temperature was 60°C.

DNA sequencing was performed in both directions, with the PCR primers using “ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit” (Applied Biosystems, UK), as recommended by the manufacturer. Analysis of the DNA sequence reactions was carried out by the Biological Sciences department of Durham University. DNA sequence electropherograms were visually inspected using Sequence Navigator™ (Version 1.01, Applied Biosystems Inc., 1994), base-call errors were corrected manually, and a consensus sequence for each product derived by comparison of forward and reverse sequences.

3.1.1.2 FASTA

The obtained rDNA sequences were used to delineate the Scottish *Pseudo-nitzschia* strains to species level, by comparison with sequences from the EMBL-PLANT database, using the program fasta3 (www.ebi.ac.uk/fasta33/nucleotide.html). Fasta3 uses the FASTA (FAST-All) algorithm (Pearson & Lipman 1988; Pearson 1990) for pairwise sequence alignment, comparing protein or DNA sequences by alignment with protein or DNA sequences from a database. The observed pattern of word (ktupel) hits is used to identify potential matches before attempting the optimised search. FASTA produces optimal local alignment scores for the comparison of the query sequence to every sequence in the database. It assigns an expectation value (E) to each hit. The E-value is a parameter that describes the number of hits that can be expected just by chance when searching a database of a particular size. An E-value of 1 assigned to a match between two sequences means that one match can be expected to be found in the searched database simply by chance. As sequence length is taken into account, shorter sequences can result in E-values close to 1, because they are more likely to match with sequences from the searched database. The lower the E-value, the closer and more significant is the match between the two sequences. In this study the default settings of the programme for DNA nucleotide search (ktupel = 6, search both DNA strands) were used.

3.1.1.3 Alignment

A phylogeny of the Scottish *Pseudo-nitzschia* strains was inferred combining the ITS and partial LSU genes. *Cylindrotheca closterium* (Ehrenberg) Lewin & Reimann (ITS1 and ITS2, 5.8S and partial LSU, GenBank AF289049) was used as an outgroup. Like *Pseudo-nitzschia* spp., it belongs to the family Bacillariaceae and according to Lundholm et al. (2002a) arises from the base of the clade grouping *Pseudo-nitzschia*

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species. After combining the ITS and partial LSU rDNA of the Scottish strains, they were aligned together with *Cylindrotheca closterium* using ClustalX (version 1.64B; Thompson et al. 1997). Adjustments to the alignment were made after visual inspection (see appendix 4 for full alignment). Redundant sequences were excluded from the analysis (using only one sequence of a set of identical sequences), leaving a data matrix (see appendix 4) with sequences of six Scottish *Pseudo-nitzschia* species plus the outgroup (*C. closterium*). The 5.8S gene sequence of *Cylindrotheca closterium* was used to identify the 5.8S gene within the Scottish *Pseudo-nitzschia* sequences and hence the end of the ITS1 and the beginning of the ITS2 region.

3.1.1.4 Partition homogeneity test

Congruence of data sets was tested with the partition homogeneity test (Farris et al. 1995) in PAUP* (Swofford 2002, version 4.0b.10). It tests the null hypothesis that data sets, which might have originated from two different genes or regions within genes (in this case ITS and LSU), underlie different evolutionary assumptions, for example, unequal rates of evolution. Most parsimonious trees are generated separately for the partitioned regions and the tree lengths resulting from each partition are added together to a total tree length. Random sampling of sites generates a randomised distribution of tree lengths from the combined original data set. If the added tree length lies in the middle region of the randomly regenerated tree length distribution, the partitioned regions provide similar topologies, indicating that the null hypothesis is rejected and data sets should be combined. If the test shows that the null hypothesis is significant (in case the tree length is outside the middle range, $p < 0.05$) it is accepted, suggesting that the data sets should not be combined.

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3.1.1.5 Maximum likelihood analysis

DNA evolution is expressed in changes of nucleotide bases between an ancestral and a recent sequence. The overall evolutionary changes that have occurred per site between those sequences are expressed as the divergence between them (e.g. Swofford et al. 1996). Maximum likelihood (ML) is one method of tree-reconstruction. First applied for gene frequency data by Cavalli-Sforza & Edwards (1967), Felsenstein (1973, 1981) later developed an ML algorithm for amino acid and nucleotide sequence data. ML algorithms attempt to estimate the amount of evolutionary change required to explain the data according to an evolutionary model. The likelihood L of a phylogenetic tree is the probability of observing the data (e.g. the nucleotide sequences) under a given tree and a specified model of character state changes (e.g. changes between the nucleotides A,C,G,T). To find an appropriate model for the ML analysis of the sequences used in this study, the computer program Modeltest (version 3.06, Posada & Crandall 1998) was used.

3.1.1.6 Modeltest

The family of General Time Reversible (GTR) nested models encompasses 64 models of DNA site substitution, of which the one that best fits the data has to be chosen prior to the ML analysis. The program Modeltest (version 3.06, Posada & Crandall 1998) is designed to compare different nested models of DNA substitution and uses likelihood scores to establish the model of DNA evolution that best fits the data. To select a nucleotide substitution model for the dataset, the program uses the hierarchical likelihood ratio test (see Posada & Crandall 1998) to compare the fits of the nested General Time Reversible family of nucleotide substitution models. It additionally calculates the Akaike Information Criterion estimate (AIC, Akaike 1974) associated with the likelihood scores. The AIC rewards models for good fit, but imposes a penalty

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for unnecessary parameters. Using those algorithms, Modeltest estimates a nucleotide substitution model for the dataset, which includes the substitution matrix for the nucleotides (Fig. 3.1) and the base frequency parameters. In addition to the models describing the rates of change from one nucleotide to another, the parameters used in modeling rate heterogeneity across sites such as the gamma shape parameter (gamma distributed site-to-site rate variation) and the proportion of invariable sites (extent of static, unchanging sites in a dataset) are suggested for the further analysis.

	A	C	G	T
A	-	a	b	c
C	a	-	d	e
G	b	d	-	f
T	c	e	f	-

Fig. 3.1 Nucleotide substitution matrix (a = A \leftrightarrow C, b = A \leftrightarrow G, c = A \leftrightarrow T, d = C \leftrightarrow G, e = C \leftrightarrow T, f = G \leftrightarrow T; a, c, d and f indicate transversions, b and e indicate transitions; in the GTR model all rates (a-f) are different: nst = 6).

3.1.1.7 Tree search in PAUP*

Trees including the Scottish *Pseudo-nitzschia* strains were searched in PAUP* (Swofford 2002, version 4.0b10) under the likelihood criterion. Gaps were treated as missing data. Initially the nucleotide substitution model evaluated in Modeltest (version, Posada & Crandall 1998) was used in a ML analysis, using a heuristic search with random addition of sequences (10 replicates) and a branch swapping algorithm (tree-bisection reconnection). The exact parameters were estimated from consecutive searches and reoptimising parameters until the values of parameters converged. Bootstrap analysis to determine the robustness of nodes (Felsenstein 1985) was conducted with 2000 replicates under a heuristic search.

3.1.1.8 Bayesian analysis

As a second approach of inferring a phylogeny of the Scottish *Pseudo-nitzschia* strains a Bayesian analysis using the computer programme MrBayes (version 3.0b4) was conducted. The programme applies the Bayesian approach, which samples trees according to their posterior probabilities, instead of searching for the single optimal tree (Huelsenbeck et al. 2001). In Bayesian analysis an evolutionary model is tested by posterior predictive simulation, which compares a test statistic with the posterior predictive distribution of that statistic generated under the assumption that the model is correct (Lewis 2001). The posterior probability is approximated by a Markov Chain Monte Carlo (MCMC, Gilks et al.1996). The MCMC takes the form of a correlated random 'walk' through parameter space, which contains the sets of all possible trees and model parameters, and approximates any probability distribution by periodically sampling. In phylogenetic analysis each step in a Markov chain involves a random modification of the tree topology, a branch length or a parameter in the substitution model (e.g. nucleotide substitution rate ratio). If the posterior that is computed for a proposed step is larger than that of the current tree topology and parameter values, the proposed step is taken (Lewis 2001). From the MCMC run a consensus tree can be constructed with the posterior probabilities of the individual clades indicated on the tree. This is roughly equivalent to performing a maximum likelihood analysis with bootstrap resampling, but much faster (Larget & Simon 1999). The generations of the chain before it reaches a peak (a "hill" in parameter space, indicating trees with high posterior probabilities) are usually discarded by the "burnin" command. However, if the number of generations is high and the number of taxa is low the burn in does often make no difference in the consensus tree. For this study the analysis was conducted under a GTR model with gamma distribution (shape parameter = 0.32) and invariable sites (I = 0.3689) as evaluated by Modeltest (version 3.06, Posada and Crandall 1998)

(see results for evaluated base frequencies). Four Markov chains were run for 10^7 generations with a sampling frequency of 10^3 , this yielded in 10^4 trees. Discarding none, the initial 100 or 1000 trees did not alter the topology or posterior probabilities of the consensus tree. The consensus tree was created by the 50% majority rule, using PAUP* (Swofford 2002, version 4.0b10). The program also calculated branch lengths as a measure of evolutionary changes between taxa.

3.1.4 Domoic acid testing of cultures

Table 3.1 (results, section 3.3.1) gives an overview of which cultures were tested for domoic acid (DA) production. DA concentrations were determined in the stationary growth phase of cultures (after 16 to 27 days of growth), using a high performance liquid chromatography (HPLC) of the fluorenylmethoxycarbonyl derivative (Pocklington et al. 1990), with a detection limit of $15 \text{ pg} \cdot \text{mL}^{-1}$. Total DA was measured in whole culture samples (cells plus medium) (Bates et al. 1989) that were first sonicated for 1 min to disrupt the cells, then filtered through a $0.2 \text{ }\mu\text{m}$ disposable acrodisc (25 mm surfactant free cellulose acetate membrane, Nalgene) to remove cell debris and frozen at -20°C prior to analysis. Samples were then sent cooled on ice to S. Bates (Fisheries and Oceans, Canada) for DA analysis.

For 25 day old, stationary phase *P. seriata* (PLY1St.16B and PLY1St.52B) and *P. australis* cultures (PLY1St.19A and PLY1St.54B), additionally extracellular DA (DA that was released by the cells, referred to as “medium DA”) was analysed: whole-culture samples were filtered under low vacuum onto 25 mm glass-fibre filters (type A/E, Pall Corporation) and the filtrate (cell-free medium) was frozen at -20°C prior to analysis. DA per cell was calculated by dividing total DA of the whole culture by the cell number.

3.3 Results

3.3.1 Cultivated species

A total of 59 *Pseudo-nitzschia* strains were isolated from Scottish waters and maintained in culture. Forty-six of these were freshly isolated within this study, the remaining 13 had previously been isolated by C. Bolch but were maintained within this study. All were identified by their morphological characteristics and partial rDNA sequences. The cultured strains comprised seven species, 19 strains of *P. australis*, two strains of *P. seriata*, nine strains of *P. fraudulenta*, two strains of *P. pungens*, one strain of *P. cf. subpacific*a, one strain of *P. calliantha* and 25 strains of *P. delicatissima*. Twenty-six of the cultures were identified by TEM and 49 by their partial rDNA sequence, including ITS, 5.8S and partial 28S LSU region.

Table 3.1 gives an overview of all culture strains, their sampling location and date of isolation. It also indicates the result of DA analysis, and gives information about methods that were used for their identification.

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Table 3.1 Scottish *Pseudo-nitzschia* strains, with sampling location, date of isolation, results of domoic acid testing (+ = positive test, - = negative test, detection limit: 15 pg · mL⁻¹) and identification. Isolates from Aug. '99 were established by C. Bolch. n.d. means not determined.

Species	Strain	Sampling Location	Date of isolation	DA production	Identification	
					TEM	ITS, 5.8S, LSU
<i>P. australis</i>	Paustralis3	DML Pontoon	Aug. '99	+	SEM	+, +
	Pmarina2.7	Dunstaffnage Marina	02.07.01	+	+	n.d.
	Pmarina2.7A	Dunstaffnage Marina	02.07.01	+	+	n.d.
	Pmarina2.7B	Dunstaffnage Marina	02.07.01	+	+	+, +
	PLY1St.19A	LY1	13.07.01	+	+	+, +
	PLY1St.20A	LY1	20.07.01	+	+	+, +
	PLY1St.24D	LY1	17.08.01	+	+	+, +
	PLY1St.27E	LY1	07.09.01	+	+	+, +
	PLY1St.33A	LY1	19.10.01	+	n.d.	+, +
	PLY1St.37A	LY1	15.11.01	-	n.d.	+, +
	PLY1St.37B	LY1	15.11.01	-	+	+, +
	PLY1St.54B	LY1	14.06.02	+	n.d.	+, +
	PLY1St.54C	LY1	14.06.02	+	+	+, +
	PLY1St.54D	LY1	14.06.02	+	+	n.d., +
	PLY1St.56B	LY1	28.06.02	+	n.d.	+, +
	PLY1St.68C	LY1	20.09.02	+	n.d.	+, +
	D257 6G	D257, station 6G	30.09.01	-	n.d.	+, +
	D257 7GA	D257, station 7G	30.09.01	-	+	+, +
	D257 10GA	D257, station 10G	30.09.01	-	+	+, +
<i>P. seriata</i>	PLY1St.16B	LY1	22.06.01	+	+	+, +
	PLY1St.52B	LY1	31.05.02	+	+	+, +
<i>P. fraudulenta</i>	PLY1St.11A	LY1	03.05.01	-	+	-, +
	PLY1St.11C	LY1	03.05.01	-	+	+, +
	PLY1St.11D	LY1	03.05.01	-	+	+, +
	PLY1St.12A	LY1	17.05.01	-	+	+, +
	PLY1St.12B	LY1	17.05.01	n.d.	+	n.d.
	PLY1St.12C	LY1	17.05.01	n.d.	+	n.d.
	PLY1St.15A	LY1	15.06.01	-	+	+, +
	PLY1St.36A	LY1	09.11.01	-	+	+, +
PLY1St.85E	LY1	04.04.03	n.d.	+	+, +	
<i>P. pungens</i>	Ppungens6	DML Pontoon	Aug. '99	-	+	+, +
	PLY1St.33C	LY1	19.10.01	n.d.	+	n.d.
<i>P. cf. subpacific</i>	D257 F	D257, station F	04.10.01	-	+	+, +
<i>P. calliantha</i>	Porkney4	Orkney	Aug. '99	n.d.	+	n.d.
<i>P. cf. delicatissima</i>	Porkney1	Orkney	Aug. '99	-	+	+, +
	Porkney2	Orkney	Aug. '99	n.d.	n.d.	+, +
	Porkney3	Orkney	Aug. '99	n.d.	n.d.	+, +
	Porkney5	Orkney	Aug. '99	n.d.	n.d.	+, +
	Porkney6	Orkney	Aug. '99	n.d.	n.d.	+, +
	Porkney7	Orkney	Aug. '99	-	+	+, +
	Porkney8	Orkney	Aug. '99	n.d.	n.d.	+, +
	Porkney9	Orkney	Aug. '99	n.d.	n.d.	+, +
	Porkney10	Orkney	Aug. '99	n.d.	n.d.	+, +
	Porkney11	Orkney	Aug. '99	-	n.d.	+, +
	Pontoon1-2	DML Pontoon	Aug. '99	n.d.	+	+, +
	PLY1St.5	LY1	09.02.01	n.d.	n.d.	+, +
	PLY1St.42A	LY1	04.02.02	-	+	+, +
	PLY1St.43C	LY1	15.02.02	n.d.	n.d.	+, +
	PLY1St.43D	LY1	15.02.02	n.d.	+	n.d.
	PLY1St.43F	LY1	15.02.02	n.d.	+	n.d.
	PLY1St.45E	LY1	18.03.02	n.d.	+	+, +
	PLY1St.46A	LY1	28.03.02	n.d.	+	+, +
	PLY1St.46C	LY1	28.03.02	n.d.	+	-, -
	PLY1St.48A	LY1	26.04.02	n.d.	+	+, +
	PLY1St.48B	LY1	26.04.02	n.d.	+	n.d.
	PLY1St.77C	LY1	06.12.02	n.d.	n.d.	+, +
	PLY1St.85A	LY1	04.04.03	n.d.	n.d.	+, +
PLY1St.85B	LY1	04.04.03	n.d.	n.d.	+, +	
PLY1St.85Z	LY1	04.04.03	n.d.	n.d.	+, n.d.	

3.3.2 Identification by morphological fine structure features

Measurements of the morphological fine structure of some of the cultured species are summarised in Table 3.2. For comparison, Tables 3.3 and 3.4 give the dimensions for *Pseudo-nitzschia* species as stated in published identification keys. For some species, differences in morphology between strains of the same species and ambiguities to identification keys were observed, this is discussed in detail below.

P. australis cultures. The morphological fine structures of frustules from cells in *P. australis* cultures Pmarina2.7A, PLY1St.24D, PLY1St.27E and PLY1St.54D matched the descriptions found in published identification keys (see Tab. 3.2)

Some differences between the Scottish *P. australis* cultures and also to the descriptions in the keys were evident. Strain D25710G for example had more interstriae per 10 μm , slightly more poroids per 1 μm and was narrower (4.6 μm) than the other cultured strains. Cells in cultures PLY1St.37A, 37B and 54C were slightly narrower and PLY1St.37A and B had more poroids within 1 μm than described in keys.

P. seriata cultures. The detailed TEM examination of the valve morphology of strains PLY1St.16B and PLY1St.52B showed that most of their morphological features (number of fibulae and interstriae per 10 μm , poroids per 1 μm) corresponded with published species descriptions of *P. seriata* f. *seriata* (Tab. 3.3). However, there were some important differences between these two strains and also with the published descriptions, as detailed below. The cell width of strain PLY1St.16B was 5.0-5.2 μm , whereas cells from strain PLY1St.52B were 4.6-6.0 μm wide. While strain PLY1St.16B had 14-20 fibulae and 14-20 interstriae in 10 μm , cells of strain PLY1St.52B had 18-19 fibulae and 16-20 interstriae in 10 μm . In both strains, the valves were slightly asymmetric and the cells lacked a central interspace. Unlike the described *P. seriata* f. *seriata*, which has two rows of poroids plus one to three extra rows, these strains had

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two rows of poroids plus rarely one third row per stria. Instead of a third row, there were sometimes just a few single poroids or no third row visible between the two rows of poroids.

P. pungens culture. Culture PLY1St.33C matched the previously published descriptions of that species.

P. fraudulenta cultures. Measurements of morphological fine structure features of the cultured strains resembled those of the keys quite well. Strains PLY1St.12C and 36A had 1-2 more interstriae per 10 μm and cells of PLY1St.12C were narrower than stated in the keys (mean width of 4.3 μm instead of 4.5 - 10 μm).

P. cf. subpacifica culture. Morphological features of cells belonging to strain D257F were ambiguous for species delineation. Cells had a similar number of fibulae in 10 μm (16-18) as *P. subpacifica* (15-20) from identification keys, but less interstriae per 10 μm (27-28 compared to 28-32), occasionally more rows of poroids (2-3 compared to 2) and less poroids per 1 μm (8-9 compared to 9-10). Their widths (3.9-4.5 μm) matched the width range reported for *P. heimii* (4-6 μm), although they had slightly more fibulae and interstriae per 10 μm and also more rows of poroids and poroids per 1 μm than that species. From the morphological analysis this strain would be delineated as either *P. cf. subpacifica* or *P. cf. heimii*.

P. delicatissima cultures. No culture except Porkney1 exactly matched the key descriptions for *P. delicatissima*. The main differences were in the number of poroids per 1 μm (cultures Pontoon1-2, PLY1St.46A, 46C and 48B) and that they exceeded the width range of 1.1-2.0 μm for *P. delicatissima* as stated in keys by up to 0.5 μm (Pontoon1-2, PLY1St.43D, 46C, 48A and 48B).

Table 3.2 Fine structure measurements of the *Pseudo-nitzschia* spp. cultures from station LY1 and cruise D257 by TEM.

Species	Culture	Date of isolation	Central interspace	Fibulae / 10 µm	Interstriae / 10 µm	Rows of poroids	Poroids /1 µm	Width [µm]
<i>P. australis</i>	Pmarina2.7A	2.7.1	absent	15	15	2	4-5	7.6
	PLY1St.24D	17.8.1	absent	15	16	2	4-5	?
	PLY1St.27E	7.9.1	absent	15	15	2	4-5	6.6
	D25710GA	30.9.1	absent	18	18-20	2	5-6	4.6
	PLY1St.37A	15.11.1	absent	17	17	2	4-7	4.8-5.4
	PLY1St.37B	15.11.1	absent	14	14-17	2	4-6	5.1-6.5
	PLY1St.54C	14.6.2	absent	14-16	14-16	2	4-5	4.9-6.8
	PLY1St.54D	14.6.2	absent	14-15	15	2	5	6.3-7.2
	PLY1St.16B	2.6.1	absent	14-20	14-20	2 (+1)	7-8	5-5.2
	PLY1St.52B	31.5.2	absent	18-19	16-20	2 (+1)	7-8	4.6-6
<i>P. pungens</i>	PLY1St.33C	19.10.1	absent	12	12	2	3-4	3.1
	PLY1St.12C	17.5.1	present	24	25	2-3	5-6	4.3
<i>P. fraudulenta</i>	PLY1St.36A	9.11.1	present	24	24-26	2-3	5-6	4.7
	PLY1St.85E	4.4.3	present	20	22	2-3	5-6	5.1
	D257F	4.10.1	present	16-18	27-28	2-3	8-9	3.9-4.5
<i>P. cf. subpacifica</i>	Porkney1	August'99	present	25	38	2	10-12	1.5
	Pontoon 1-2	August'99	present	24-26	37-38	2	8-9	2.4-2.5
<i>P. delicatissima</i>	PLY1St.42A	4.2.2	present	20	39	2	9	1.85
	PLY1St.43D	15.2.2	present	21	34-40	2	9-10	2.2-2.5
	PLY1St.43F	15.2.2	present	22	40	2	9-10	2.0
	PLY1St.45E	18.3.2	present	20	38	2	9-11	2.0
	PLY1St.46A	28.3.2	present	22-24	35-40	2	10-14	1.7-1.8
	PLY1St.46C	28.3.2	present	22	40	2	8-10	2.1-2.2
	PLY1St.48A	26.4.2	present	23-27	35-37	2	9-12	2.4
	PLY1St.48B	26.4.2	present	23	35-39	2	8-11	2.3-2.8
<i>P. calliantha</i>	Porkney4	August'99	present	15-22	34-39	1	4-6	1.3-1.8

Table 3.3 Morphological fine structural features of the valve for identification of the *Pseudo-nitzschia seriata* group and *P. americana*

Species, source	Valve shape	Central interspace	Fibulae / 10 µm	Interstriae / 10 µm	Rows of poroids	Poroids / 1 µm	Width [µm]
<i>P. australis</i> Skov et al. 1999, Hasle 1965,	linear to lanceolate, asymmetrical (valve view), linear to spindle-shaped, (girdle view), tips rounded	absent	12-18	12-18	2	4-5	6.5-8
<i>P. seriata</i> f. <i>seriata</i> Skov et al. 1999, Hasle 1965,	linear to lancet-shaped, asymmetrical (valve view), linear to lanceolate, symmetric (girdle view)	absent	14-18	14-18	3-5, often 2x2 rows	7-8	5.5-8
<i>P. seriata</i> f. <i>obtusata</i> Skov et al. 1999, Hasle 1965,	linear to lancet-shaped, asymmetrical (valve view), linear to lanceolate, symmetric (girdle view), obtuse-shaped tips	absent	15-20	15-20	2	7-8	4.5-5.5
<i>P. pungens</i> Skov et al. 1999, Hasle 1965,	linear to lanceolate, symmetrical (valve and girdle view),	absent	9-16	9-16	1-2	3-4	2.4-5.3
<i>P. fraudulenta</i> Skov et al. 1999, Hasle 1965,	spindle-shaped, symmetric (valve view)	present	12-24	18-24	2-3	5-7	4.5-10
<i>P. subpacifica</i> Skov et al. 1999, Hasle 1965,	one straight, one convex side, asymmetric	present	15-20	28-32	2	9-10	5-7
<i>P. heimii</i> Skov et al. 1999, Hasle 1965,	linear to lanceolate obtuse-shaped tips (valve view), sigmoid ends (girdle view)	present	11-16	19-26	1-2	5-6 (7-8)	4-6
<i>P. americana</i> Lundholm & Moestrup 2002; Hasle 1964	linear with truncate ends (in girdle view), often as epiphyte on diatoms e.g. <i>Chaetoceros</i> spp., only single cells have been observed	absent	18-24	26-31	2-3	8-10	2.5-4

Table 3.4 Morphological fine structural features of the valve for identification of the *Pseudo-nitzschia delicatissima* group.

Species, source	Valve shape	Central interspace	Fibulae / 10 µm	Interstriae / 10 µm	Rows of poroids	Poroids / 1 µm	Sectors of poroid hymen	Width [µm]
<i>P. delicatissima</i> Skov et al. 1999, Hasle 1965,	linear to lanceolate (valve view), linear (girdle view), straight cut ends	present	19-25	36-41	2	10-12	-	1.1-2
<i>P. calliantha</i> Lundholm et al. 2003	linear (in valve and girdle view)	present	15-22	34-39	1	4-6	7-10	1.3-1.8
<i>P. pseudodelicatissima</i> Lundholm et al. 2003	linear, symmetrical (valve view), lanceolate (girdle view)	present	20-25 (28)	36-43	1	5-6	two, hexagonal pattern	0.9-1.6
<i>P. cuspidata</i> Lundholm et al. 2003	lanceolate (valve view)	present	19-25	35-44	1	4-6	two	1.4-2.0
<i>P. cacialantha</i> Lundholm et al. 2003	lanceolate, slightly asymmetrical	present	15-19	28-31	1	3.5-5	4-5	2.7-3.5
<i>P. sp. strain Hobart 5</i> Lundholm et al. 2003	lanceolate	present	20-22	38-40	1	3-5	differs	1.4-1.6

3.3.3 Remaining cultures

A few cultures were identified together with N. Lundholm (then Botanical Institute, Department of Phycology, University Copenhagen) under the TEM without detailed measurements. Cells were recognised from their distinct morphological fine structure (for example such as the structured large poroids of *P. fraudulenta*). They included *P. australis*, *P. pungens*, *P. fraudulenta* and *P. delicatissima* (Tab. 3.5).

Table 3.5 Cultures identified by TEM together with N. Lundholm, without detailed measurements.

Species	Culture
<i>P. australis</i>	Pmarina2.7A
	Pmarina2.7B
	PLY1St.19
	PLY1St.20
<i>P. pungens</i>	Ppungens6
<i>P. fraudulenta</i>	PLY1St.11A
	PLY1St.11B
	PLY1St.11C
	PLY1St.12A
	PLY1St.12B
<i>P. delicatissima</i>	Porkney5
	Porkney7

Figures 3.2 and 3.3 show examples of TEM micrographs of Scottish *Pseudo-nitzschia* strains belonging to the *P. delicatissima* and *P. seriata* groups. They were taken from cultured species and used for morphological identification.

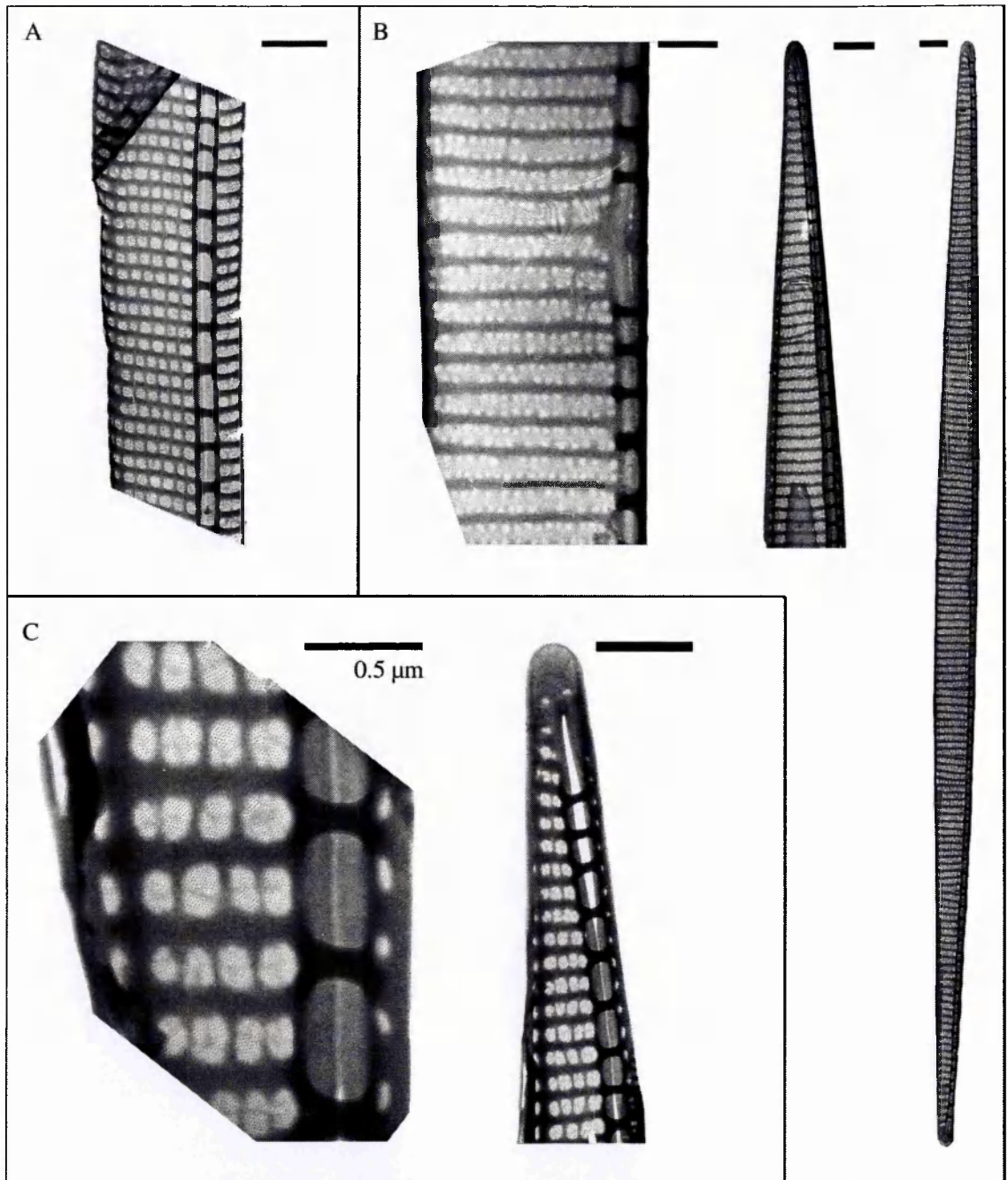


Fig. 3.2 TEM micrographs of frustules of Scottish *Pseudo-nitzschia* strains (B from culture, A and C from field samples) belonging to the *P. delicatissima* group: (A) *P. calliantha*, (B) *P. delicatissima*, (C) *P. pseudodelicatissima*. Scale bars represent 1 μm if not stated otherwise.

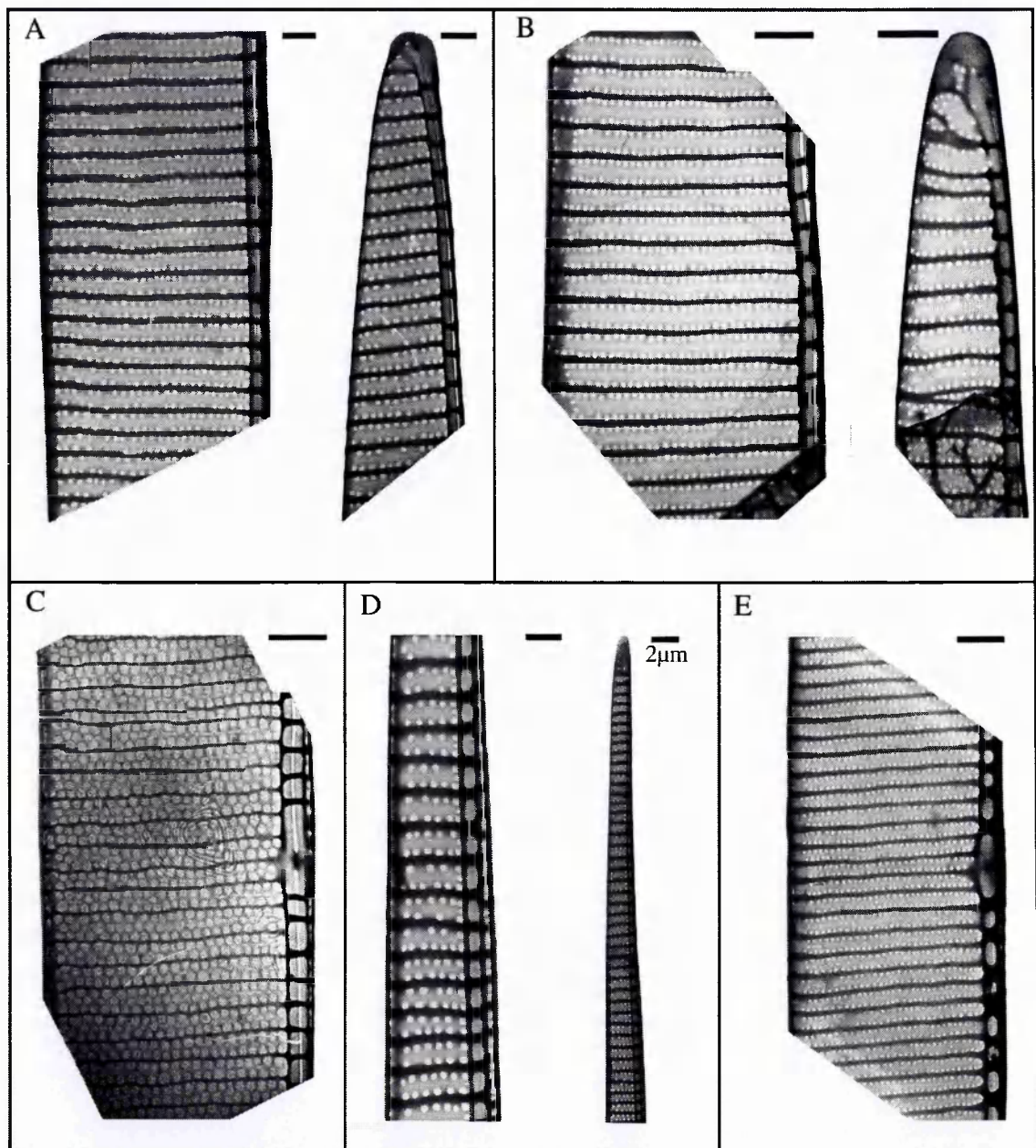


Fig. 3.3. TEM micrographs of frustules of Scottish *Pseudo-nitzschia* strains (all from cultures) belonging to the *P. seriata* group: (A) *P. australis*, (B) *P. seriata*, (C) *P. fraudulentata*, (D) *P. pungens*, (E) *P. cf. subpacificata*. Scale bars represent 1 μm if not stated otherwise.

3.3.4 Field sample identifications

Species that were identified by TEM from field samples (station LY1 and cruise D257) include *P. australis*, *P. seriata* f. *seriata*, *P. pungens*, *P. cf. subpacificata*, *P. americana*, *P. cf. delicatissima* (at least two morphotypes or species) and *P. cf. pseudodelicatissima*. For all species identifications, the morphological measurements do not always exactly match those from the keys. Especially for cells here named *P. cf.*

pseudodelicatissima no definite species names (e.g. *P. pseudodelicatissima*, *P. cuspidata* or *P. cacciantha*) could be assigned (see Tab. 3.7).

The results from field samples showed that different *Pseudo-nitzschia* species (toxic and non-toxic) that belong to one group and could not be distinguished by light microscopy (LM), can co-occur. *P. australis*, *P. seriata* and *P. pungens* (all belonging to the *P. seriata* group) were, for example, found together in samples from 1G (together with *P. fraudulenta*), 2G and LY1St.70. *P. australis* (toxic) and *P. fraudulenta* (non-toxic), that are easy to distinguish by TEM, but difficult by LM, co-occurred in LY1St.20. In LY1St.70 all five species identified within this study belonging to the *P. seriata* group, were observed together, further indicating the co-occurrence of toxic and non-toxic *Pseudo-nitzschia* with very similar with LM indistinguishable, morphologies. TEM identification using morphological fine structure features was ambiguous for *P. australis* from field samples. Numbers of fibulae and interstriae per 10 μm , rows of poroids, poroids per 1 μm and width differed between cells assigned as *P. australis* within the samples. Some *P. australis* and *P. seriata* cells identified from field samples might have been mistaken for each other. The decision on whether a species found in a field sample was designated as *P. australis* or *P. seriata* in this study depended mainly on the number of rows of poroids (*P. seriata* had to have at least 2 + 1 rows) and the number of poroids in 1 μm (≤ 6 for *P. seriata*).

Table 3.6 Fine structure measurements of the *P. seriata* group and *P. americana* from some plankton net field samples. Dates indicate the sampling day. Rows of poroids: numbers in brackets mean additional single poroids.

Species	Station/ sampling	Date	Central interspace	Fibulae / 10 μ m	Interstriae / 10 μ m	Rows of poroids	Poroids / 1 μ m	Width [μ m]
<i>P. australis</i> also found at LY1St 23 (10.8.1) LY1St 65 (29.8.2)	LY1St.20	20.7.1	absent	14-15	14-15	2	4-5	7-7.4
	D257 1G	29.9.1	absent	14-18	14-18	2	4-5	6.8-7.5
	D257 2G	29.9.1	absent	16	16	2 (+1)	5-6	7.2
	D257 4G	29.9.1	absent	18-20	18	2 (+1)	5-6	7.5
	D257 6G	30.9.1	absent	15	18-20	2	5-6	6.8-7.5
	LY1St.55	21.6.2	absent	15-16	15-16	2 (+1)	5-6	8.0
<i>P. seriata</i> f. <i>seriata</i> also found at LY1St 23 (10.8.1)	LY1St.58	12.7.2	absent	15-16	15-16	2	4-5	7.2-7.6
	LY1St.61	2.8.2	absent	13	13	2 (+1)	4-5	7.2-7.6
	LY1St.70	4.10.2	absent	15	16	2	5-6	?
	D257 1G	29.9.1	absent	16	16	2	5-6	5.6-6.4
	D257 2G	29.9.1	absent	17-18	17-18	2 (+1 or 2)	6-7	? (>6)
	LY1St.55	21.6.2	absent	18	18	2 (+1)	6-7	? (>6)
<i>P. pungens</i>	LY1St.70	4.10.2	absent	18	18	2 (+2)	7-8	5.4
	D257 1G	29.9.1	absent	16	16	2 (+1)	6-7	5.6-6.4
	D257 2G	29.9.1	absent	12-14	12	2	3-4	2.4-2.6
	D257 6G	30.9.1	absent	14	14	2	3-4	2.8
	LY1St.60	26.7.2	absent	14	14	2	3-4	2.8
	LY1St.70	4.10.2	absent	11	12	2	3-4	2.7
<i>P. fraudulent</i> also found at: LY1St 20 (20.7.1) LY1St 65 (29.8.2) LY1St 70 (4.10.2)	LY1St.70	4.10.2	absent	10	10	2	3	2.2
	D257 1G	29.9.1	present	24	22-24	2-3	6-7	5.1
	LY1St.70	4.10.2	present	17-18	26-28	2	8-9	5
	D257 1G	29.9.1	absent	16-22	24-34	2 (+1)	9-10	2.3-2.5
<i>P. cf. subpacific</i> <i>P. americana</i>	D257 4G	29.9.1	absent	18-20	25-27	2	8-12	3.3-3.6
	D257 6G	30.9.1	absent	16-22	24-34	2 (+1)	9-10	2.3-2.5
	D257 7G	30.9.1	absent	15-18	25-27	2 (+1)	9-10	2.6-3
	D257 10G	30.9.1	absent	19	24	2	8-9	3.3
	LY1St.61	4.10.2	absent	16	25	2 (+1)	8	1.8

Table 3.7 Fine structure measurements of the *P. delicatissima* group from some plankton net field samples. Dates indicates the sampling day.

Species	Station/ sampling	Date	Central interspace	Fibulae / 10 µm	Interstriae / 10 µm	Rows of poroids	Poroids /1 µm	Sectors of poroid hymen	Width [µm]
<i>P. cf. delicatissima</i> also found at: LY1St.7 (9.3.1) LY1St.9 (10.4.1) LY1St.47 (26.4.2)	LY1St.55	20.7.1	present	30	40	2	9-10	-	1.5
	D257 10G	30.9.1	present	20	32-34	2	6-7	-	2.2-2.3
	D257 M	3.10.1	present	20-24	28-34	2	6-8	-	2.2-2.3
	D257 F	4.10.1	present	17-20	28-30	2	5-8	-	2.1-2.8
	LY1St.60	21.6.2	present	30	40	2	9-10	-	1.5
	LY1St.61	2.8.2	present	28	40	2	10	-	1.5
	D257 1G	29.9.1	present	22-26	40-42	1	5-6	4-6	1.3
<i>P. cf. pseudodelicatissima</i>	D257 7G	30.9.1	present	22	40-42	1	4-6	4-6	1.3
	D257 M	30.9.1	present	?	40	1	5	4-6	1.6
				18	31-32	1	4-5	2	1.8-2
	D257F	4.10.1	present	15-22	34-42	1	4-5	2	1.1-2
	LY1St.62	7.8.2	present	24	40-42	1	6-7	2	1.1
	LY1St.70	4.10.2	present	24	40	1	6-7	4-6	1.6

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Morphological fine structure features of cells identified as *P. americana* (Tab. 3.6) often did not exactly match the descriptions in published keys (Tab. 3.3), however *P. americana* cells can be well distinguished from other *Pseudo-nitzschia* species by their short length and round shape of the valve tips. It is not clear though if the cells found in Scottish waters belong to a different species within the *P. americana* complex (Lundholm et al. 2002b). However, from their fine structural measurements they were delineated to *P. americana* rather than to the species *P. brasiliana* or *P. linea*, which have similar features (as e.g. the distinct round tips).

3.1.5 Sequences

Alignments of partial rDNA sequences showed that they were identical within each species. Even in those strains of *P. delicatissima* which showed variations in their morphology, ITS and partial LSU rDNA sequences were identical. Therefore one representative sequence of each species was used in a fasta3 search to find homologue sequences on the EMBL-PLANT database. Results (matching sequences/ strains, their accession numbers and expectation values) are presented in Table 3.8.

Table 3.8 Species identification using fasta3.

Sequence of Scottish strain	Gene	Species Match	Strain of matched species	EMBL-PLANT Accession number	E-value
PLY1St.54B	ITS	<i>P. australis</i>	OEM1	AY257842	1e 10 ⁻⁷⁷
	LSU		OM1	AF417651	6.1e 10 ⁻²¹⁶
PLY1St.52B	ITS	<i>P. seriata</i>	Nissum3	AY257841	2.8e 10 ⁻⁷⁸
	LSU		Lynaes8	AF417653	4.6e 10 ⁻²⁰⁶
Ppung6	ITS	<i>P. pungens</i>	P-24	AY257845	7.3e 10 ⁻⁷¹
	LSU		KBH2	AF417650	5.9e 10 ⁻²⁰⁵
PLY1St.11C	ITS	<i>P. fraudulenta</i>	Limens1	AY257840	8.1e 10 ⁻⁸¹
	LSU		Limens1	AF417647	6.9e 10 ⁻²¹²
D257F	ITS	<i>P. cf. subpacific</i>	Limens8	AY257859	2e 10 ⁻⁷⁸
	LSU		Zhenbo7B	AF417644	1.5e 10 ⁻²⁰³
Porkney1	ITS	<i>P. delicatissima</i>	Laesoe5	AY257849	3.9e 10 ⁻⁷⁵
	LSU		NWFSC 002	AF440767	1.2e 10 ⁻²⁰⁹

The results agreed with the species identifications that were delineated by morphological methods for those strains with unambiguous morphological identifications. For culture strains PLY1St.16B, PLY1St.52B and D257F morphological identifications were ambiguous. Sequences of strain PLY1St.16B and PLY1St.52B were identical, the fasta 3 search was undertaken using the ITS and LSU sequences of PLY1St.52B. The search resulted in high similarity of PLY1St.52B sequences with those of other *P. seriata* clones (Nissum3 for ITS and Lynaes8 for LSU), identifying the Scottish strains unambiguously as *P. seriata*. Strain D257F was thought to belong to either *P. cf. heimii* or *P. cf. subpacifica*, based on morphological measurements. In the fasta3 search the lowest E-values and hence best match were found for sequences of *P. cf. subpacifica* (clones Limens 8 for ITS: $E = 2e 10^{-78}$, and Zhenbo7B for LSU: $E = 1.5e 10^{-203}$), therefore, and assuming that the identification of the species in the database was accurate and correct, it was decided that culture strain D257F represented *P. cf. subpacifica* rather than *P. cf. heimii*.

3.1.5.1 Special case of *P. seriata*

Prior to this study *P. australis* was the only confirmed DA producer in Scottish waters. In this study *P. seriata* was isolated from Scottish waters, identified and positively tested for DA production. However, due to its ambiguous morphology and morphological similarity to *P. australis*, its ITS and LSU sequences were compared to those of *P. australis* to verify that it was indeed *P. seriata*.

From its morphology the Scottish *P. seriata* strain PLY1St.16B was initially mistaken for *P. australis*. However, the ITS and partial LSU rDNA sequences of both *P. seriata* strains PLY1St.16B (GenBank AY452523, AY452525, respectively) and PLY1St.52B (GenBank AY452524, AY452526, respectively) were identical to one another and also to the ITS and partial LSU rDNA sequences of the Danish *P. seriata* f. *seriata* strains

(GenBank AY257841, AF417653, AF417652). A total of 20 nucleotide differences across the ITS and partial LSU rDNA regions were observed between the Scottish *P. seriata* strains (PLY1St.16B and PLY1St.52B) and *P. australis* strains (PLY1St.19A, GenBank AY452527, AY452529 and PLY1St.54B, GenBank AY452528, AY452530, respectively) (Fig. 3.4). The only base difference between the *P. seriata* and *P. australis* partial LSU rDNA (total length of 838 base pairs) was one transition (position 189), which represents a molecular dissimilarity (number of base differences divided by sequence length) of less than 0.002. Within the ITS1 region (275 bases), 10 base differences were found between *P. seriata* and *P. australis*: seven transitions, two transversions and one indel (insertion or deletion). This corresponds to a dissimilarity between the sequences of only 0.036. No difference was found between the sequences of the 5.8S rDNA gene (162 bases, not shown). The dissimilarity between *P. seriata* and *P. australis* sequences in the ITS 2 region (330 bases) was 0.027. They differed in nine bases: six transitions and three transversions. Taking into account the full ITS1, ITS2, 5.8S and LSU sequences (1605 bases), only 20 nucleotide differences were observed, which describes a finite but small sequence dissimilarity of 0.012 between *P. seriata* and *P. australis*. Figure 3.4 shows the regions of sequence with base differences between *P. seriata* and *P. australis* ITS and LSU rDNA (Scottish strains and GenBank strain).

ITS1	..110	...	130	140	150	160...	270..
P.seriata_Nissum	..GTCGCTG...		CTATTTTCAGTGATATGCTATTTACTAGTGTATCAC...				ATCA..
P.seriata16B	..GTCGCTG...		CTATTTTCAGTGATATGCTATTTACTAGTGTATCAC...				ATCA..
P.seriata52B	..GTCGCTG...		CTATTTTCAGTGATATGCTATTTACTAGTGTATCAC...				ATCA..
P.australis19A	..GTCTCTG...		CT-TTTTLAGTGATGGCTAGCTACTAGCGTATTAC...				ACCA..
P.australis54B	..GTCTCTG...		CT-TTTTLAGTGATGGCTAGCTACTAGCGTATTAC...				ACCA..

ITS2	..	30...	110..	140	...	170	...	240...	270..
P.seriata_Nissum	..CCGGTC...		AGTTA...	GACACTC...		TTTAT...		GTGTCA...	CCTGTATT..
P.seriata16B	..CCGGTC...		AGTTA...	GACACTC...		TTTAT...		GTGTCA...	CCTGTATT..
P.seriata52B	..CCGGTC...		AGTTA...	GACACTC...		TTTAT...		GTGTCA...	CCTGTATT..
P.australis19A	..CCAGTC...		AGAGA...	GACATTC...		TTCAT...		GGGTCA...	CTAGTACT..
P.australis54B	..CCAGTC...		AGAGA...	GACATTC...		TTCAT...		GGGTCA...	CTAGTACT..

LSU	..180	190	200..
P.seriata_Lynaes	..CCGCCTGGTAGAGTGAGTCAT..		
P.seriata_Nissum	..CCGCCTGGTAGAGTGAGTCAT..		
P.seriata16B	..CCGCCTGGTAGAGTGAGTCAT..		
P.seriata52B	..CCGCCTGGTAGAGTGAGTCAT..		
P.australis19A	..CCGCCTGGTAGAGTGAGTCAT..		
P.australis54B	..CCGCCTGGTAGAGTGAGTCAT..		

Fig. 3.4 ITS and partial LSU rDNA sequence alignments of *Pseudo-nitzschia seriata* f. *seriata* and *P. australis*. Danish strains: P.seriata_Nissum (GenBank AY257841, AF417652), P.seriata_Lynaes (GenBank AF417653) and Scottish strains: P.seriata16B (PLY1St.16B, GenBank AY452523, AY452525), P.seriata52B (PLY1St.52B, GenBank AY452524, AY452526), P.australis19A (PLY1St.19A, GenBank AY452527, AY452529), P.australis54B (PLY1St.54B, GenBank AY452528, AY452530). Only sequence sections that include differences (indicated by grey bars) between both species are shown.

3.1.5.2 Partition homogeneity test

The partition homogeneity test showed that ITS and LSU sequences of all Scottish *Pseudo-nitzschia* strains were congruent. The evolutionary assumptions drawn from both genes were not significantly different (p value = 0.895), hence for each species ITS and LSU sequences were combined.

3.1.5.3 Modeltest

For the combined dataset the 'General Time Reversible' model (GTR) plus invariable sites and gamma distribution was selected by the Akaike information criterion (AIC, Akaike 1974). The likelihood settings from the best-fit model were the following: base frequencies : A = 0.2512, C = 0.2044, G = 0.2651, T = 0.2794; substitution model rate

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matrix: $a = 0.7853$, $b = 1.1278$, $c = 1.1608$, $d = 0.561$, $e = 2.6966$, $f = 1.0$; gamma distribution shape parameter = 0.32, proportion of invariable sites (I) = 0.3689.

3.1.5.4 Maximum likelihood and Bayesian analysis

Starting with the model selected by AIC (Akaike information criterion, Akaike 1974) in Modeltest, the ML model parameters were estimated via consecutive heuristic searches and re-optimised until their values stabilised. The following parameters were applied in the ML analysis: base frequencies: A = 0.25049, C = 0.20389, G = 0.26636, T = 0.27927; substitution model rate matrix: $a = 0.869219$, $b = 1.178685$, $c = 1.246156$, $d = 0.635066$, $e = 2.688958$, $f = 1.0$; gamma distribution shape parameter = 0.203093, proportion of invariable sites (I) = none. The log likelihood of the tree was 5783.69. The maximum likelihood 50% majority rule consensus tree of 2000 bootstrap replicates showed the same tree topology as the 50% majority consensus tree from 10001 trees generated in the Bayesian analysis, therefore they are represented together as a phylogram created in MrBayes (Fig. 3.5). It includes branch lengths as a measure of evolutionary changes between the sequences of the taxa. A mean of 19 changes for example between *P. seriata* and *P. australis* indicates their very close relation. This close relation is further expressed in the 98% bootstrap support and a posterior probability of 100 assigned to the *P. seriata*-*P. australis* clade. The *P. seriata*-*P. australis* clade clustered with *P. pungens* (bootstrap support of 96%, posterior probability of 100). All Scottish species belonging to the *P. seriata* group (width > 3 μm : *P. seriata*, *P. australis*, *P. pungens*, *P. fraudulenta* and *P. cf. subpacificica*) built one clade (74% bootstrap support, posterior probability of 68), separating them from *P. delicatissima*, representing the *P. delicatissima* group (width < 3 μm).

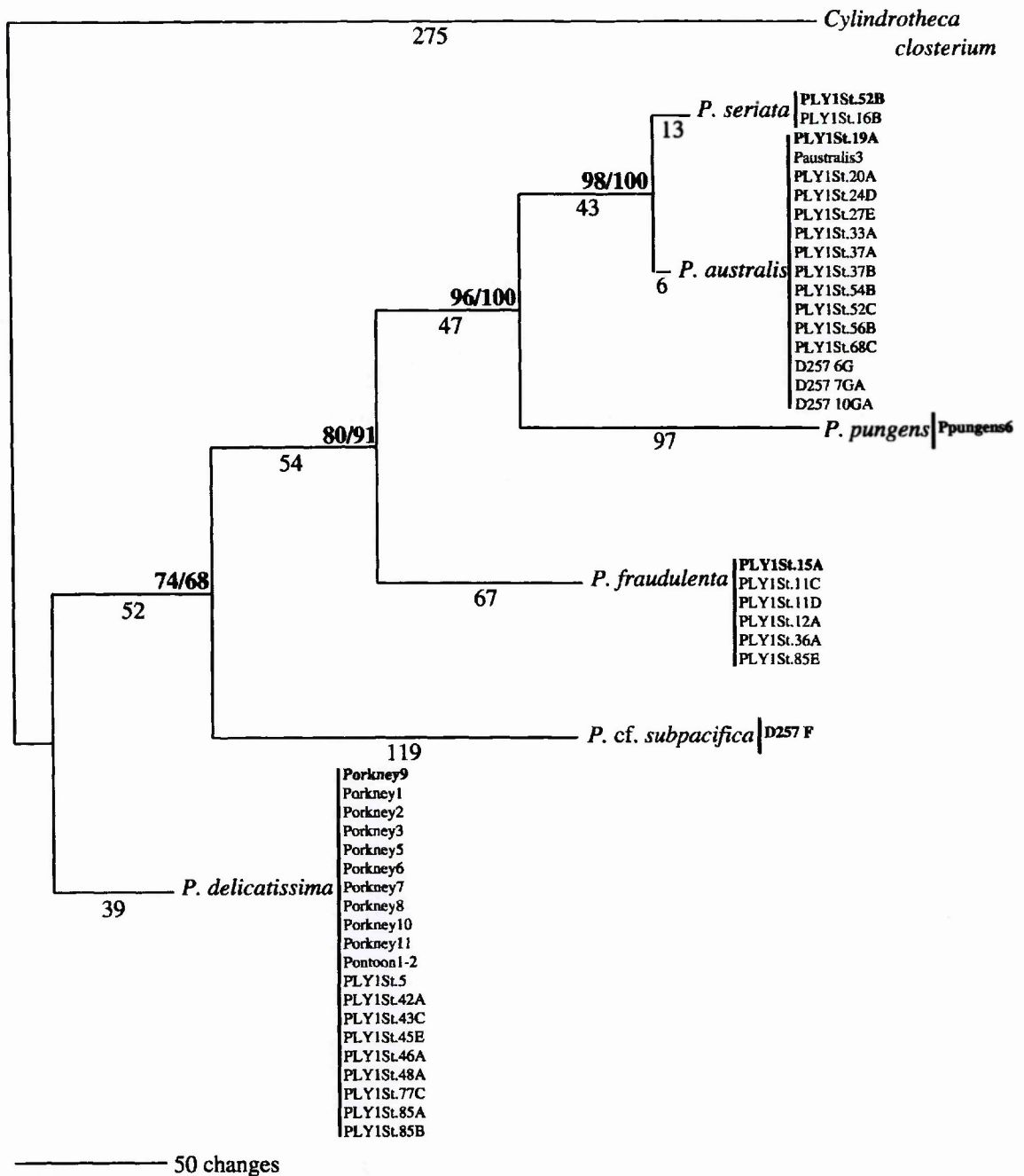


Fig. 3.5 50% majority rule consensus tree based on combined ITS and partial LSU rDNA sequences. The consensus tree was combined from 2000 bootstrap replicates in ML analysis and 10001 trees in MrBayes, both analyses led to the same tree topology. Bootstrap values (before slash) and probabilities (behind slash) are shown at the internal nodes. Branchlengths are indicated below each branch and posterior probabilities are shown in bold at the internal nodes. Redundant sequences of the cultured strains representing the species are indicated behind the species names. Strains in bold are the actual strains whose sequences were used for the analysis. *Cylindrotheca closterium* (AF289049) was used as an outgroup.

3.1.6 Domoic acid results

Fourteen *P. australis* cultures (at time of analysis in stationary phase) were positively tested for DA production (Tab. 3.1), while tests of cultures PLY1St.37A, 37B and D2576G, 7GA and 10GA were negative. As those five cultures showed very little growth at time of analysis, it may be that not enough material was tested to detect DA. However, as samples for cell counts were not taken prior to DA testing this cannot be confirmed. D2577GA was contaminated with another species when DA was tested and was later re-isolated. Apart from the 14 *P. australis* strains, the two *P. seriata* strains also produced toxin. None of the other tested *Pseudo-nitzschia* cultures contained DA. For detailed study of DA production of *P. seriata* culture PLY1St.52B under phosphate and silicate limitation during a full growth cycle see chapter 5.

Table 3.9 Domoic acid content of *Pseudo-nitzschia seriata* and *P. australis* strains after 25 days (stationary phase) in batch culture.

Strain, species	DA (ng · mL ⁻¹)		DA (pg · cell ⁻¹)		Cell number (mL ⁻¹) × 10 ³
	Whole culture	Medium	Whole culture	Medium	
PLY1St.16B, <i>P. seriata</i>	41.5	31.8	0.16	0.12	266
PLY1St.52B, <i>P. seriata</i>	28.9	9.9	0.23	0.08	127
PLY1St.19A, <i>P. australis</i>	26.5	20.5	0.15	0.12	172
PLY1St.54B, <i>P. australis</i>	217.2	114.8	1.68	0.89	129

To compare the toxin production of *P. australis* and *P. seriata*, two *P. australis* (PLY1St.19A and PLY1St.54B) and *P. seriata* strains (PLY1St.16B and PLY1St.52B) were again tested for DA after 25 days in culture (stationary phase). Those strains were chosen because they were the only isolated Scottish *P. seriata* strains and the most healthy looking *P. australis* strains. All strains contained DA (Tab. 3.9). The *P. australis* strain PLY1St.54B produced considerably more whole-culture DA (217.2 ng

DA · mL⁻¹) and medium DA (114.8 ng DA · mL⁻¹) than did the other *P. australis* strain (PLY1St.19A), which was isolated a year later, or the *P. seriata* strains. *P. seriata* strains and *P. australis* strain PLY1St.19A all produced similar amounts of whole culture and medium DA · cell⁻¹.

3.4 Discussion

Within this study *Pseudo-nitzschia* species were isolated, cultivated, identified and tested for domoic acid production. It hence presents the first detailed assessment of *Pseudo-nitzschia* species in Scottish waters, identifying two DA producers, *P. australis* and *P. seriata*, and the presence of the following other species: *P. fraudulenta*, *P. pungens*, *P. cf. subpacifica*, *P. americana*, *P. delicatissima*, *P. calliantha* and *P. pseudodelicatissima*. The species *P. americana*, *P. calliantha* and *P. cf. subpacifica* were for the first time reported from Scottish waters.

Pseudo-nitzschia seriata had previously been implicated in DA toxin production, but never before in Scottish waters. Lundholm et al. (1994) documented the first instance of DA production by three isolates of *P. seriata* from Danish waters (although no ASP events have been recorded in that region). This is in contrast to an earlier study in which an isolate from PEI, Canada, identified as *P. seriata*, was found to be non-toxic (Bates et al. 1989). High levels of DA in the digestive glands of scallops from the Magdalen Islands (Gulf of St. Lawrence, Canada) were associated with the presence of *P. seriata*, which was shown to produce DA in culture (Couture et al. 2001). In the spring of 2002, for the first time ever, most of the southern Gulf of St. Lawrence was closed due to DA produced by *P. seriata* (Bates et al. 2002). In Scotland (Gallacher et al. 2001, Campbell et al. 2001, 2003) and Ireland (Cusack et al. 2000), *P. seriata* has been observed within the phytoplankton community at times when these shellfisheries were closed due to DA contamination. However, cultures of *P. seriata* were not established from these events,

preventing any toxicity testing and unambiguous species identification. Furthermore, as toxic *P. australis* was identified during these events, the spotlight was directed at this latter species. This study therefore presents the first identification of *P. seriata* f. *seriata* from UK waters as a DA producer and suggests its potential to act as a causative organism for ASP toxin in molluscan shellfish from those waters.

3.4.1 Morphological identification of ambiguous species

3.4.1.1 *P. seriata*

Pseudo-nitzschia seriata has long been known to have a close morphological similarity to *Pseudo-nitzschia australis*. First described as *Pseudonitzschia australis* by Frenguelli (1939), *P. australis* was transferred to the genus *Nitzschia*, but because the name *Nitzschia australis* was already taken, it was proposed to be called *Nitzschia pseudoseriata* (Hasle 1965) because of its strong resemblance to *Nitzschia seriata* (today known as *Pseudo-nitzschia seriata*). Indeed, their morphological similarity has resulted in the misidentification of *P. australis* as *P. seriata* in the past (Garrison et al. 1992, Villac et al. 1993b, Fryxell et al. 1997).

The morphological fine structure of *Nitzschia seriata* (Cleve) (type locality: Tindlingen, Greenland), which is today known as *Pseudo-nitzschia seriata* (Cleve) Peragallo (Peragallo and Peragallo 1900), was described by Hasle (1965). Two rows of poroids per stria are evident, which are, in addition, joined in most cases by two, but sometimes one or three, more rows of poroids. For *P. seriata* f. *seriata*, Skov et al. (1999) describe three to five rows of poroids per stria, often grouped as two times two rows. In contrast, for *Nitzschia seriata* f. *obtusa*, today known as *Pseudo-nitzschia seriata* f. *obtusa*, Hasle (1965) observed only two rows of poroids per stria and obtuse valve ends.

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The phenotypic appearance of the two Scottish *P. seriata* strains (PLY1St.16B and PLY1St.52B) was ambiguous when compared to other described *P. seriata* f. *seriata* strains, differing mainly by the observation of only two rows of poroids per stria, with occasionally a third row. Moreover, the number of fibulae and interstriae per 10 μm differed between the two strains.

Although lacking the obtuse valve ends characteristic of *P. seriata* f. *obtusa* (as listed in Skov et al. 1999), the morphological features described above could lead to the identification of these strains as either *P. seriata* f. *obtusa*, or (because of two rows of poroids) *P. australis*, rather than *P. seriata* f. *seriata*. Indeed, after strain PLY1St.16B was isolated into culture, it was initially thought that it may be *P. australis* because the frustules lacked a central interspace and had two rows of poroids.

The unusual morphology exhibited by those strains may be related to the fact that the cells had been in culture for ca. six months before they were examined by TEM. In culture, the cell length would have diminished due to cell division (e.g. Pan et al. 2001), but this would not necessarily have affected the fine structural features. Furthermore, the cells were not lobed or otherwise deformed as has previously been observed in some older *Pseudo-nitzschia* clones (e.g. Subba Rao & Wohlgeschaffen 1990; Pan et al. 2001; S. Bates and J. Fehling, personal observations).

3.4.1.2 *P. cf. subpacific*

In Europe, *P. cf. subpacific* has been isolated from Portuguese and Spanish waters (Lundholm et al. 2002a). The strain established in this study is the first strain identified from Scottish waters. Recently *P. subpacific* has been detected in the Bay of Fundy (Canada) (Kaczmarska et al. 2004). The presence of *P. subpacific* in Gulf Stream samples (Kaczmarska et al. 1986) led Kaczmarska et al. (2004) to the suggestion of a northward expansion of the species via that current. A molecular analysis of *P.*

subpacifica from the Bay of Fundy would determine if the Scottish and Canadian strains belong to the same species. If the Scottish *P. cf. subpacifica* is similar to *P. subpacifica*, the suggestion of its northward expansion would be confirmed.

P. subpacifica was first described as *Nitzschia subpacifica* by Hasle (1965). It has some similar morphological characters to *P. heimii*, such as striated bands (Hasle 1965; Hasle & Syvertsen 1996), but is in general described as wider and more delicate in structure. Although in this study *P. cf. subpacifica* was found to be narrower than *P. subpacifica* described in keys, some of its morphological features such as the number of fibulae and interstriae approximate the numbers given for *P. subpacifica* or *P. heimii*. Its identification just by morphological features is ambiguous and *P. cf. subpacifica* could be delineated as *P. cf. heimii*, or even as some non-described species. This is another example of ambiguous morphology in *Pseudo-nitzschia* and emphasises the need to combine molecular identification with classical morphological characters.

3.4.1.3 *P. americana*

P. americana, originally described as *Nitzschia americana* (Hasle 1964), is often found as an epiphyte on other diatoms such as *Chaetoceros* species (Lundholm 2002b), this was the case in this study too. It had previously been reported from tropical to temperate waters all over the world, including the North Sea and Skagerrak. This study presents the first record of *P. americana* from the North Atlantic, although its identification is so far only based on morphological features, as it was not isolated and cultured (for positions of sampling locations at which *P. americana* was observed, see chapter 2, Table 2.2). It was not isolated, because it was not recognised in non-fixed field samples. Furthermore, as it was mainly found sitting on *Chaetoceros* spp. cells, it would have been difficult to separate it from those cells and grow it in a clonal culture without *Chaetoceros* spp.. From its morphological fine structure, the cells examined in this

study matched recently published descriptions of *P. americana* (Lundholm, 2002b). One difference noted was that *P. americana* was observed to occur in chains of up to three cells, in some samples, whereas the species is described as occurring only as single cells (Hasle 1964). Further investigation involving its cultivation and identification by molecular methods would be needed to confirm its species delineation. A Californian strain of *P. americana* tested negative for DA production (Villac et al. 1993a) and hence this species might not be a concern for ASP in Scottish waters. However, as DA production may be strain specific, further study on Scottish *P. americana* is required.

3.4.1.4 *P. delicatissima*

The morphology of strains of *P. delicatissima* (Cleve) Heiden, first described as *N. actydropbila* (Hasle 1965), differed considerably. Most of the cultivated strains and cells from field samples were wider than described in published keys, some of them possessed less poroids than the type description. From their morphology it is likely that some strains belong to a new species close to *P. delicatissima* that is described in a paper by N. Lundholm which is not yet in press (N. Lundholm, personal communication). Morphological features might change in culture or with environmental conditions. However, the differences in morphology observed in cultured strains, were not shared in the molecular data, the partial rDNA sequences were identical. The example of *P. delicatissima* again shows the importance and advantage of identification by the molecular approach, morphological ambiguities that might lead to misidentification can be overcome by unambiguous species delineation applying molecular techniques. While it was shown that the cultured strains all belonged to one species, the lack of molecular evidence means it will remain unknown if the cells identified from field samples were delineated correctly.

3.4.1.5 *P. calliantha* and *P. pseudodelicatissima*

Before the description of *P. calliantha* as a new species (Lundholm et al. 2003), it was often identified as *P. pseudodelicatissima*. *P. pseudodelicatissima*, was first described as *Nitzschia delicatula* (Hasle 1965), until the species complex (also including *P. cuspidata*) was recently re-examined (Lundholm et al. 2003). The cultivation and identification of *P. calliantha* again represents the first occurrence of this species in Scottish waters. Unfortunately the culture died before it was tested for domoic acid production and before its DNA was extracted, but the distinct morphological fine structure clearly identified the cultured strain as *P. calliantha*. This species might be of ecological interest in Scottish waters, as some strains have been observed to produce DA: *P. calliantha* was responsible for high DA concentrations in blue mussels (*Mytilus edulis*) in the Bay of Fundy, Canada in 1988 (Martin et al. 1990). Another strain from Danish waters had also been positively tested for DA production (Lundholm et al. 1997). However, other cultures of *P. calliantha* did not appear to produce the toxin (Lundholm et al. 1994; Lundholm et al. 2003).

In field samples, various *P. cf. pseudodelicatissima* cells were observed and identified by TEM. In at least some of those cells (e.g. from D257M, D2577G, LY1St.70) the valve structural features were ambiguous and did not exactly match the species descriptions for *P. pseudodelicatissima*, *P. calliantha*, *P. cacialantha* or *P. cuspidata*, indicating that further undescribed species probably exist. More study is required to isolate, cultivate and identify some of those ambiguous strains with morphological and molecular methods. This could be of interest as that species complex includes some potential DA producers (Lundholm et al. 2003) that might contribute to ASP events in Scottish waters.

3.4.2 Possible reasons for morphological differences in strains belonging to the same species

Difference in the number of fibulae and interstriae between strains (e.g. in *P. seriata* PLY1St.16B and PLY1St.52) or width (e.g. *P. delicatissima* strains Porkney1 and PLY1St.48B) might be explained in three ways. First, many of the strains were isolated from the same place (LY1) but in successive years. It is possible that different populations had been sampled, with slightly divergent morphological appearances. Second, the difference in age of the cultures may be important, adaptation to laboratory conditions could have an effect on the morphological valve fine structure. However, there is no other evidence to show that ageing in culture could result in a change in the number of fibulae and interstriae. Third, there is evidence that temperature can have an impact on some frustule structures. Similar to the findings of this study, Lundholm et al. (1997) noted a reduction in the number of rows of poroids in *P. seriata* from four to two (or three) as temperature was increased from 4° C to 15° C. Lewis et al. (1993) observed a difference in the number of rows of poroids in *P. multiseriata*, when grown in batch culture at different temperatures. Cells grown at 5-15°C had three to four rows of poroids (as is common for *P. multiseriata*), whereas cells grown at 25°C had only two to three rows. As diatom taxonomy is mainly based on valve morphology, the impact of temperature on *Pseudo-nitzschia* species morphology requires more study.

3.4.3 ITS and LSU sequences

3.4.3.1 *P. seriata* and *P. australis*

The high similarity in the ITS and LSU regions indicates that *P. seriata* and *P. australis* are closely related; this is also supported by their close morphological similarity. Discrimination between these two species is more problematic than for others from the

genus *Pseudo-nitzschia*. For example, Manhart et al. (1995) aligned ITS1 sequences of *P. multiseriata* and *P. pungens* and found 57 point mutations and three indels; many more differences than the nine between the ITS1 sequences of the Scottish *P. seriata* and *P. australis*. Using the ITS2 regions, Cangelosi et al. (1997) compared *P. multiseriata*, *P. pungens* and *P. australis*. The *P. multiseriata* sequence exhibited 17% and 21% divergence from *P. pungens* and *P. australis*, respectively, again a much higher divergence than the 2.7% that was observed in the ITS2 region between *P. seriata* and *P. australis*.

Within *Pseudo-nitzschia* species, identification based only on morphology and partial LSU sequences can be ambiguous and may lead to misidentification. With only one base difference, the LSU region on its own can be insufficient to distinguish between *P. seriata* and *P. australis*. After TEM examination of strain PLY1St.16B, it was erroneously concluded to be *P. australis*. The LSU region was then sequenced and compared to other *P. australis* sequences. The finding of only one base difference in the sequences was at first interpreted to be population-level variation. However, after sequencing the ITS region and making more detailed measurements on the TEM images, it was concluded that the species was not *P. australis*. By aligning the ITS and LSU regions of the Scottish strain with other *P. seriata* strains conducting a FASTA search, its identity was shown to be *P. seriata* f. *seriata*. In this case it proved essential to sequence more than one gene region.

3.4.3.2 *P. cf. subpacificata* and *P. cf. heimii*

As discussed above, using a morphological approach, culture D257F could have also been identified as *P. cf. heimii*. Searching ITS and LSU sequences against the EMBL-PLANT database, the highest match was found with *P. cf. subpacificata*. For *P. cf. heimii* only one LSU sequence is published on databases (as *P. cf. heimeii*, GenBank

AF440777). Aligning the D257F LSU rDNA sequences with the sequences of *P. cf. subpacifica* (strain Zhenbo7BL, GenBank AF417644) and *P. cf. heimii* (GenBank AF440777; see appendix 5 for alignment) showed the number of differences between them, detailed in Table 3.10.

Table 3.10 Differences in partial LSU sequences between *P. cf. subpacifica* and *P. cf. heimii* *

	<i>P. cf. subpacifica</i>	<i>P. cf. heimii</i>	D257F
<i>P. cf. heimii</i>	4 (2 ts, 2 tv)	-	4 (1 ts, 3tv)
D257F	2 (2ts)	4 (1 ts, 3tv)	-

*ts = transition, tv = transversion;

Given the differences in sequences between the three strains it is possible that strain D257F is an intermediate form of *P. cf. heimii* and *P. cf. subpacifica*. This view was supported by comparing ITS sequences. The ITS sequences of D257F and *P. cf. subpacifica* (strain Limens8, AY257859; see appendix 5 for alignment), showed 10 differences (9 ts, 1 indel) between the two sequences. Still, that strain was the highest match among all *Pseudo-nitzschia* strains listed in that database (an E-value of $2e^{-78}$). However, 10 is quite a high number of differences, indicating that those two species variants might be indeed different species, leaving the delineation of strain D257F uncertain.

3.4.3.3 Intra-specific variation

LSU sequences did not show intra-specific divergence within *P. australis*, *P. seriata*, or *P. fraudulenta*. For *P. delicatissima*, the strains examined potentially represent a species-complex. This indicates that the LSU region of the rDNA operon is not sufficient to distinguish strains within species of *Pseudo-nitzschia*. However, some differences between ITS sequences from world-wide strains were found for example in

P. pungens, *P. fraudulenta* and *P. delicatissima*, which might be useful as regional population markers.

The observed consistency between partial rDNA sequences of species is an advantage in using gene probes for monitoring toxigenic *Pseudo-nitzschia* species.

3.4.4 Phylogenetic analysis

In both, ML and Bayesian analyses *P. seriata* and *P. australis* were grouped together in a clade with high bootstrap (98) and posterior probability (100) values. Their close phylogenetic relationship was expected, due to their similar morphology, both are domoic acid producers and only one difference was found between their LSU rDNA sequences. In other studies, using either partial LSU rDNA (Lundholm et al. 2002a, b; Lundholm & Moestrup 2002) or the ITS region (Lundholm et al. 2003), these two species were also identified as sister taxa, confirming the result of this study. Similar to those other studies, of the taxa used, *P. pungens* was most closely grouped to the *P. seriata* - *P. australis* clade. *P. seriata*, *P. australis* and *P. pungens* all lack a central interspace and have 2 (in case of *P. seriata* 2 +1) rows of poroids, justifying a close phylogenetic relationship between the three. This shows, that the valve morphology reliably indicated the taxonomic relationships. The next closest taxon was *P. fraudulenta* and then *P. cf. subpacific*, relating all taxa belonging to the *P. seriata* group to each other. This result was different to studies that used either partial LSU rDNA or ITS rDNA, where *P. cf. subpacific* was found separated from the *P. seriata* group cluster, in a group with *P. delicatissima* (Lundholm et al. 2002ab; Lundholm & Moestrup 2002; Lundholm et al. 2003). However, as *P. cf. subpacific* strains differed, they might have been different species. In other studies ML, parsimony and distance methods were used and fewer bootstrap replicates were conducted.

In combining ITS and LSU rDNA a better resolved phylogeny, distinguishing the *P. seriata* and *P. delicatissima* group was achieved. ML and Bayesian analysis resulted in the same tree topologies, both were supported by high bootstrap and posterior probability values, indicating a high confidence in the established phylogenetic relationships between the used taxa. The identical topology of trees obtained from both methods recommends the use of ML and Bayesian methods in future analysis, rather than distance and parsimony methods, although they might be costly in computational time.

A combination of SSU, ITS and LSU rDNA sequences to build a phylogeny including Scottish strains and *Pseudo-nitzschia* strains from round the world is currently in preparation. This might give more insight in the evolutionary relationships of *Pseudo-nitzschia* spp. in general and in particular the relation of the Scottish *P. cf. subpacific* to the other taxa.

3.4.5 Domoic acid production

This study confirmed the toxicity of Scottish *P. australis* strains and hence the results of Campbell et al. (2001) who tested two *P. australis* cultures isolated from Scottish waters.

This is the first documentation of DA production by a Scottish isolate of *P. seriata*. The first report of DA production by *P. seriata* was in Danish strains (Lundholm et al. 1994). It is difficult to compare the toxicity of the Scottish and Danish *P. seriata* strains because of the rapid daily increase in DA concentration as the cultures age in stationary phase. In our study, the DA content was 0.16 to 0.23 pg DA cell⁻¹ (for both strains) on day 25 and at 15° C. This compares with 0.31 to 1.6 pg DA cell⁻¹ (for three strains) at unspecified days during the stationary phase, but also at 15° C (Lundholm et al. 1994). Temperature may be an important factor for DA production in *P. seriata*. The Danish

cultures produced more DA at 4° C than at 15° C. Furthermore, the affect of photoperiod is unknown, but the Danish cultures grew under a 16:8 h L:D cycle, compared to our 12:12 h L:D cycle. Interestingly, two Canadian isolates of *P. seriata* produced the identical amount of total DA (0.16 pg · cell⁻¹) as reported here for strain PLY1St.16B, but at 8° C, on day 17, and with a 10:14 h L:D cycle (Bates et al. 2002). At the end of the experiment (day 64), the cellular DA content had increased to 3.4 pg · cell⁻¹.

Even when grown under the same culture conditions, *P. australis* strain PLY1St.54B exhibited greater toxin production at the same stage in culture than *P. australis* strain PLY1St.19A. The difference in toxicity might be explained by the fact that the latter was isolated a year after the former. Both strains may therefore have been collected from different planktonic populations; many plankton taxa are known to exist in temporally and spatially highly variable sub-populations (e.g. Bolch et al. 1998; Medlin et al. 1996; Ryneerson & Armbrust 2000). Further genetic analyses of additional strains and genetic markers with a higher level of polymorphism than displayed by the ITS and LSU rDNA regions are needed to test this hypothesis for *Pseudo-nitzschia* spp. However, the results of this study support the finding that *Pseudo-nitzschia* strains of the same species can vary in their toxin production, as observed for *P. seriata* (Lundholm et al. 1994), *P. australis* (Villac et al. 1993a, b), *P. multiseriata* (Bates et al. 1989, Villareal et al. 1994), *P. multistriata* (Rhodes et al. 2000), and *P. pungens*, *P. delicatissima* and *P. fraudulenta* (Rhodes et al. 1998).

The discovery of DA production by *P. seriata* in Scottish waters indicates that *P. australis* is not the only toxic species of concern in Western Europe. The latter species was thought to be the causative organism of the 1999 – 2002 closures of king scallop harvesting across offshore regions of northern and western Scotland (Campbell et al. 2003). However, because of their similar appearance and morphology, it is likely that *P.*

seriata was also present but often unnoticed. Strains of *P. fraudulenta*, *P. pungens* and *P. delicatissima* isolated from Scottish waters did not produce DA, nor did the *P. cf. subpacific* strain from the open Atlantic (D257 F, 57°30'N, 12°15'W). *Pseudo-nitzschia multiseri* has previously been observed in eastern Scottish waters in low numbers (Gallacher et al. 2001), but cultures were not established so they could not be tested for DA.

3.4.6 Implications and conclusions

This study indicates that at least two *Pseudo-nitzschia* species (*P. seriata* and *P. australis*) can be responsible for ASP toxicity in Scottish waters. A third potentially toxic species, *P. calliantha*, was first observed in these waters, but not tested. Further isolation, identification and toxicity testing of the resident phytoplankton community is needed to determine if other species may also contribute to DA production in this region.

This study also demonstrated the importance of combining morphological and genetic approaches for identifying closely related *Pseudo-nitzschia* species. Identification can be ambiguous in phytoplankton monitoring programmes where harmful species are counted with the light microscope. Significant economic and health consequences can result, should there be confusion between toxic and benign species. The advancement of such monitoring programmes requires the development and implementation of molecular probes capable of differentiating among similar species that may display variable, or no toxicity. The ITS data might be sufficient in most cases for probe development. For example, ITS1 data contain a region of 20 base pairs (at position 126-148), that has 6 differing nucleotides between the closely related species *P. australis* and *P. seriata*. This region should be sufficiently different to provide a template for a probe distinguishing between the two.

4 Chapter 4: Laboratory experimental studies

4.1 Introduction

4.1.1 Studies on DA production in *Pseudo-nitzschia* species

Considerable effort has been expended on studying the growth and DA production of *P. multiseriis*. In particular, detailed laboratory experiments on isolates under a range of defined conditions have shown that toxin production is most prevalent in inorganic nutrient limited stationary phase (e.g. Bates et al. 1989; Pan et al. 1991; Bates et al. 1991; Douglas & Bates 1992; Douglas et al. 1993; Lewis et al. 1993; Pan et al. 1993; Smith et al. 1993; Bates et al. 1995; Pan et al. 1996a, b, c, d; Zhiming & Subba Rao 1998; Bates 2000).

Field observations and laboratory studies led to the suggestion that environmental stress such as nutrient limitation influences DA production in *Pseudo-nitzschia* species (e.g. Scholin et al. 2000). In eastern Canada, during a *P. multiseriis* bloom in the field, the maximum cellular DA concentration was found about one week after the maximum of *P. multiseriis* cells in the water column (Smith et al. 1990a). In this case cells might have been exposed to nutrient limitation, resulting in the cessation of growth and in enhanced DA production.

The particular nutrient that limits cell yield is thought to be of importance to toxin production (as stated in section 1.8). Nitrogen limitation is thought not to induce toxin production (Bates et al. 1991). However, Si and P have been shown to enhance DA production in *P. multiseriis*, when they became limited in the growth medium (section 4.1.2). Detailed analysis of those studies showed that DA production by *P. multiseriis* occurs mainly in Si limited stationary phase. When Si limited cultures were re-supplied with the limiting nutrient, DA production was suspended, but resumed when Si in the medium became low (Pan et al. 1996b, c). For P, Pan et al. 1996a associated high DA

production in steady-state continuous and batch cultures with high cellular N:P ratios, and hence P limitation or at least P stress on the cells.

Of all *Pseudo-nitzschia* species isolated from western Scottish waters as part of this study (chapter 3), only *P. australis* and *P. seriata* produced DA. As noted in chapter 1, section 1.8.1, two studies (Garrison et al. 1992; Cusack et al. 2002) have investigated toxin production by *P. australis*. However, results were ambiguous and the experiments were not conducted with a defined limited nutrient.

Only one study has been conducted with *P. seriata*, indicating elevated DA production under low temperature (Lundholm et al. 1994) (see chapter 1, section 1.8.3).

These studies therefore provide some, but inconclusive, support for the hypothesis that *Pseudo-nitzschia* species (at least *P. multiseriata* and *P. australis*) have different physiological mechanisms governing DA production. Furthermore, while the temporal variation in toxin production is potentially related to nutrient stress, it may also be strain specific (section 1.4) and additionally may be modulated by other factors such as temperature or photoperiod. This has obvious implications for monitoring *Pseudo-nitzschia* blooms and predicting their toxicity.

Field observations at the monitoring site LY1 (chapter 2) suggested that the distribution of toxic and non-toxic *Pseudo-nitzschia* species might be influenced by seasonal, environmental factors such as nutrient availability and photoperiod. As *P. seriata*, which can be confused with *P. australis*, was isolated and identified from Scottish waters, it was necessary to test its toxicity. Furthermore, the experiments were undertaken with defined P and Si limitation as had previously been conducted for *P. multiseriata* (e.g. Bates et al. 1991; Pan et al. 1996a, b, c).

Other factors that are not easily monitored in the field, such as interactions with bacteria might also play a role in the toxin production and seasonal occurrence of *Pseudo-*

nitzschia species. Hence, this study goes on to investigate the effects of some basic interactions between the diatoms and bacteria, on growth dynamics of *P. seriata*.

The following hypotheses were tested in laboratory experiments:

- A) The toxin production and growth dynamics of *P. seriata* is influenced by the yield limitation of silicate or phosphate.
- B) *P. seriata* shows differences in growth, when grown on NH₄ or NO₃ based media.
- C) *P. delicatissima* and *P. seriata* have different light length preferences.
- D) The presence of bacteria enhances growth of *P. seriata*.

4.1.2 Experiment A: Growth and toxin production of *P. seriata* under Si- and P limitation

In this study the effects of inorganic P or Si limitation on a Scottish *P. seriata* isolate were investigated. Experiments were conducted in controlled laboratory batch cultures under both inorganic Si and P limitation allowing the investigation of nutrient stress on cell numbers and both carbon (C) and N biomass growth and yield, chl *a* concentrations and both intracellular and extracellular DA production.

For frustule formation of diatoms Si is essential (e.g. Paasche 1973), but it also plays an important role in the cell-cycle. Cells take up Si during a specific stage of the cell cycle (Brzezinski 1992), in *P. multiseriata* during the light period, presumably at the end of the G1 phase (Bates 1998). Si limitation impedes the progress of the cell-cycle division by interfering with the synthesis of DNA (Brzezinski et al. 1990). Si might affect the regulation of gene expression in diatoms, as its limitation may indirectly inhibit DNA polymerase and may therefore arrest cells at a particular phase in the cell division cycle that is conducive to DA production (Pan et al. 1998; Bates 1998 and references therein).

Silicate is the limiting nutrient in the commonly used F/2 growth medium (Guillard 1975), being in relatively low supply compared to the other nutrients. In batch cultures of diatoms (Subba Rao et al. 1988b; Bates et al. 1991; Pan et al. 1991) the stationary growth phase is often initiated by silicate limitation and with it DA production starts, as was shown for *P. multiseriis* (Bates 1998 and references therein). Lower initial Si concentrations in the medium resulted in higher cellular DA levels in stationary phase (Bates et al. 1991). Furthermore, as shown by Pan et al. (1996b), the amount of DA production seems to be inversely correlated with the Si concentration in the culture.

In coastal waters the Si concentration is either decreasing or relatively constant, while N is potentially increasing. Hence, as the N:Si ratio is normally 1:1 for optimal phytoplankton growth, this would lead to Si limitation (Gilpin et al. 2004).

In batch and continuous culture experiments *P. multiseriis* has been shown to produce DA under P limitation (Bates et al. 1991; Pan et al. 1996a). Pan et al. (1996a) associated high DA production in batch and steady-state continuous cultures with high cellular N:P ratios, and hence P limitation or at least P stress on the cells. In the marine environment P is only occasionally a limiting nutrient in coastal waters. It is not known by what 'mechanism' other than placing cells under physiological stress, P limitation can effect DA production.

4.1.3 Experiment B: *P. seriata* growth on different N sources

Nitrate (NO_3^-) and ammonium (NH_4^+) are the main nitrogen (N) sources for primary production, they occur in the world oceans in concentrations commonly less than 40 μM , sometimes higher concentrations are observed due to local eutrophication (Sommer 1998). In times of high primary productivity (e.g. during a phytoplankton bloom), the NO_3^- and NH_4^+ concentration in the water can decrease below the detection limit. At LY1 between May and September, when *Pseudo-nitzschia* spp. abundance was high

(chapter 2), NO_3^- concentrations were low, between 0.3 μM and detection limit. However, NH_4^+ concentrations were elevated with a maximum concentration of 1.5 μM at times when *Pseudo-nitzschia* blooms occurred (chapter 2). It is known that ammonium (as NH_4^+) is preferred to NO_3^- by some algae as a N source (e.g. Dugdale & Goering 1967). This preference for NH_4^+ uptake is attributed to its more reduced state (Levasseur et al. 1993) and the implication of a higher energy cost for growth on NO_3^- as it must be reduced prior to use (Syrett 1981).

Smith et al. (1990a) observed that blooms of *P. multiseriis* in the field are closely associated with NO_3^- pulses. Hence, NO_3^- and other inorganic N sources probably play an important role in controlling growth and DA production in *Pseudo-nitzschia* in the field and laboratory. To better understand its cell physiology, it is important to investigate which nitrogenous nutrient conditions favour toxic *Pseudo-nitzschia* growth and DA production (Bates et al. 1993). Previous experiments have been undertaken on *P. multiseriis* (Bates et al. 1993, as described in chapter 1). Although in Scottish waters *P. seriata* and *P. australis* seem to be the main toxin producing species (chapter 3), little is known about their N source preferences. As the *P. seriata* strain PLY1St.16B was isolated at a time when the NO_3^- concentration in the water was zero, its ability to grow on NH_4^+ as a N source was tested by conducting an experiment, comparing growth rates in media based on NO_3^- , NH_4^+ and a mix of both.

4.1.4 Experiment C: *Pseudo-nitzschia* growth under different L:D cycles

The phytoplankton community composition is influenced by light (Hobson & McQuoid 1997). Light influences the phytoplankton in particular due to seasonal variation in irradiance and day length, depending on the latitude. The daily light:dark (L:D) cycle is one of the strongest and most predictable of all environmental parameters affecting phytoplankton. It is thought to affect the content of algal pigments, growth rate and dark

respiration rate (Gilstad et al. 1993). However, the growth response to day length seems to be species dependent (Brand & Guillard 1981), and depending on the environmental conditions they are exposed to, phytoplankton may have evolved adaptations to the daily fluctuation of light. While oceanic phytoplankton may mainly be affected by daily and seasonal changes in irradiance levels and light availability, the light regime for coastal species can also change due to mixing caused by tidal and bottom topography induced turbulences and is therefore less stable. Light specific adaptations are for example found in some oceanic phytoplankton, of which some species require a L:D cycle for asexual reproduction, while many coastal species were found to divide more rapidly when exposed to long photoperiods or to continuous light (Brand & Guillard 1981).

L:D cycles are also known to influence sexual reproduction, for example affecting the auxosporulation and auxospore length, in some pennate diatoms (see Hiltz et al. 2000 and references therein). Many pennate diatoms require a particular light cycle, and in some species continuous light can inhibit sexual reproduction (e.g. *Nitzschia lanceolata*). In a study on sexual reproduction in *P. multiseriis*, a L:D cycle of 10:14 h, corresponding in nature to the day length of autumn, was shown to yield the highest number of initial cells per female gamete (Hiltz et al. 2000).

The L:D cycle impacts DA production by influencing the cell cycle, with increasing DA production during the light phase for *P. multiseriis* (Bates et al. 1991). This is related to the cell division cycle. During light periods cells are in the G1 phase of the cell cycle, a phase of active Si uptake. This then promotes DNA synthesis in the G2 phase. The DA production seems to be highest in the G1 phase. It is assumed that, in Si limited conditions, cells in stationary phase, are arrested in their G1 phase, unable to proceed to the G2 phase (Chisholm & Brand 1981; Brzezinski 1992). As cells continue to

photosynthesis, the excess energy that cannot be used for DNA synthesis, may be used for DA production (see Bates et al. 1991).

Studies on the influence of light on growth and/or DA production of *Pseudo-nitzschia* species have mainly been conducted under 12:12 h L:D cycles or continuous light with changing light intensities (e.g. Miller & Kamykowski 1986a, b; Pan et al. 1991; Lewis et al. 1993; Pan et al. 1996a, b, c, d). Laboratory studies investigating the effect of varying duration of the L:D cycles on growth in *Pseudo-nitzschia* species have not previously been conducted.

While light cycles clearly influence DA production in *P. multiseriis*, irradiance levels do not seem to affect it much. When *P. multiseriis* was grown at different temperatures and two different photonflux densities (80 and 180 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) (Lewis et al. 1993), no significant differences in toxin production were measured, except from cultures grown at the highest irradiance and temperature, that produced higher DA concentrations. However, in that case it was impossible to distinguish between the impacts on DA production of each single factor.

When a strain of *P. australis* was grown under a very low (12 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) and a moderate (115 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) irradiance at different light cycles (16:8 and 12:12 h L:D, respectively), DA was not produced for 40 days and then only in trace amounts under the low irradiance/long-light treatment (Cusack et al. 2002). In the other light conditions DA production started during late exponential phase and reached maximal 26 $\text{pg DA} \cdot \text{cell}^{-1}$ during stationary phase. However, that study did not resolve if the irradiance, light cycle or both had the main impact on DA production.

From the phytoplankton monitoring programme (chapter 2) it was obvious that toxic and non-toxic *Pseudo-nitzschia* species occurred in Scottish waters during different seasons of the year. One of the main factors determining the seasons in Scottish waters is the duration of light availability. The field observations from the monitoring site LY1

(chapter 2) suggested a preference of *P. delicatissima* for spring conditions while *P. seriata* showed highest cell densities during the summer months. While changes in the light irradiance were not measured during the phytoplankton monitoring and are not known for the local Scottish waters, day length is known for the location where the *Pseudo-nitzschia* cultures were isolated. In February 2002, when *P. delicatissima* strain PLY1St.42A was isolated, the day length at approximately the latitude of LY1 (56°N) was 9 h. While in June 2001, at times of isolation of *P. seriata* strain PLY1St.16B, daylight was approximately 18 h. To investigate the effect of day length on the two *Pseudo-nitzschia* species, a growth study was conducted. *P. delicatissima* and *P. seriata* were grown in laboratory batch cultures under spring and summer simulating L:D cycles, a constant temperature and irradiance.

As the *P. delicatissima* strain (PLY1St.42A) was not toxic (chapter 3), DA concentrations were measured only in *P. seriata* cultures.

4.1.5 Experiment D: *P. seriata* - bacteria interactions

Bacteria-algal interactions can be highly variable in natural plankton communities. It is known that bacteria and phytoplankton interact in several ways (as referenced in Grossart 1999), with bacteria having been found to live attached to diatoms (e.g. Bell et al. 1974; Cole 1982; Rosowski 1992). One of the advantages for the bacteria in being closely associated with a diatom cell is that the algae surface might provide nutrients that are absent or deficient in the surrounding fluid (Sutherland 1983). The protoplast of the diatom might secrete an array of substances influencing the activity of the bacteria in positive and/or negative ways (see Rosowski 1992). In this kind of symbiosis bacteria utilise phytoplankton exudates while the algae benefit from bacterial nutrient remineralisation, vitamins or growth factors. However, bacteria may compete with the algae for nutrients, and under certain environmental conditions they can inhibit algal

growth (Grossart 1999). Bacteria can be comensalic, by benefiting from the algae without affecting them, or live parasitically on phytoplankton and lead to their cell lysis and death. In general, bacterial-microalgal interactions can be highly variable.

As reported in the general introduction (chapter 1), most experiments on *Pseudo-nitzschia* have been undertaken with xenic cultures, as were experiments A-C in this chapter. While it has been shown that bacteria may enhance DA production in toxic *Pseudo-nitzschia* species (Bates et al. 1995), the effect of bacteria on growth of single *Pseudo-nitzschia* cells had not been studied previously.

To test the hypothesis that *P. seriata* associated bacteria affect the growth of the diatoms, growth of an initially single cell in each petri dish was monitored in presence and absence of bacteria and/or bacterial exudates and some bacterial strains associated with *P. seriata* were isolated and identified.

4.2 Material and Methods

4.2.1 General methods

4.2.1.1 Culture strains used for laboratory experiments

All experiments used *P. seriata* strain PLY1St.16B. The L:D experiment was additionally conducted on *P. delicatissima* strain PLY1St.42A. Both xenic, clonal cultures were initially isolated from surface waters (0-20 m), collected using 20 µm mesh plankton net samples from LY1 (chapter 2). Strain PLY1St.16B was isolated on 22 June 2001 and PLY1St.42A on 4 February 2002.

Cultures were isolated and maintained as described in chapter 3 and identified with morphological and molecular methods.

Chapter 4

4.2.1.2 Cell counts

Cells counts were conducted with a 1 ml Sedgewick-Rafter counting chamber using a Zeiss Axiovert S100 microscope on 100 x magnification. Maximum cell number or chamber area counted are detailed in the individual results sections. Growth rates were calculated by determining the maximum coefficient (r^2) achievable when fitting straight lines to semi-logarithmic plots of cell density. In experiment A, growth rates were calculated using C equivalent biomass. A general linear model (GLM) was used for statistical comparison of growth rates between different treatments.

4.2.1.3 Nutrient concentrations

Samples for the determination of inorganic nutrient concentrations (silicate, phosphate nitrate in experiment A and additionally ammonium, in experiments B and C), were collected by filtration through A/E glass fibre filters and stored frozen at -20°C prior to analysis. Two pseudo replicates were analysed with a LCHAT Quick Chem 8000 autoanalyser by T. Brand (SAMS), using flow injection after standard autoanalyser methods.

4.2.1.4 Domoic acid analysis

Domoic acid concentrations were determined (experiments A and C) using a high performance liquid chromatography (HPLC) of the fluorenylmethoxycarbonyl derivative by S. Bates and C. Léger (Fisheries and Oceans Canada) (Pocklington et al. 1990). Total DA was measured in whole culture samples (cells plus medium) (Bates et al. 1989). Samples were first sonicated for 1 min to disrupt the cells, filtered through a 0.2 µm disposable acrodisc (25 mm surfactant-free cellulose acetate membrane, Nalgene) to remove cell debris and frozen at -20°C prior to analysis. For extracellular

DA (DA that was released by the cells), cell-free medium samples (from the filtrate obtained from inorganic nutrient samples) were frozen at -20°C prior to analysis. Cellular DA concentrations were computed from the total cell number and the difference in DA concentration between the whole culture and the cell-free medium (Bates 1998; Kotaki et al. 2000). Total produced DA per cell was calculated by dividing the DA content of the whole culture sample by the cell density.

4.2.2 Experiment A: Growth and domoic acid production of *P. seriata* under Si and P - limitation

P. seriata was grown in duplicate 10 litre polycarbonate carboys containing autoclaved, filtered (GF/F Whatman) seawater continuously aerated with $0.2\ \mu\text{m}$ filtered air, at 15°C under a light intensity of $120\ \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (12:12 h L:D cycle). The seawater was enriched with F/2 nutrients (Guillard 1975), modified to achieve two different initial phosphate (P) and silicate (Si) concentrations, such that the cells would experience P- or Si-yield limitation, respectively (Tab. 4.2, section 4.3.1.1). Nitrate, vitamins and trace elements were present in excess, at F/2 concentrations. To minimise toxin carryover into the experiment, cells used for the inoculum were taken from early exponential-phase stock cultures (e.g. Douglas & Bates, 1992), grown in F/2 medium plus $107\ \mu\text{M}$ Si (Guillard 1975).

Carboys were sampled for 21 days. Following gentle shaking to disperse the cells, aliquots were collected by siphon, daily for the first 15 days and thereafter every second day. At the end of the experiment, each carboy still contained at least half of the initial volume of medium. All results are reported as the mean of duplicate carboys.

For cell counts, whole-culture samples were fixed with glutaraldehyde (2.5% v/v final concentration) and counted in at least triplicate (100-1500 diatom cells per count).

Cells for chl *a* analysis were filtered under low vacuum onto 25 mm glass-fibre filters (type A/E, Pall Corporation) and stored frozen at -20°C. Once thawed, pigments were extracted in the dark at 4°C overnight into 8 mL of 90% acetone. Filters were sonicated and after centrifugation chl *a* was measured with a Turner TD-700 fluorometer.

Cells for particulate C and N determinations were collected under low vacuum onto pre-ashed 13 mm A/E glass-fibre filters (Pall Gelman Laboratory) and stored frozen at -20°C. Once thawed, they were air dried at 60°C for 4 h and analysed (by K. Davidson) using a 20-20 ANCA GSL mass spectrometer (PDZ Europa), calibrated with isoleucine. Inorganic nutrient and domoic acid concentrations were determined as described in sections 4.2.1.3 and 4.2.1.4.

4.2.3 Experiment B: *P. seriata* growth on different N sources

P. seriata strain PLY1ST.16B, isolated and maintained as described above (section 4.2.1.1), was used as the stock culture. At inoculum 20 ml of the stock culture were added to three types (Treatments A, B and C) of modified in terms of N, silicate-enriched F/2 medium (Guillard, 1975), two replicate flasks of 250 ml were used for each treatment. The three treatments were based on media composed of different nitrogen sources (Tab. 4.1). The cultures were grown for 15 days, 2 ml samples for cell counts were taken on day zero (day of the inoculum) and days two, five, eight, twelve and 15 and fixed with Lugol's Iodine, 1% final concentration. For cell counts of each replicate at least 100 cells or ten fields were counted per sample.

Table 4.1 Initial NO₃⁻ and NH₄⁺ concentrations in Treatments A, B and C.

Treatment	NO ₃ ⁻ [μM]	NH ₄ ⁺ [μM]
A	100	-
B	-	100
C	50	50

4.2.4 Experiment C: *Pseudo-nitzschia* growth under different L:D cycles

4.2.4.1 Experimental cultures

This experiment used both *P. seriata* and *P. delicatissima*. To precondition the cells to the experimental conditions, 17 days prior to the start of the experiment, batch culture replicates of *P. seriata* and *P. delicatissima* (one replicate of each species) were transferred to 2 incubators that were set to a temperature of 10°C, approximately 100 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ light and L:D cycles of 18:6 h (treatment hereafter called L, for "long light") and 9:15 h (treatment hereafter called S, for "short light"), representing spring and summer day lengths. The temperature of 10°C was chosen as a compromise between surface temperatures that occurred at times of isolation of *P. delicatissima* (PLY1St.42A) and *P. seriata* (PLY1St.16B) (7.5°C and 10.8°C, respectively). To inoculate the experiment with exponential phase culture, the replicate cultures from both incubators were subsampled 5 days prior to inoculation to become the inoculi cultures for the experimental cultures (*P. delicatissima* L, *P. delicatissima* S, *P. seriata* L and *P. seriata* S).

4.2.4.2 Experiment inoculation

To achieve similar initial cell numbers, preconditioned medium was used to dilute the inoculum cultures of both species. The preconditioned medium consisted of the either long or short light cycle adapted inoculum culture, filtered through a 0.2 μm acrodisc to exclude algae and bacteria. For inoculation the cells were then added in 50 ml volumes to Erlenmeyer flasks containing 500 ml F/2 +Si medium (Guillard, 1975) (see Table 4.3, section 4.3.3.1). Two replicate flasks were studied for each species in each of the two L:D cycles (8 flasks total).

4.2.4.3 Cell counts.

For cell counts, 3 ml subsamples were taken aseptically from each replicate flask every day from day 0 to 20 and then every second day until day 30. Cells were preserved with Lugol's Iodine solution (~ 1% final concentration) and counted in duplicate. Some samples had to be diluted (1/4, 1/2, 1/10 or 1/20) with sterile medium prior to the counts. At least 50 fields or 300 cells were counted per replicate. Additionally to the counts, cells were sized at the end of the experiment. The length and widths of 10 cells from each species was measured to approximate the volume based on a rectangular box ($V = \text{length} \cdot \text{width}^2$). From those volume measurements carbon per cell was determined for both species applying the following formula for diatoms: $\text{pg C} \cdot \text{cell}^{-1} = 0.288 \cdot \text{volume}^{0.811}$ (Menden-Deuer & Lessard, 2000).

4.2.4.4 Nutrients

As the replicate experiments received identical nutrients as inoculum and the experiment did not seek to study nutrient utilisation as such, time course measurements of inorganic nutrient concentrations were not made. Rather, samples for inorganic nutrient analysis were taken on days 19 and 30. Only concentrations of inorganic nitrate, phosphate, silicate and ammonium were determined.

4.2.4.5 Domoic acid

On day 30 the *P. seriata* cultures were tested for DA production.

4.2.5 Experiment D: *P. seriata* - bacteria interactions

Single *Pseudo-nitzschia seriata* cells were isolated using a micropipette from culture strain PLY1St16B. After washing them in sterile medium, a single cell in a small

volume of medium (~ 5 μL) was placed into fifteen 5.5 cm diameter petri-dishes containing 10 ml sterile silicate enriched F/2 medium (Guillard 1975).

The first set of five petri-dishes (Treatment 1) functioned as the control, no bacteria or culture filtrate were added. The next five petri-dishes were supplemented with 850 μl of culture PLY1St.16B, filtered through a 0.2 μm acrodisc (Treatment 2), excluding algal cells and bacteria, but containing bacterial and algal exudates. To exclude additional *Pseudo-nitzschia* cells, but to add associated bacteria, 650 μl of 3 μm filtrate of the same culture were added to the next five petri-dishes (Treatment 3). The dishes were incubated at 15°C, a light photon flux rate of ca. 100 $\mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and a 12:12 h L:D cycle.

Over twelve days (on days 0, 1, 3, 4, 6, 8, 10, 12) the algae in the petri-dishes were enumerated by observation under an inverted Zeiss Axiovert S100 microscope and 100 x magnification. Division rates were calculated when exponential growth was observed (between days four and ten; four and twelve in two cases) for each petri-dish in which algal growth was observed.

After twelve days bacteria from petri-dishes, in which bacteria were observed and/or in which algal cells had successfully divided, were grown for two weeks on marine Agar. Single colonies were then isolated and grown for a further three weeks. They were then passed through purity, transferred into liquid broth and grown in a 25°C incubator (Gallenkamp cooled orbital incubator) at 180 $\text{rev} \cdot \text{min}^{-1}$ for six days. Samples were frozen in liquid nitrogen and stored at -80°C prior to further analysis.

Bacterial genomic DNA was extracted from defrosted, re-grown bacteria using a method based on cetyltrimethylammonium bromide purification (CTAB) (Ausubel et al. 1999). The small subunit rDNA (SSU rDNA) gene from chromosomal DNA was amplified in a polymerase chain reaction (PCR) using the primer pair 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (ACGGCTACCTTGTTACGACTT)

(Weisburg et al. 1991). PCR was carried out on a MJ Research PTC200 DNA Engine thermocycler and used 1 U of *Taq* polymerase (ABgene, UK) in a 50 µl reaction containing a final concentration of 1.8 mM Mg²⁺, 20 mM NH₄SO₄, 75 mM Tris-HCl (pH 8.8) and 0.01% Tween 20. Cycling parameters were as follows: 94°C for 2 min, 26 cycles of 55°C for 30 s, 72°C for 2.5 min, and 94°C for 10 s, followed by 72°C for 10 min. The PCR products were purified through Centricon-PCR Ultrafilters (Millipore, UK) according to the manufacturers instructions and sequenced in the forward direction using the 27F primer and ABI-PRISM “Big-Dye” terminator chemistry (Applied Biosystems, USA) according to the standard protocols. Sequence reactions were electrophoresed on an ABI 377 DNA sequencer (Applied Biosystems), and resulting sequences aligned and manually checked for consistent base-calling, using Sequence Navigator (Version 1.0.1, Applied Biosystems, USA). Three bacterial strains were isolated and their SSU was sequenced. Their SSU sequences were compared with the empro database in a FASTA (Pearson 1990) search using fasta3 for strain identification.

4.3 Results

4.3.1 Experiment A: Si- and P limitation

4.3.1.1 Inorganic nutrients

Inorganic nutrient concentrations in the medium on day zero are presented in Table 4.2. As N is required for DA production by *Pseudo-nitzschia* (Bates 1998), dissolved inorganic nitrogen (DIN) was added in excess to all cultures to prevent N-limitation of growth rate or biomass yield. DIN concentrations always remained > 630 µM throughout the experiments (data not shown), resulting in N:P and N:Si ratios at least an order of magnitude greater than those which would result in N-yield limitation (Redfield 1963). The dissolved inorganic phosphate (DIP) and dissolved silicate (DSi)

concentrations at inoculation (day zero) gave DIP:DSi ratios of 1:37 and 1:7, hence generating conditions that would be expected to result in P- and Si-yield limitation, respectively. This was confirmed by the pattern of uptake of DIP and DSi, as described below.

In P-limited cultures, the DIP concentration on day zero was $\sim 3 \mu\text{M}$ (Fig. 4.1A), and decreased to a threshold concentration of $\sim 1 \mu\text{M}$ on day five. The DSi concentration (Fig. 4.1B) decreased to $\sim 62 \mu\text{M}$ on day six; the cessation in decrease of DSi coincided with the exhaustion (threshold concentration) of DIP. In Si-limited cultures, the initial DSi concentration decreased to exhaustion on day nine. The initial DIP concentration of $\sim 15 \mu\text{M}$ decreased to a threshold of $\sim 5 \mu\text{M}$ on day nine, coinciding with the exhaustion of DSi.

Table 4.2 Measured nutrient concentrations in treatments A and B on day zero (immediately after inoculation), values are means of duplicate carboys.

Treatment	DSi [μM]	DIP [μM]	DIN [μM]
A: P limitation	103.0	2.8	759.9
B: Si limitation	103.5	14.5	973.3

4.3.1.2 Cell numbers

Cell densities at inoculation were $\sim 2 \times 10^3 \text{ cells} \cdot \text{mL}^{-1}$ in all cultures (Fig. 4.1C). The duration of the exponential phase was estimated by determining the maximum coefficient of determination (r^2) achievable when fitting straight lines to semi-logarithmic plots of cell density. Exponential growth was evident from days one to five for all cultures, with mean specific growth rates of 0.55 d^{-1} and 0.58 d^{-1} for P-limited and Si-limited cultures, respectively. The cessation of exponential cell division in P-limited cultures coincided with the reduction of DIP to its low threshold concentration on day five, when a mean cell density of $2.24 \times 10^4 \text{ cells} \cdot \text{mL}^{-1}$ was achieved.

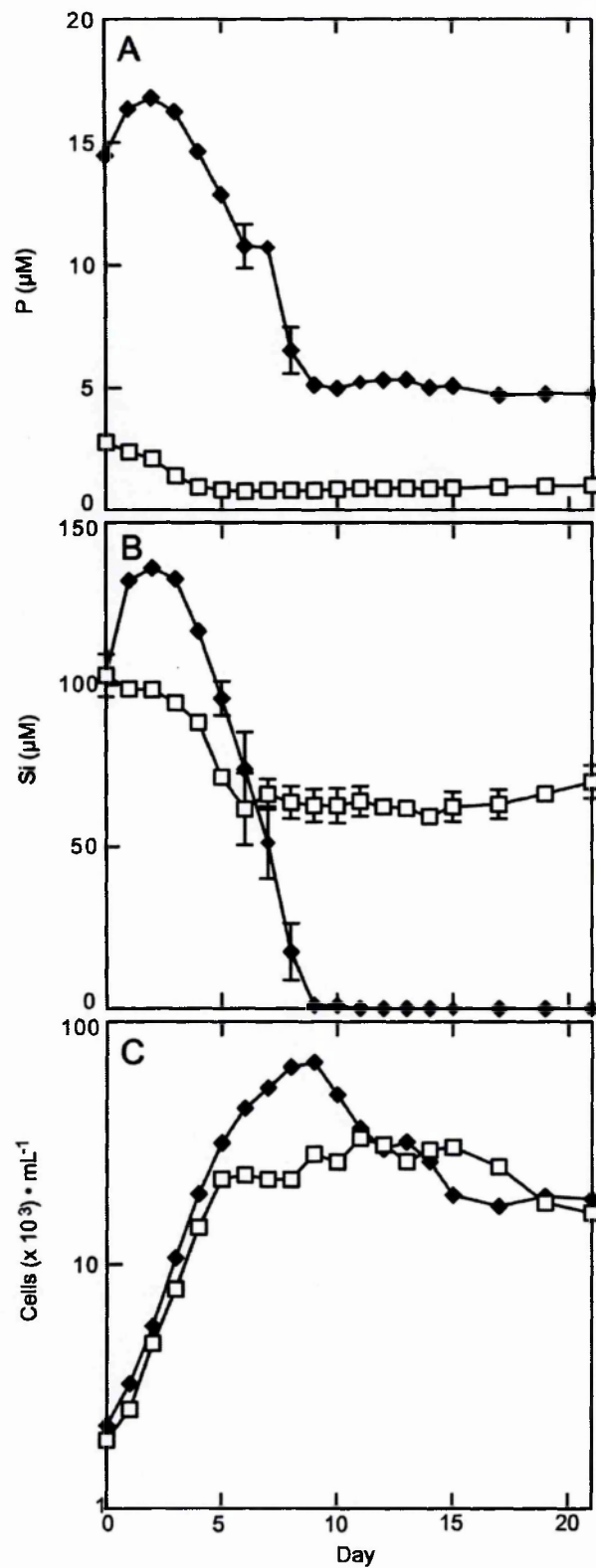


Fig. 4.1 Dissolved inorganic P (A) and Si (B) concentrations and cell number (C) in *P. seriata* batch cultures. P-limited cultures (squares); Si-limited cultures (diamonds). All results are means of duplicate carboys. Error bars represent the standard error.

A short stationary phase then followed, until day eight, after which there was a slow increase in cell density up to 3.04×10^4 cells \cdot mL⁻¹ on day 15, and then a decline until

the end of the experiment. The exponential phase was characterised by cells full of cytoplasm. Subsequent to nutrient exhaustion, many cells showed the typical features of nutrient-stressed diatoms, such as larger vacuole areas (e.g. Davidson et al. 2002). Those cells completely devoid of cytoplasm, and hence present as empty frustules, were considered dead and not included in the counts.

In Si-limited cultures, the rapid period of exponential cell division ceased on day five, even though the DSi concentration still remained high ($> 95 \mu\text{M}$). A period of slower exponential cell division (0.24 d^{-1}) then followed, until day eight. A maximum cell density of $6.79 \times 10^4 \text{ cells} \cdot \text{mL}^{-1}$ was then achieved on day nine, coincident with the exhaustion of DSi (Fig. 4.1C). No classical stationary phase was observed, except for possibly between days eight and nine; cell density thereafter rapidly declined. From day 17 onward, cells ceased to decline and some “fresh viable cells” were observed in the cultures. These cells were easily distinguished from the more numerous “dead” cells under the microscope, being full of cell plasma and lacking the large vacuole areas, which were evident in all other nutrient-stressed cells.

4.3.1.3 Cellular carbon and nitrogen

Particulate C showed growth dynamics qualitatively similar to that of cell numbers for all cultures (data not shown). However, the stationary phase indicated by cell numbers in P-limited cultures between days five and eight was not observed for $\text{C} \cdot \text{mL}^{-1}$; instead, particulate C gradually increased. Spearman Rank correlation analysis confirmed the generally good correspondence between the dynamics of $\text{cell} \cdot \text{mL}^{-1}$ and $\text{C} \cdot \text{mL}^{-1}$ throughout the experiment and in both sets of conditions ($r = 0.898$, $p = 0.000$ for P-limited cultures, and $r = 0.554$, $p = 0.019$ for Si-limited cultures). However, an important difference was evident from plots of $\text{C} \cdot \text{cell}^{-1}$ (Fig. 4.2A), which decreased in both conditions during fast exponential growth (days one to five), indicating lower

maximum cell-specific growth rates compared to the C-specific values, until the limiting nutrient was exhausted. Variation in $C \cdot \text{cell}^{-1}$ was also particularly marked in Si-limited cultures, especially after day 13, which reflects renewed positive net C fixation in those cells that remained viable.

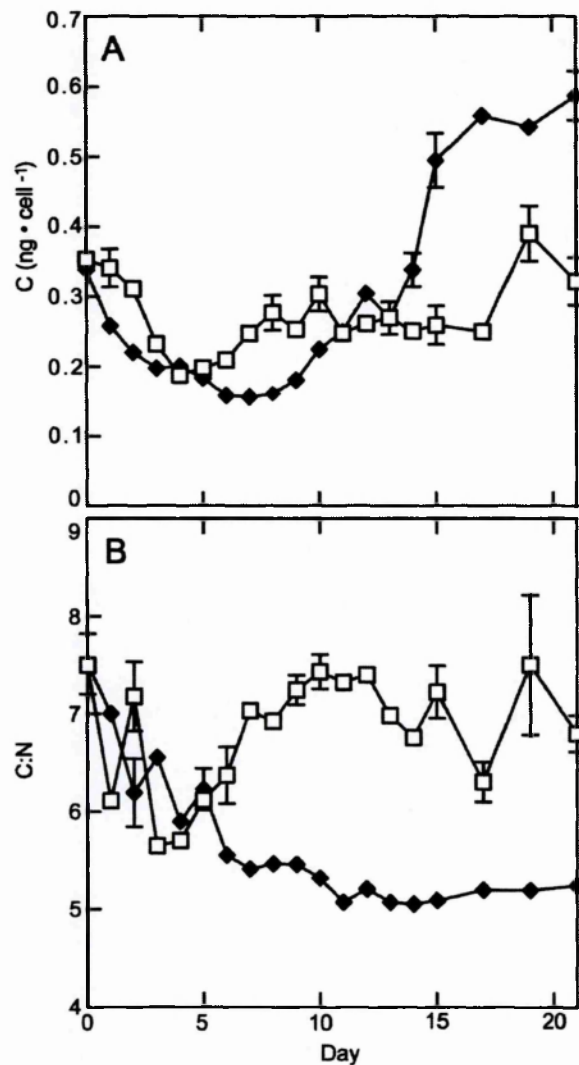


Fig. 4.2 C per cell (A) and C:N ratio (B) in *P. seriata* batch cultures. P-limited cultures (squares); Si-limited cultures (diamonds). All results are means of duplicate carboys. Error bars represent the standard error.

Cellular N exhibited generally similar dynamics to those of C (data not shown). However, in the post-exponential phase the C:N ratios gradually decreased and levelled off in Si-limited cultures, whereas they increased prior to levelling off in P-limited cultures (Fig. 4.2B).

4.3.1.4 Chlorophyll *a*

Chl *a* · mL⁻¹ correlated well with cell numbers ($r = 0.865$, $p = 0.000$ for P-limited cultures and $r = 0.950$, $p = 0.000$ for Si-limited cultures). Chl *a* · mL⁻¹ was also correlated with C · mL⁻¹ ($r = 0.588$, $p = 0.013$ for P-limited cultures and $r = 0.711$, $p = 0.003$ for Si-limited cultures). However, in contrast to C · cell⁻¹ in both conditions, chl *a* · cell⁻¹ increased during the exponential phase (Fig. 4.3A).

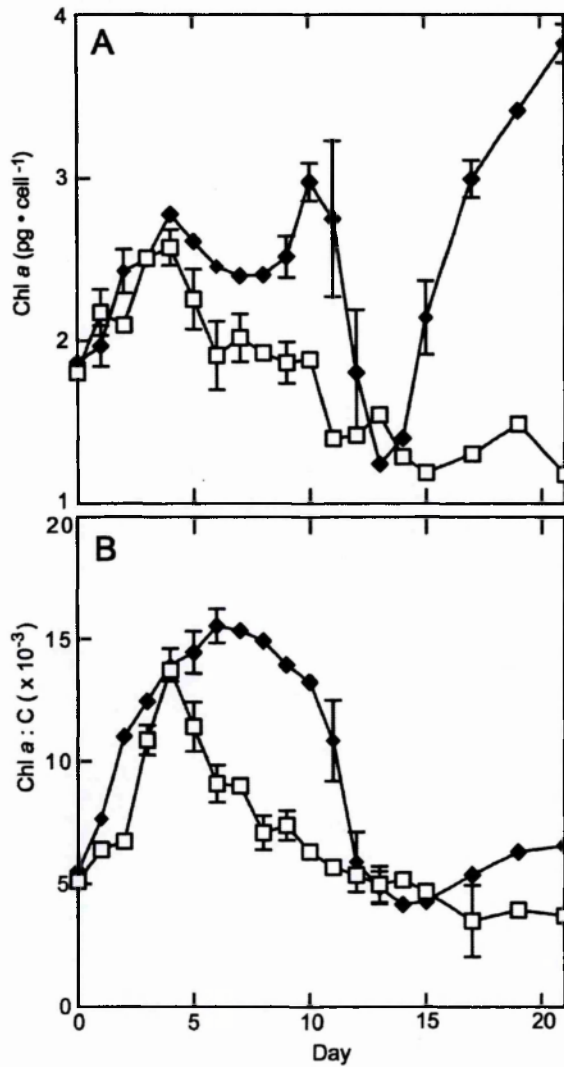


Fig. 4.3 Chl *a* per cell (A) and chl *a*:C ratio (B) in *P. seriata* batch cultures. P-limited cultures (squares); Si-limited cultures (diamonds). All results are means of duplicate carboys. Error bars represent the standard error.

Then, upon DIP reaching its threshold on day five in P-limited cultures, it decreased until the end of the experiment. In Si-limited cultures, chl *a* · cell⁻¹ decreased during

days five to nine, coincident with the period of slower exponential cell division. This was followed by a short increase and then, from days ten to day 13, chl *a* steeply declined in parallel with the decline in cell numbers.

Finally, chl *a* · cell⁻¹ increased rapidly until the end of the experiment, coincident with the appearance of some viable cells, reaching a higher cellular chl *a* level than during the exponential phase. A similar pattern was evident for the chl *a*:C ratio (Fig. 4.3B), although the smaller relative increase in chl *a*:C compared to chl *a* · cell⁻¹ near the end of the experiment for Si-limited cultures indicated a better coupling between chl *a* and C at this time.

4.3.1.5 Domoic acid

In P-limited cultures, a low concentration of DA (2 ng DA · mL⁻¹) was present in the whole culture (cells plus medium) on day zero (Fig. 4.4A), although no DA was detected in the medium. During the exponential phase, whole culture DA initially increased slowly to 7.8 ng DA · mL⁻¹ on day four, and then more rapidly to 28.4 ng DA · mL⁻¹ by day five. Thereafter, it increased approximately linearly during days five to ten, when DIP became reduced to its threshold concentration. The concentration of DA then further increased to a maximum of 108 ng DA · mL⁻¹ on day 19. Extracellular DA also increased from day four onwards, reaching a maximum of 68 ng DA · mL⁻¹ on day 21 (Fig. 4B). Total produced DA · cell⁻¹ (Fig. 4.4C) increased markedly near the end of the exponential phase (day five), until the end of the stationary phase, reaching 2.9 pg DA · cell⁻¹ on day eight. This was followed by a plateau, corresponding to a gradual increase in cell numbers. It then further increased, reaching maximum of 5.9 pg DA · cell⁻¹ on day 19, before decreasing slightly on day 21.

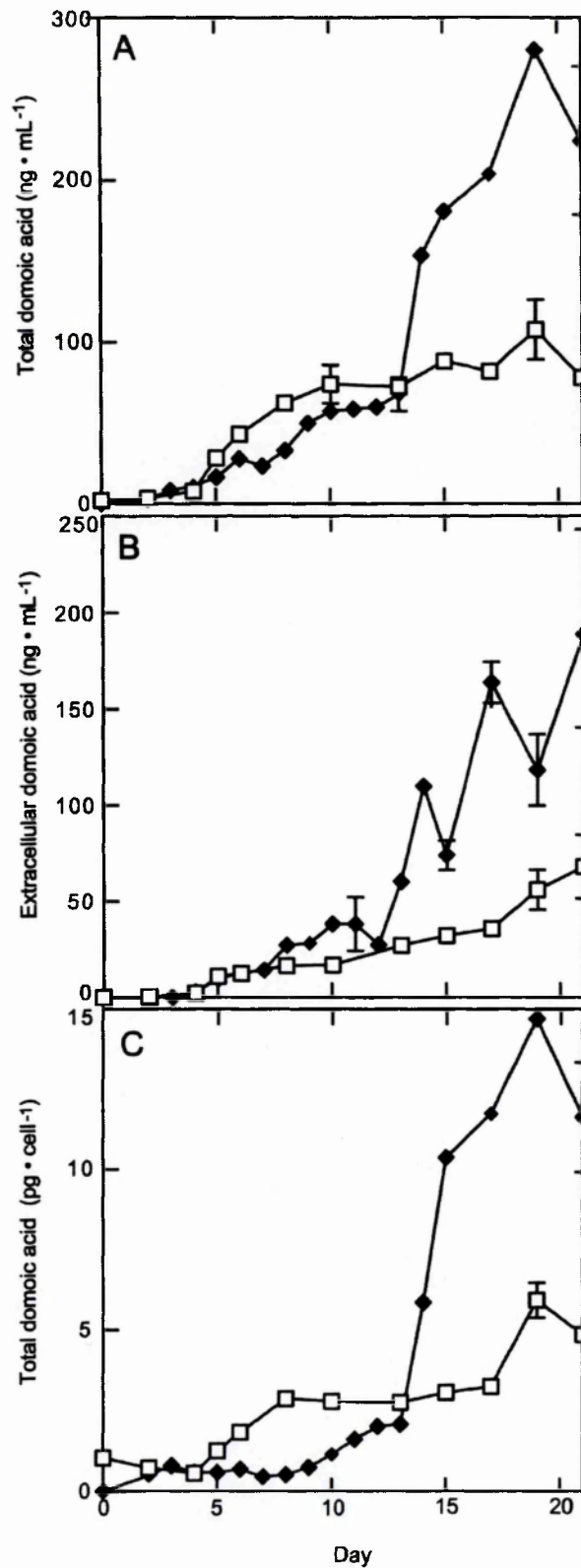


Fig. 4.4 Domoic acid concentration in the whole culture (A), medium (B) and total produced domoic acid per cell (C), expressed as whole culture domoic acid divided by cell number, in *P. seriata* batch cultures. P-limited cultures (squares); Si-limited cultures (diamonds). All results are means of duplicate carboys. Error bars represent the standard error.

In Si-limited cultures, no DA was detected on day zero (Fig. 4.4A). However, by day two, there was $\sim 3 \text{ ng DA} \cdot \text{mL}^{-1}$, which steadily increased to $16.5 \text{ ng DA} \cdot \text{mL}^{-1}$ on day

five, at the end of the fast exponential cell division phase. As in P-limited cultures, no extracellular DA was detected until near the end of the exponential phase (day four) (Fig. 4.4B), when cell growth began to slow. The linear increase in whole-culture DA concentration continued until day 13, when it reached $67.6 \text{ ng DA} \cdot \text{mL}^{-1}$ (Fig 4A).

Thereafter, it rapidly increased, reaching $280 \text{ ng DA} \cdot \text{mL}^{-1}$ on day 19, prior to a slight decline in concentration on the last day. This DA was both intra- and extracellular. Hence, whereas P-limited cells did not release DA into the medium prior to DIP being reduced to a threshold, considerable amounts of DA (up to $190 \text{ ng} \cdot \text{mL}^{-1}$, or 84% of the whole culture DA value) were detected in the medium in Si-limited cells by day 21 (Fig. 4.4B). Total DA per cell showed a similar pattern (Fig. 4.4C), with slowly increasing values during the exponential phase (to $0.6 \text{ pg} \cdot \text{cell}^{-1}$ on day five) and markedly increasing values from day 13, onward (to $14.7 \text{ pg} \cdot \text{cell}^{-1}$ on day 19).

Between days five to ten, whole-culture and total cellular produced DA were slightly greater under P-limiting conditions. However, thereafter more DA was produced under Si-limiting than P-limiting conditions, whether expressed per mL (Fig. 4.4A), per cell (Fig. 4.4C) or per unit carbon (not shown).

4.3.2 Experiment B: *P. seriata* growth on different N sources

Growth of *P. seriata* was evident in all treatments. A two days lag phase was observed in the NH_4^+ treatments before exponential growth commenced, this was not evident in the other treatments. In NO_3^- and NO_3^- - NH_4^+ -mixed treatments, cells divided exponentially until day eight, while the NH_4^+ culture entered the stationary growth phase on day twelve. Highest cell yield were $3.9 \times 10^3 \text{ cells} \cdot \text{mL}^{-1}$ in NO_3^- (day twelve), $3.4 \times 10^3 \text{ cells mL}^{-1}$ in NH_4^+ (day fifteen) and $4.0 \times 10^3 \text{ cells} \cdot \text{mL}^{-1}$ in NO_3^- - NH_4^+ -mix cultures (day twelve). The corresponding growth rates were 0.287 (NO_3^- -based, $r^2=0.98$, days 0-8), 0.238 (NH_4^+ -based, $r^2=0.99$, days 2-8) and 0.280 ($\text{NO}_3^-/\text{NH}_4^+$ -based,

$r^2=0.99$, days 0-8). No significant differences were found between the exponential phase growth rates observed in the different treatments (GLM, $p > 0.05$). Semi-logarithmic plots (Fig. 4.5) of cell density illustrate the similarity of both, the magnitude and duration of growth phases in all treatments. This is further illustrated in Fig. 4.5B by removing the lag phase of the NH_4^+ based cultures and plotting the growth curve again with this two days shift.

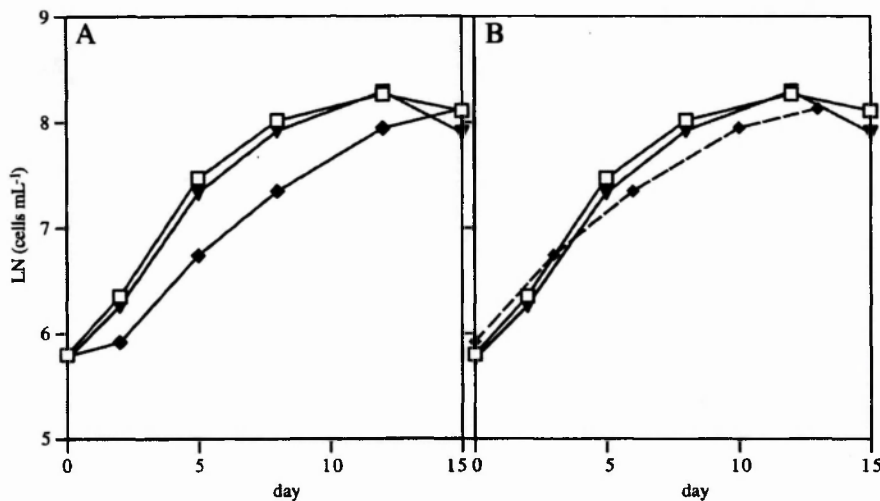


Fig. 4.5 Cell density of *P. seriata* in LN cell numbers. A: NO_3^- -treatment (squares), NH_4^+ -treatment (diamonds), NO_3^- - NH_4^+ -mix treatment (triangles). B: same symbols for treatments as in (A), for NH_4^+ -treatment (diamonds, broken line) cell density is plotted with a two days shift.

4.3.3 Experiment C: *Pseudo-nitzschia* growth under different L:D cycles

4.3.3.1 Inoculation of experiment cultures

Cell densities and volumes used for the inoculation are presented in Table 4.3. Apart from the short light *P. seriata* culture, all other cultures had to be diluted with preconditioned medium prior to inoculation, to achieve similar cell densities in cultures at the start of the experiment.

Table 4.3: Cell numbers in inoculum cultures and volumes of cultures and preconditioned medium (0.2 μm filtered inoculum culture) added to inoculate the replicate cultures. S= 9:15 h L:D cycle; L= 18:6 h L:D cycle.

Treatment Light duration	Species	cells ($\times 10^3 \text{ mL}^{-1}$) in inoculum culture	vol. of inoculum culture [ml]	vol. of preconditioned medium [ml]
S	<i>P. delicatissima</i>	427	2	48
L	<i>P. delicatissima</i>	808	1	49
S	<i>P. seriata</i>	19.7	50	-
L	<i>P. seriata</i>	37.6	25	25

4.3.3.2 Cell numbers

Inoculum cell density in all cultures was ~ 1.2 to $1.6 \times 10^3 \text{ cells} \cdot \text{mL}^{-1}$. Cultures exhibited the classical "logistic" growth with an exponential and stationary growth phase. A lag phase was not apparent (Fig. 4.6). In both cases the short light treatment exhibited a longer exponential phase ceasing on days 12 (*P. delicatissima*) and 9 (*P. seriata*). Under long light conditions exponential phases lasted for 7 days.

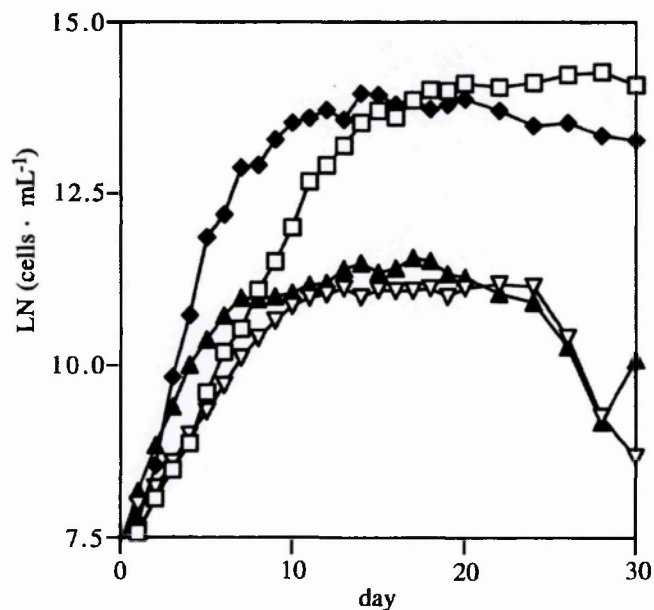


Fig. 4.6 Cell density of *P. delicatissima* (open squares: short light, diamonds: long light conditions) and *P. seriata* (open triangles: short light, closed triangles: long light conditions).

Chapter 4

The highest cell yield with a maximum cell density of 1.6×10^6 cells \cdot mL⁻¹ and an equivalent biomass of 25.5 μ g C \cdot mL⁻¹ was found in *P. delicatissima*, grown under short light after 28 days of growth (Tab. 4.4). *P. seriata* gained higher maximal cell numbers and biomass in long rather than short light. However, maximal yields in *P. seriata* (cell numbers and biomass) were substantially lower than in any treatment of *P. delicatissima*.

4.3.3.3 Maximal growth rates

During exponential growth, both species had higher division rates when grown under long light, than when exposed to short light, with a maximal rate of 0.89 div \cdot d⁻¹ for *P. delicatissima* L (Tab. 4.4). The growth rate of *P. delicatissima* L was about twice as high as that of *P. delicatissima* S (0.89 cf. 0.49 div \cdot d⁻¹). *P. delicatissima* S, and *P. seriata* L, had similar maximal growth rates (around 0.5 div \cdot d⁻¹). The growth rates differed significantly between species and treatments in all cases (GLM $p < 0.05$).

Table 4.4 Max. cell numbers, max. biomass, and max. growth rates in *P. delicatissima* and *P. seriata* grown under short (S) and long (L) light conditions.

Treatment	Species	Day	max. cell density [cells \cdot mL ⁻¹]	max. biomass [μ g C \cdot mL ⁻¹],	max. growth rate [div \cdot d ⁻¹]
S	<i>P. delicatissima</i>	28	1.6×10^6	25.5	0.49
L	<i>P. delicatissima</i>	14	1.1×10^6	18.5	0.89
S	<i>P. seriata</i>	22	7.1×10^4	8.1	0.36
L	<i>P. seriata</i>	17	10.4×10^4	12.0	0.51

4.3.3.4 Nutrient concentrations

Based on known stock concentrations inoculum nutrient concentrations should have been 883 μ M N, 36.6 μ M P and 107 μ M Si.

Inorganic macronutrient concentrations (ammonium, phosphate, and silicate) in the growth medium from day 19 and 30 are presented as average concentrations of both

replicates for each culture (Fig. 4.7 and Fig. 4.8). For measured concentrations that were below detection limit in one of the two replicate cultures, only the result above detection limit is given.

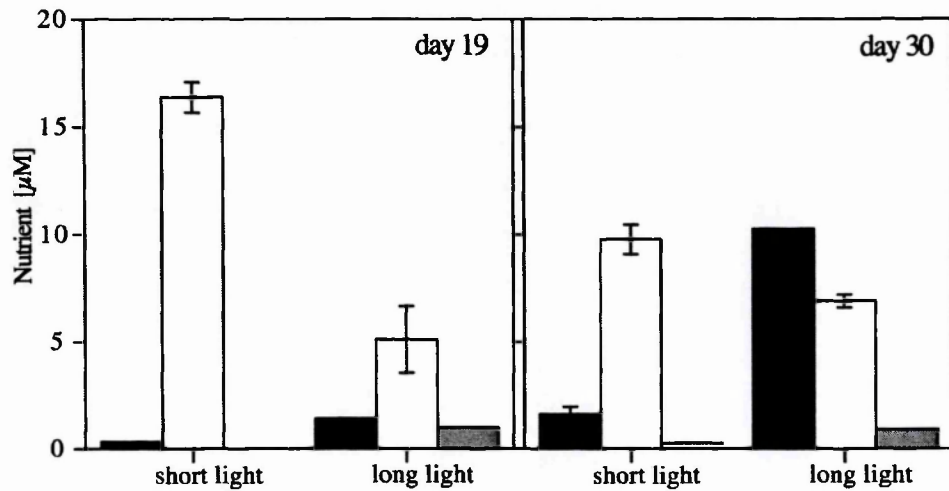


Fig. 4.7 Ammonium (black), phosphate (white) and silicate (grey) concentrations in *P. delicatissima* short and long light treatments on days 19 and 30.

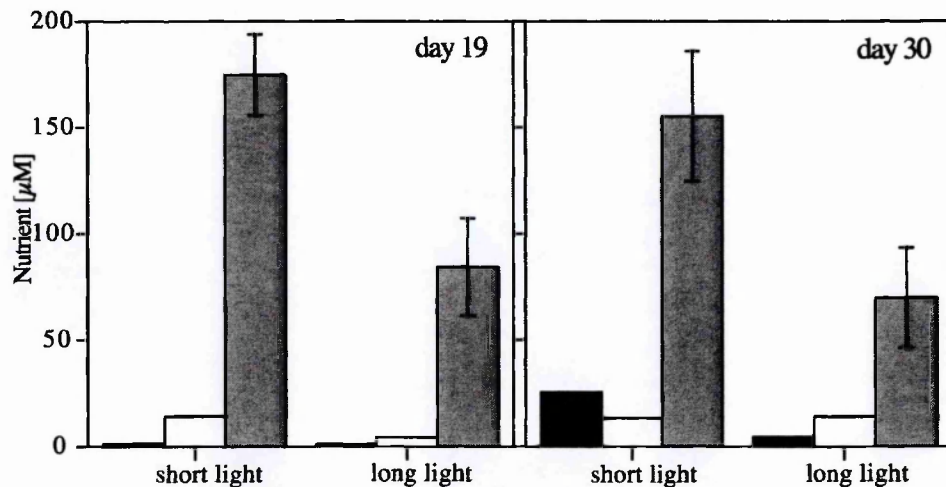


Fig. 4.8 Ammonium (black), phosphate (white) and silicate (grey) concentrations in *P. seriata* short and long light treatments on days 19 and 30.

4.3.3.5 Ammonium

For both species the concentration of ammonium increased from day 19 to day 30 in L and S treatments. Concentrations were higher in long light compared to short light treatments. For both light periods highest concentrations were found in *P. seriata* cultures.

4.3.3.6 Phosphate.

In S treatments, for both species, the inorganic phosphate concentration (P) decreased between day 19 and 30, while it increased in L treatments. While the decrease in P (from day 19 to day 30) was small for *P. seriata*, it was more marked for *P. delicatissima*. The increase in P between day 19 and 30 in treatments L was greater in *P. seriata* than in *P. delicatissima* cultures.

4.3.3.7 Silicate

On days 19 and 30 silicate (Si) was depleted in *P. delicatissima* cultures in both treatments, with Si concentrations in treatment S lower than in treatment L.

In *P. seriata* cultures Si concentrations were substantially higher than in *P. delicatissima* cultures. Here concentrations decreased from day 19 to 30 with higher concentrations in treatment S than in L. F/2 medium by Guillard (1975) enriched with silicate usually contains 107 μM . A concentration of about 200 μM in one *P. seriata* culture S (replicate B, day 19) indicates that initial Si concentrations in the medium must have been higher than this in all cultures, as the medium for all replicates was prepared in one container. Calculations based on the Redfield ratio (Redfield et al. 1963) of C:N:P = 106:16:1, assuming N:Si around 1:1, indicate that to achieve the observed carbon yield of *P. delicatissima* S on day 19 (1614 μM C), cells must have taken up about 244 μM Si. Furthermore, the yield of 565 μM C in *P. seriata* S on day 19 would have required 85 μM Si. On that day, 174 μM Si were detected in the medium, which leads to the conclusion that initial Si concentrations was approximately 260 μM (85 μM + 174 μM = 259 μM).

4.3.3.8 Nitrate

Inorganic nitrogen (N) was abundant in all cultures ($> 490 \mu\text{M N}$). In *P. delicatissima* cultures, N concentrations were lower than in *P. seriata* cultures. Between days 19 and 30 the amount of N taken up by *P. delicatissima* was greater than in *P. seriata* cultures. While more N was utilised between day 19 and 30 in *P. delicatissima* treatment L ($\sim 145 \mu\text{M N}$) than in treatment S ($\sim 132 \mu\text{M N}$), in *P. seriata*, cultures of treatment S more N ($\sim 55 \mu\text{M N}$) was used than in treatment L ($\sim 14 \mu\text{M N}$) during the same period of time.

4.3.3.9 DA concentration in *P. seriata* cultures

In both treatments, cells grown under long light conditions produced more DA than those grown under a short light cycle (Fig. 4.9).

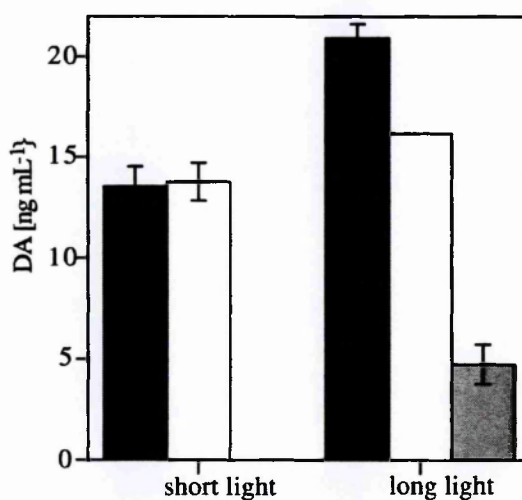


Fig. 4.9 DA content of *P. seriata* short and long light treatments in whole culture (black), medium (white) and cell fraction (grey) samples on day 30.

In *P. seriata* cultures treatment S, whole culture samples and culture medium contained about $13.6 \text{ ng DA} \cdot \text{ml}^{-1}$ after 30 days growth, which indicates that most of the DA from the cells was released into the culture medium in the late stationary phase of the growth cycle. Whole culture DA per cell was greater ($2.36 \text{ pg DA} \cdot \text{cell}^{-1}$) in S, than in

treatment L ($1.36 \text{ pg DA} \cdot \text{cell}^{-1}$) on day 30. The high calculated DA concentration per cell in S is also a result of very low cell numbers on day 30. Significantly (\pm standard error level) higher DA concentrations in whole culture samples ($21 \text{ ng DA} \cdot \text{ml}^{-1}$) than in the medium ($16 \text{ ng DA} \cdot \text{ml}^{-1}$) were found in treatment L, which indicated that DA was still produced by that culture in late stationary phase.

4.3.4 Experiment D: *P. seriata* - bacteria interactions

4.3.4.1 Algal growth in different treatments

Numbers of algal cells in dishes of the three treatments are presented in Table 4.5.

In Treatment 1, to which no filtrate was added (control), only one of the five replicates exhibited positive net growth. Diatom cells appeared to lack cell content after four (dish 2) and six days (dishes 1, 3, 5). In dishes 2 and 3 the single cells were not observed after day four and eight, respectively. In dish 4 *P. seriata* had divided once by day three and thereafter exponential growth was observed with a mean rate of 0.68 div d^{-1} between days four and twelve (Fig. 4.10).

In Treatment 2 ($0.2 \mu\text{m}$ filtrate added) no algal growth was observed. The single diatom cells in dishes 2, 3, 4 and 5 were not found after days four (dish 2) and three (dish 3, 4 and 5) and the cell in dish one appeared to be empty after day two. However, bacterial growth was observed in dishes 3 and 4 from day four.

In contrast to the other treatments, net positive division was evident in most of the dishes of Treatment 3 ($3 \mu\text{m}$ filtrate added). Division of the initial single *P. seriata* cell occurred in all dishes except dish 3, where the cell was not found after day four. In the other dishes exponential growth started after day two (dishes 2 and 4), three (dish 5) and four (dish 1) (Fig. 4.10). On day twelve the bottoms of dishes 1, 2 and 5 were covered

by cells and numbers could only be estimated. A maximal mean growth rate of $0.99 \text{ div} \cdot \text{d}^{-1}$ between days four and ten was observed in dish 5.

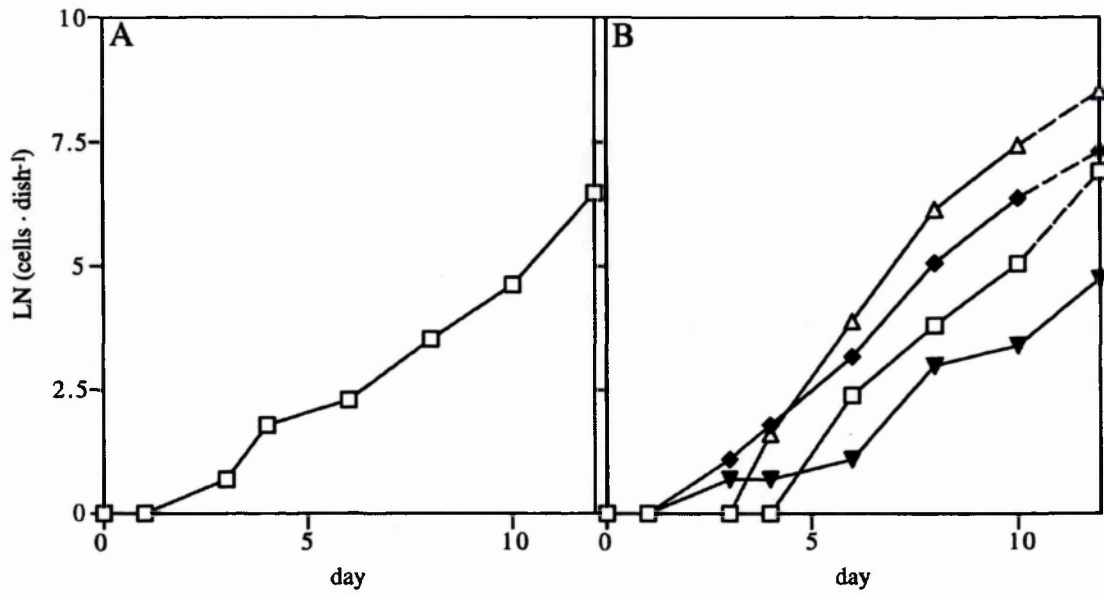


Fig. 4.10 (A) LN of cell numbers of *P. seriata* in dish 4, Treatment 1 (no added bacteria); (B) LN of cell numbers from Treatment 3 ($3 \mu\text{m}$ filtrate added): dishes 1 (squares), 2 (diamonds), 4 (filled triangles) and 5 (empty triangles). Broken lines: estimated cell numbers on day 12.

Table 4.5 Numbers of *P. seriata* cells per petri-dish* under different treatments and growth rates per day.

day	Number of cells per dish															max. growth rate [div · d ⁻¹]	r ²	start/end of exponential phase (days)
	Treatment 1: no filtrate added					Treatment 2: 0.2 µm filtrate added					Treatment 3: 3 µm filtrate added							
	dish 1	dish 2	dish 3	dish 4	dish 5	dish 1	dish 2	dish 3	dish 4	dish 5	dish 1	dish 2	dish 3	dish 4	dish 5			
0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
2	1	1	1	1	2	1	1	1	1	1	1	1	1	1	1			
3	1	1	3	2	3	1	1	1	1	1	1	3	1	2	1			
4	1		3	6	3	1	1				1	6	1	2	5			
6	1		2	10	3	1					11	24		3	49			
8	1			34	3	1					45	159		20	465			
10	1			102	3	1					156	586		30	1691			
12	1			652	3	1					>1000	>1500		115	>5000			
				0.68							0.83	0.78		0.52	0.99			
				0.99							0.97	0.995		0.96	0.99			
				4-12							4-10	4-10		4-12	4-10			

*Grey shading indicates the observation of either unhealthy looking cells, empty *P. seriata* cells, or that no cells could be found.

4.3.4.2 Comparison of treatments

In general algal growth was positively influenced by addition of bacteria. Results of the GLM indicated that there was no significant ($p > 0.05$) difference between algal growth rates in dishes where division of *P. seriata* was evident (dish 4, Treatment 1 and dishes 1, 2, 4 and 5, Treatment 3).

4.3.4.3 Identification of bacteria

Although no filtrate or bacteria were added to dishes of Treatment 1, bacterial and algal growth were evident in dish 4. Similarly bacterial growth was found in dishes 3 and 4 of Treatment 2, although no bacteria had deliberately been added. Bacteria were not enumerated, but some were isolated and identified. Bacteria named strains JF027, JF031 and JF033 were identified from dishes with algal growth (Treatments 1 and 3), while JF031 was additionally found in dish 3, Treatment 2.

The highest match from the FASTA search ($E = 1 \text{ e}^{-84}$) showed strain JF027 to be similar to an unidentified Alpha-*Proteobacteria*, family *Rhodobacteraceae*. The bright red colour of this organism might indicate that it could be a type of a phototrophic *Roseobacter*-like bacterium.

Strain JF031 is thought to belong to the *Gamma-Proteobacteria*, order *Vibrionales*, family *Vibrionaceae*, genus *Vibrio*. The most similar sequences were from *Vibrio* sp. LMG 200 (AJ316192, $E = 1.6 \text{ e}^{-72}$) and *Vibrio* sp. R-1556 (AJ316203, $E = 3 \text{ e}^{-72}$).

Bacteria representing strain JF033 seem to belong to the *Gamma-Proteobacteria*, order *Pseudomonadales*, family *Pseudomonadaceae*, genus *Pseudomonas*. The highest hit was an unknown marine gamma proteobacterium NOR5 (AY007676, $E = 1.6 \text{ e}^{-71}$), but as with the other strains, there were no significant type strain hits.

4.4 Discussion

In Scottish waters *Pseudo-nitzschia seriata* is most prevalent in summer months when water temperatures reach at least 14°C (chapter 2, Fehling et al. 2004b). Hence, an experimental temperature of 15°C was chosen for all experiments (except experiment C, for the reasons detailed in section 4.2.4) to provide suitable conditions for growth and potential toxin production. The results indicate that *P. seriata* is capable of producing the neurotoxin DA at this temperature.

4.4.1 Experiment A: Si- and P-limitation

4.4.1.1 Cell growth dynamics

The cell growth dynamics of *P. seriata* under either P- or Si-yield-limiting conditions (Fig. 4.1C) generally follow a logistic model (section 4.3.3.2). However, some significant differences were evident in each case, as discussed below.

In both conditions, cell growth commenced after a one-day lag phase. The short duration of this lag phase is a result of using early exponential phase cells as the inoculum (to prevent DA carry over from stock to experiment; Douglas & Bates 1992; Garrison et al. 1992). Exponential growth then commenced, as one would expect in nutrient-sufficient conditions. Statistical comparison of fitted lines using a General Linear Model (GLM) indicated that the specific rate of cell division was not significantly different in the two sets of conditions ($p > 0.05$) from day one to five. After day five, a difference was evident in the growth response of *P. seriata* to the two nutrient conditions. When DIP was the yield-limiting nutrient, exponential growth expressed as either cell number or C biomass was only evident prior to DIP becoming exhausted to a threshold level (of $\sim 0.8 \mu\text{M}$; Fig. 4.1A). The subsequent, short approximately linear growth phase, after a short stationary phase, is unusual for batch

culture growth. These changes in cell number were unrelated to any measurable change in extracellular DIP concentration, which had already decreased to its threshold.

In contrast, under Si-limiting conditions, the first (faster) exponential phase was followed by a second (slower) exponential phase, which occurred prior to DSi exhaustion. This slowing in growth rate is not unusual in batch cultures, and can be related to some cells stopping growth, while others are still dividing. Nevertheless, this response was not easily related to inorganic nutrient stress; silicate concentrations still exceeded 50 μM at the beginning of this phase (Fig. 4.1B). However, the onset of the second exponential phase coincided with a stabilisation of the C:N ratio (Fig 4.2), and may therefore simply be indicative of a better metabolic balance within the cells.

No stationary phase was observed under the Si-limiting condition. Rather, cell numbers declined immediately and rapidly upon DSi exhaustion (Fig. 4.1C). In diatoms, Si uptake and the cell cycle are coupled, as Si is essential for valve formation and DNA replication (Martin-Jézéquel et al. 2000). Because diatoms cannot sequester and store DSi, it is necessary for the cells to acquire Si just before its deposition in the frustule. Hence, cell division ceased almost immediately once DSi became exhausted. Apart from the observed stabilisation in the cell counts (Fig. 4.1C), DSi exhaustion also led rapidly to cell death (also observed by Davidson et al. 2002) and the decrease in cell numbers of viable plasma-filled cells.

An unusual feature of the Si-limited cultures, not evident under P-limitation, was the slight increase in cell numbers after day 15 (Fig. 4.1C), following a precipitous decline in cell density. Microscopic observation indicated the presence of some potentially viable cells, full of cell plasma, amongst the empty cells normally expected in late stationary phase. This observation corresponds to a rapid increase in both C and chl *a* per cell (Figs. 4.2A, 4.3A). This active metabolism in late stationary phase Si-limited cultures suggests that a DSi supply was available. The dissolution of empty *P. seriata*

frustules likely provided the DSi, which was rapidly taken up by the new cells and therefore not detected in the medium. Like *P. multiseriata* (Sommer 1994), *P. seriata* may therefore be a good competitor at low Si:N ratios. Furthermore, the immediate cessation in cell division after day ten suggests that *P. seriata* was particularly stressed by Si limitation; blooms under these conditions are likely to be short lived, but highly toxic. The DA produced during late stationary phase likely originated from the Si-stressed cells, rather than from the potentially viable cells that began to grow at that time.

4.4.1.2 Chlorophyll as an index of *Pseudo-nitzschia* biomass

Chlorophyll is a commonly used index of biomass for many phytoplankton monitoring programmes, as the measurements can be made relatively quickly and cheaply and, if necessary, by means of remote sensing. One might therefore suggest chl *a* as an index of potentially harmful *Pseudo-nitzschia* biomass, at least in areas and at times of the year when it is expected to dominate a phytoplankton bloom. However, a number of authors (Davidson et al. 1991, Geider et al. 1998) have noted the variability of chl *a*:C ratios and hence have cautioned against the indiscriminate use of chl *a* when quantitative estimates of phytoplankton C biomass are required. This is confirmed by the approximately three-fold variation of the chl *a*:C ratio observed during the growth cycle under both nutrient conditions (Fig. 4.3B). The rapid decrease in chl *a* · cell⁻¹ and chl *a*:C (Fig. 4.3) in Si-limited conditions during the decline in cell numbers between days ten to 13 indicates a rapid breakdown of chl *a* under nutrient-stressed conditions, when toxin production might be expected.

4.4.1.3 Magnitude of toxin production

Pseudo-nitzschia seriata produced DA under both nutrient conditions, although more was produced when DSi, rather than DIP, was limiting during the stationary phase (Fig. 4.4). This is the first study that directly compares P and Si limitation with respect to DA production in one experiment. Comparisons of DA production are difficult to make in different studies, where various growth conditions and sampling times were used (cf. Cusack et al. 2002). Pan et al. (1996a) found a maximum of ~ 4.5 pg DA \cdot cell⁻¹ in P-limited batch cultures of *P. multiseriata*, compared to our maximum of ~ 6 pg DA \cdot cell⁻¹ for *P. seriata*. In a separate batch culture experiment, Pan et al. (1996b) found substantially smaller amounts of cellular DA (maximum of 0.3 pg DA \cdot cell⁻¹) in *P. multiseriata*. However, (Pan et al. 1996c) found a maximum of 11.9 pg DA \cdot cell⁻¹ in Si-depleted continuous cultures of *P. multiseriata*. The maximum in the present study (14.6 pg DA \cdot cell⁻¹) was similar to that of Danish *P. seriata* strains (~ 13.7 pg \cdot cell⁻¹, at 4°C; Lundholm et al. 1994).

Relatively few reports are available for the kinetics of DA production by *Pseudo-nitzschia* species in batch culture. For *P. multiseriata*, toxin production is low or below detection during the exponential phase of growth (Subba Rao et al. 1990, Bates et al. 1991). The situation for other species is less well understood. Garrison et al. (1992) suggested that *P. australis* produced relatively high amounts of DA during the exponential phase. However, Cusack et al. (2002), working on a different strain of the same species, were unable to confirm this result. In further contrast, for *P. cf. pseudodelicatissima*, Pan et al. (2001) observed net DA production in batch cultures only during the exponential phase, not in stationary phase (see section 1.8.1).

Results for *P. seriata* in the present study, in combination with those of Lundholm et al. (1994), confirm that finite but low amounts of toxin (< 1.3 pg DA \cdot cell⁻¹) are produced during the exponential phase. This low magnitude of DA production suggests that this

species is not an environmental hazard under conditions of nutrient sufficiency, but only subsequent to the onset of nutrient (P or Si) limitation.

Under both nutrient conditions, most DA was produced post-exponential phase, as is common for *P. multiseriis* (Bates 1998) and as reported for one study of *P. australis* (Cusack et al. 2002). Once the cultures began to experience nutrient limitation, differences in toxin production patterns under the two nutrient conditions reflected the growth dynamics. More DA was produced in P-limited than in Si-limited cultures during the period immediately after the exponential phase (days five to 12; Fig. 4.4A). Particulate C continued to increase during this period in the P-limited cultures, indicating continued photosynthesis. In late stationary phase (after day 13), more DA was produced in the Si-limited cultures, on a per cell basis (Fig. 4.4C) and also per unit biomass, expressed as particulate carbon (not shown). During that time, there was a large increase in total produced $\text{DA} \cdot \text{cell}^{-1}$ (Fig. 4.4C) and in total $\text{DA} \cdot \text{C}^{-1}$ (not shown), which coincided and correlated with similarly large increases in both $\text{C} \cdot \text{cell}^{-1}$ (Fig. 4.2A; $r = 0.943$, $p = 0.035$) and $\text{chl } a \cdot \text{cell}^{-1}$ (Fig. 3A; $r = 0.886$, $p = 0.048$). The observed patterns of toxin production are consistent with the energy hypothesis of Pan et al. (1998) that, following single nutrient-yield limitation by either P or Si, cells may still photosynthesise but the energy normally used for cell growth is now channelled towards DA biosynthesis.

Bates et al. (1991) pointed out that $\text{chl } a$, responsible for energy harvesting and transfer in photosynthesis, is essential for DA production. Pan et al. (1998) also observed that DA production in *P. multiseriis* stopped after the cellular $\text{chl } a$ concentration had dropped to a critical level ($< 0.05 \text{ pg} \cdot \text{cell}^{-1}$) during late stationary phase. Bates et al. (2001) further documented that both DA production and cellular $\text{chl } a$ declined when *P. multiseriis* was grown with low concentrations of iron. Thus, the increase in cellular $\text{chl } a$ observed at the end of the experiment in Si-limited cultures would facilitate the

potential for greater capture of energy for photosynthetic C assimilation, evidenced in our cultures as an increase in C · cell⁻¹. However, in the present study the possibility cannot be excluded, that a degree of self-shading, of either the individual cells or the population, may have acted to cause a net decrease in photosynthetic capacity. The relative lack of DSi in stationary phase prevented substantial cell division, such that any excess energy could potentially be diverted to DA production (cf. Pan et al. 1998).

Results for both nutrient conditions indicate that *P. seriata* releases DA into the medium, as does *P. multiseriata* (Bates et al. 1991); no data are available for *P. australis*. Similar to Bates et al. (1991) and Pan et al. (1996a, b), in the present study no significant amounts of DA were found in the medium during the exponential phase. During the post-exponential phase, more DA was released by Si-limited than by P-limited cells (Fig. 4.4B), even when the DA in the medium was calculated as a percentage of the whole-culture DA (e.g. on day 10, 23% of the total DA was released in the P-limited cultures and 67% in the Si-limited cultures). The reasons why more DA was released in the Si-limited cultures remain unclear. In the case of P-limited cultures, the reduced supply of DIP may impair the formation of the lipid bilayer, consisting mainly of phospholipid, thereby allowing the DA to leak more easily through the cell membrane and into the medium (Pan et al. 1996a). In addition, the approximately constant DA concentration evident during the decline of cell numbers indicates that severe P limitation may suppress the production of DA in *P. seriata*.

4.4.1.4 Intracellular properties

Comparison of the mean C- and cell-number-specific growth rates during days one to five indicates no significant difference between nutrient conditions (GLM, $p > 0.05$). However, within each nutrient condition these growth rates were significantly different from each other (GLM, $p < 0.05$), although the specific rate of net C fixation was lower

than the cell-specific value, resulting in the observed marked decrease in $C \cdot \text{cell}^{-1}$ (Fig. 4.2A). This indicates that *P. seriata* did not achieve true balanced growth during that period. This was confirmed, in both conditions, by a similar reduction in the ratios of C:N (Fig. 4.2B) and chl *a*:C (Fig. 4.3B) during exponential growth. The results suggest that *P. seriata* is able to regulate its C demand to achieve optimal nutrient stoichiometry, while still dividing at an optimal or near-optimal rate.

The P- and Si-stressed cells continued to fix C during the post-exponential phase, as demonstrated by the increase in $C \cdot \text{cell}^{-1}$. This was most marked under Si-limiting conditions, when C biomass increased throughout the remainder of the experiment, reaching a value approximately twice that at inoculation. Such an increase in $C \cdot \text{cell}^{-1}$ in nutrient-starved diatoms is not uncommon and may result from continued accumulation of carbohydrate or lipid storage products (Myklestad 1974; Gilpin et al. 2004). The low and constant C:N ratio in Si-limited cultures coincided with a three-fold increase in $C \cdot \text{cell}^{-1}$, indicating balanced uptake of C and N by those cells that remained viable during the late-stationary phase.

Meso-zooplankton can be vectors for the trophic transfer of DA in marine food webs (Bargu et al. 2002). Furthermore, it has been shown both through experiment and modelling (John & Davidson 2001; Jones et al. 2002) that the intracellular nutrient imbalance (e.g. in the C:N ratio) of nutrient-stressed phytoplankton cells may make them non-optimal prey items for micro- or meso-zooplankton predators. This can result either in prey rejection or significant voiding of ingested material. Comparison of grazing on toxic and non-toxic *P. multiseriata* cells provides no evidence that toxicity, alone, deters zooplankton predators (Lincoln et al. 2001). Therefore, the intracellular nutrient imbalance in toxic cells stressed by P or Si limitation may not reduce the nutritional quality of toxic cells to the point that grazers reject them. The C- and N-rich,

but C:N balanced, cells that characterise the Si-limited cultures may thus promote particularly efficient toxin transfer to higher trophic levels.

4.4.1.5 Implications for ASP prediction

Currently, a reactive strategy is used to prevent ASP outbreaks, through the monitoring of toxin levels in shellfish tissue. The fishery is closed when tissue toxin levels exceed the permitted threshold. Such an approach, however, does not allow for advanced planning to safeguard commercial shellfish farming or fishing. An alternative approach is to develop a proactive system for predicting *Pseudo-nitzschia* growth and DA production, and hence of ASP events, prior to their occurrence. This may be accomplished by using mathematical models capable of simulating the appearance and toxicity of *Pseudo-nitzschia* species, based on environmental conditions.

Simple models such as those of Monod (1942) that relate biomass to extracellular nutrient concentration are unlikely to be successful, as these predict an immediate cessation of cell division or net biomass synthesis upon exhaustion of the yield-limiting nutrient. This criterion is obviously failed by *P. seriata* under P-limiting conditions. More realistic “cell quota” models relate growth rate to the concentration of intracellular nutrient per unit biomass (Droop 1968; Caperon 1968). However, their ability to simulate cell numbers as well as biomass (a necessary criterion for modern food web models) relies on the observation of constant C biomass per cell (Droop 1979), a criterion which was not observed for *P. seriata* under either P or Si limitation.

Therefore to simulate *Pseudo-nitzschia* growth and toxicity, it is necessary to derive models that are mechanistic in nature (Geider et al. 1998; Davidson & Gurney 1999; John & Flynn 2002), i.e. the models must be based on biological evidence, rather than relying on empirical relationships. Only then will they be able to simulate *Pseudo-nitzschia* species in a dynamic environment and across a spectrum of environmental

conditions. Such an approach, however, requires considerable further experimentation to determine the necessary biochemical relationships and parameter values.

4.4.2 Experiment B: *P. seriata* growth on different N sources

The results suggest that *P. seriata* might have a preference for NO_3^- compared to NH_4^+ uptake. The preference is expressed in the two days lag phase that was evident when cells were grown on NH_4^+ (100 μM) as the sole N source. Furthermore, the NH_4^+ -grown cultures expressed a lower growth rate than those cultures that were grown on NO_3^- , however their growth rates were not significantly different (GLM, $p > 0.05$). Such a lag and difference in growth rate has been suggested (Dortch 1990) to be indicative of a nutrient preference. In her review about the interactions of ammonium and nitrate uptake in phytoplankton, although phytoplankton usually exhibited a preference for NH_4^+ , Dortch (1990) noted that in some algae, growth on NO_3^- has been found to be often as good or better than that of NH_4^+ . Supporting the results of this experiment, studies on *Nitzschia* spp., which could potentially have included *Pseudo-nitzschia* species, showed a preference for NO_3^- compared to NH_4^+ uptake, expressed in the ratio of $\mu_{\max} \text{NO}_3^- : \mu_{\max} \text{NH}_4^+ > 1$, where μ_{\max} is the maximum growth rate (Eppley et al. 1971).

It cannot be discounted that, as cells in stock cultures were grown on NO_3^- , the observed lag was related to an adaptation period. However, this phenomenon cannot easily explain the lower observed growth rate.

The concentration of NH_4^+ used in the mixed medium (50 μM), did not have a toxic effect on *P. seriata*. Similarly, when growing *P. multiseriis* in media based on different NH_4^+ concentrations, Bates et al. (1993b) found that in treatments with initial N concentration of less than 110 μM , growth rate and DA production yield were equivalent between treatments. NH_4^+ only appeared to be toxic to *P. multiseriis* at

initial concentrations higher than 110 μM . For *Nitzschia seriata* (which was presumably *P. seriata* or *P. australis*) NH_4^+ toxicity was observed at a concentration of 100 μM , which resulted in inhibition of growth (Guillard 1963). Similarly, Hillebrand & Sommer (1996) found that NH_4^+ when supplied in concentrations $> 200 \mu\text{M}$ did not support growth in *P. multiseriata*, which was interpreted as a combined effect of inhibition of NO_3^- uptake and NH_4^+ toxicity.

No difference in growth rate was evident (GLM, $p > 0.05$), when grown on media that were solely NO_3^- based or contained the mix of NO_3^- and NH_4^+ .

Another study on microalgae (Lourenço et al. (2002) showed the tolerance of many microalgae to use different N sources, although preferences seemed to be strongly species-specific.

In conclusion, this laboratory experiment showed that *P. seriata* can grow on ammonium as a nitrogen source, at least at initial concentrations of 100 μM . However, a repetition of this experiment is required to verify a potential inhibition of growth at high NH_4^+ concentration, as there was no significant difference in the observed growth rates, to determine if preconditioning would lead to a different result and to assess if the concentration of NH_4^+ (100 μM) had a toxic effect on *P. seriata*.

4.4.3 Experiment C: *Pseudo-nitzschia* growth under different L:D cycles

4.4.3.1 Diel periodicity

The division rate of *P. delicatissima* was $\sim 0.5 \text{ div} \cdot \text{day}^{-1}$, when grown under 9 h light (short light), and $\sim 1 \text{ div} \cdot \text{day}^{-1}$, when the duration of the light phase was doubled (long light). This may be explained in two ways. Firstly, the growth response to the short light might indicate rate limitation by light. Nine hours light at an intensity of $100 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ might not have provided sufficient photochemical energy for faster cell division.

Secondly, it might indicate that cell division, and hence the cell cycle, in *P. delicatissima* may be coupled with the L:D cycle. However, for *Pseudo-nitzschia* spp. it is not known how the light period may effect the cell cycle.

While this study might suggest diel periodicity in *P. delicatissima*, this was not observed for *P. seriata*. An elongated light phase enhanced cell division in both species, but in *P. seriata* it was did not seem to be directly related to the light period. For *P. seriata* other factors, one of them potentially light limitation (in form of a short duration), had a suppressing effect on the growth rate.

Brand et al. (1981) suggested that coastal phytoplankton might be adapted to utilise light for unpredictably long time periods. Phytoplankton in coastal regions may experience light fluctuations on time scales much less than a day due to vertical mixing (tidal currents, effects of bottom topography in shallow water), while oceanic species experience a light regime dominated by the day:night cycle. Results from Discovery cruise D257 (see chapter 2) indicated that in early autumn *Pseudo-nitzschia* belonging to the *P. delicatissima* group occurred in high numbers in open ocean waters, while species belonging to the *P. seriata* group were more abundant on coastal waters at that time of the year. This might suggest that *P. delicatissima* might have preferences for oceanic conditions, and be more adapted to a stable L:D cycle than coastal *P. seriata*.

4.4.3.2 Growth rates

Reports of growth rates of *Pseudo-nitzschia* species are scarce. When *P. delicatissima* isolated from the Barents Sea was grown at -0.5°C , $70 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and 10 h light, the growth rate was $0.13 \text{ div} \cdot \text{d}^{-1}$. It increased to $0.44 \text{ div} \cdot \text{d}^{-1}$, when the light phase was extended to 19 h (Gilstad & Sakshaug 1990). Growth rates in *P. delicatissima* in this study were higher (0.49 in S and $0.89 \text{ div} \cdot \text{d}^{-1}$ in L), which can probably be explained by the higher temperature (10°C) the cultures were exposed to.

Chapter 4

In a study on *P. seriata*, isolated from the bottom 5 cm of landfast sea ice near Resolute, Northwest Territories, Canada, a strain was grown at 10°C and 25 $\mu\text{mol photons m}^{-2} \cdot \text{s}^{-1}$, presumably under continuous light (no L:D cycle was specified) (Smith et al. 1994). Although the light intensity was just 25% of the intensity used in this study, the growth rate of 0.57 $\text{div} \cdot \text{d}^{-1}$ compares well with the growth rate of *P. seriata* grown under the 18:6 L:D cycle (0.51 $\text{div} \cdot \text{d}^{-1}$). The longer light phase (e.g. continuous light) might have compensated for the lower light intensity. Compared to growth rates of the same *P. seriata* strain grown at 15°C and a 12:12 h L:D cycle (exponential phases in experiment A, P - and Si-limitation, section 4.3.1.2), the rates were about the same (long light: 0.51 $\text{div} \cdot \text{d}^{-1}$; P-limitation: 0.55 $\text{div} \cdot \text{d}^{-1}$; Si-limitation: 0.58 $\text{div} \cdot \text{d}^{-1}$).

4.4.3.3 Biomass

Interestingly, *P. delicatissima* grown under short light conditions showed the highest biomass yield (25.5 $\mu\text{g C} \cdot \text{ml}^{-1}$) of all experimental cultures, including *P. seriata*. This result suggests an adaptation to short light phases, and may be a mechanism which allows *P. delicatissima* to occur in high abundance in Scottish waters in early spring, when the light phase is relatively short. In contrast, *P. seriata* may not be adapted to short light phases, the biomass yield being higher when grown under long light conditions.

4.4.3.4 Nutrients

As carbon fixation is coupled to photosynthesis, while respiratory carbon losses occur continually, diatoms accumulate cell carbon and nitrogen during daylight. Si uptake is not dependent on light, Brzezinski et al. (1990) were observing that Si per cell increased prior to division as the cell deposited new frustules. They concluded that diatoms do not

store sufficient Si for new valve formation and must accumulate most of the requisite amount immediately before cell division. When facing Si depletion, a general feature of all diatoms that deposit siliceous frustules is to reach a silicon-dependent arrest point in the cell cycle (Brzezinski et al. 1990). This phenomenon was observed in experiment A under Si-limitation, where cell division ceased immediately once DSi was exhausted. When measured after 19 days in this study, Si was depleted in the medium of *P. delicatissima*, but not in *P. seriata*. This might be due to the higher cell yield in *P. delicatissima*, but could also suggest higher Si uptake rates in *P. delicatissima* than in *P. seriata*, which can not just be explained by higher growth rates in *P. delicatissima*. It is possible that *P. delicatissima* requires more Si per cell than *P. seriata*. Field observations show that in spring Si concentrations in the water are higher than in summer (chapter 2). Hence, it might be assumed that *P. seriata* could be adapted to take up lower Si concentrations than *P. delicatissima*, while *P. delicatissima* might be able to take up Si more quickly and in greater amounts. Experiment A showed the appearance of new viable cells in late stationary phase in the Si-limited treatment, and it was assumed that those cells might have taken up low amounts of re-dissolved Si from frustules of dead cells.

Increasing P concentrations between day 19 and 30 in both species grown under long light may indicate the release of P due to lysis of dying cells. While *P. delicatissima*, grown under short light, continued growth, as indicated by decreasing P concentrations in the medium, cells in the other treatments had ceased division. The elevation of NH_4 in all cultures supports the suggestion that as cells ceased division and at least some of the cells started to lyse and die. As some diatoms would preferably take up NH_4^+ to NO_3^- due to its lower energetic costs, as long as it is present in low concentrations, the ammonium concentration would decrease as a consequence. Here the ammonium

concentration increased in all cultures, which might indicate that cells had stopped nitrogenous uptake.

4.4.3.5 Domoic acid production in *P. seriata*

The DA results suggest that the total amount of DA in a culture might be enhanced under a long L:D cycle, while cellular levels might be higher in cultures grown under short light phases. The total amount of DA per cell (whole culture DA divided by cell number) in L (1.36 pg DA · cell⁻¹) was similar to DA in a Danish *P. seriata* strain grown under a 16:8 L:D cycle at 15°C (strain 1877A: 0.99-1.6 pg DA · cell⁻¹), although it was not specified after how many days of culture growth DA was tested in 1877A (Lundholm et al. 1994). The per cell DA concentration in treatment S on day 31 (2.36 DA · cell⁻¹) is higher than concentrations measured for Danish strain 1877C, when grown under a 16:8 L:D cycle and at 4°C (after 30 days ~ 0.5 DA · cell⁻¹, from Fig. 5 in Lundholm et al. 1994). However, compared to DA concentrations of the same strain under P- and Si-limitation (experiment A) the amounts of DA per cell are relatively low, suggesting that nutrient rather than light limitation is the most important factor influencing DA production in *P. seriata*.

Similar concentrations of DA in whole culture samples and medium in treatment S suggested that on day 30 cells had released all their DA, or while still producing DA, were releasing it at the same time. Bacteria can enhance cell lysis of diatoms (e.g. Kogure et al. 1982) and might have had an impact. As bacteria were neither enumerated nor identified, this cannot be proved. Increased P and NH₄ concentrations in the medium support the theory of cell leakage in the late stationary phase cultures.

Bates & Richards (1996) showed for *P. multiseriata* that cells divided in the light phase. A short light phase would therefore give the cells less time for division, subsequently cell numbers per culture would be lower and so would total DA per mL of culture,

assuming the DA production rates per cell are equal in long and short light. However, as Bates & Richard (1996) observed, DA production was lowered during the light phase in *P. multiseriata*, indicating that DA was produced during the dark phase. Should the same apply for *P. seriata*, an extended dark phase might elevate the DA concentration in the cells. To be certain that the length of L:D cycles affect DA production, an experiment measuring DA during the full time-course would be required.

4.4.4 Experiment D: *P. seriata* - bacteria interactions

Results of the experiment with *P. seriata* with and without added bacteria, suggested that in general bacteria associated with *P. seriata* have a growth enhancing effect on the diatoms in terms of survival and division. However, one strain of the isolated bacteria associated with the algae appeared to have a negative effect on *P. seriata* growth.

4.4.4.1 Bacteria associated with *P. seriata*

When cells were isolated into the petri-dishes they were washed in sterile culture medium beforehand and care was taken that only single cells in very small volumes of medium (~ 5 µL) were transferred. However, washing the algal cells prior to transfer may not have removed all attached bacteria from the diatom cell, and may have led to the appearance of bacteria in some control experiments. Nevertheless, sufficient replicates remained uncontaminated for conclusions to be drawn (4.4.5).

One of the bacteria strains, that was potentially attached to algal cells, belonged to the Rhodobacteriaceae, which are included into the purple nonsulfur bacteria. They are anoxygenic phototrophs that grow phototrophically, obtaining carbon from organic sources. Rhodobacteriaceae can be highly physiologically diverse and may play a significant ecological role (Madigan et al. 2003). Their effect on *P. seriata* is not

known. However, as they were only isolated from dishes with diatom growth, their presence might be beneficial for *P. seriata*.

Bacteria belonging to the genus *Vibrio* were found in all treatments and in high (visible) density in the dishes without algal growth. This genus contains facultatively aerobic rods and curved rods that possess a fermentative metabolism (Madigan et al. 2003). As the other strains, they might be able to attach to the diatom cells. As *Vibrio* was the only genus observed in dishes without algal growth, it is likely that their effect on the algal cells is not beneficial, potentially providing substances inhibitory to *P. seriata* growth.

The bacterial genus *Pseudomonas* includes straight or slightly curved chemoorganotrophic and anaerobic rods. Pseudomonads are known to grow chemoorganotrophically at neutral pH and mesophilic temperatures. Some Pseudomonads can use a wide range of organic compounds as carbon and energy sources, although they generally lack the necessary enzymes to break down polymers into their component monomers. Pseudomonads are ecologically important in soil and water where they are possibly responsible for the degradation of soluble compounds derived from the breakdown of plant materials in oxic habitats (Madigan et al. 2003). In this study they seemed to be associated with *P. seriata* cells and might have enhanced their growth through a symbiotic relationship. It can be hypothesised that the bacteria utilise organic compounds produced by the algae and in return provide them with degraded compounds that have a beneficial effect on diatom growth. As with the other results in this section, this hypothesis requires further testing.

Bibel et al. (1983) found evidence that bacteria that arrive first at a substrate may exclude later arrivers. It is possible that bacteria belonging to the *Vibrio* group were initially attached to algal cells in Treatment 2 and were occupying the surface of the cells without leaving space for other bacteria. However, they were also found in one

dish of Treatment 1 together with Pseudomonads and Rhodobacteriaceae, potentially indicating that all three strains had been attached to the algal cell.

Kogure et al. (1982) found bacteria of the genus *Vibrio* and *Pseudomonas* attached to phytoplankton cells in culture. The number of epiphytic *Vibrio* bacteria gradually decreased within four days, while *Pseudomonas* numbers on the algal cells increased. Similarly, in this study isolated *Vibrio* bacteria from dishes of Treatment 2 occurred in high numbers on the bottom of the dish in the medium after a few days. While bacteria belonging to the Pseudomonads were not visible on the bottom of the dishes, but might have occurred attached to the algae. As both bacteria genera appeared in Treatment 1 (no deliberately added bacteria), they were possibly mainly attached to the diatoms.

Kogure et al. (1982) suggest that, depending on the type of bacteria, once bacteria have colonised an algal cell, they can destroy it within several days. This might have been the case in some of the petri-dishes (e.g. dishes 2 and 3 in Treatment 1, dishes 2, 3, 4, 5, in Treatment 2 and dish 3 in Treatment 3; see Tab. 4.5) and could explain why the initial single cells were not found after some days. Bacteria of the genus *Vibrio* might have this effect on *P. seriata*, as they were present in all dishes in which cells were not found after a few days. However, further experiments are needed to test that hypothesis. Furthermore, as all the bacterial strains associated with the algal cells might not have been isolated, the results and the experiment should therefore be regarded as preliminary until more complete characterisation of the bacterial fauna is possible.

4.4.5 Conclusions

All laboratory experiments were derived from field observations at the monitoring site LY1. Low inorganic nutrient concentrations of DIP and DSi and NO₃ in the water column during the summer months, when toxic *P. australis* and *P. seriata* occurred in highest densities (during summers 2001, 2002 and 2003, see chapter 2). This led to

studies of *P. seriata* growth and toxin production dynamics during conditions simulating temperate summer (experiments A and B). The results of experiment A showed that patterns of cell growth and metabolism differed depending on the yield limiting nutrient. Only trace amounts of DA were produced and released during exponential phase, representing the earlier stages of a bloom in the field. Significant toxin production occurred during post exponential enhanced growth phases in P and Si-limited conditions, and hence prior to DSi exhaustion in Si limited cultures. Total produced DA per cell and per unit biomass was highest in the Si-limited cultures during late stationary phase. At that time, new viable cells with increased cell metabolism were evident. Comparison of the magnitude of toxin production in the two nutrient regimes indicates a greater threat of *P. seriata*-generated ASP events under Si- rather than P-nutrient limitation.

In the field P and Si were both exhausted in Scottish waters during the summer month. Blooms of potentially toxic *Pseudo-nitzschia* can be preceded by other diatoms which would decrease the Si and P concentrations in the water. As shown in experiment A, *P. seriata* seems to have a good ability to take up DSi even when it is present in only low concentrations. This ability would facilitate a prolonged duration of a *P. seriata* bloom and increase the toxicity of the algae the more the cells would be stressed by Si-limitation. When the toxigenic bloom of *P. multiseriata* occurred in PEI in December 1987, Si concentrations were low (0.67 μM) at the peak of the bloom (Subba Rao et al. 1988). Highest toxin concentrations were detected ten days later (Smith et al. 1990a; Silvert & Subba Rao 1992), at that time DSi must have been exhausted.

Field observations also showed low NO_3 concentrations in Scottish waters at times when potentially toxic *Pseudo-nitzschia* densities were highest. As experiment B showed, *P. seriata* is capable of utilising NH_4^+ in concentrations of at least 100 μM , hence it is likely that in summer, when nitrate concentrations are low, *Pseudo-nitzschia*

species take up NH_4 as a N source. However, implications on potential increases in *P. seriata* toxin production due to ammonium need further study.

Regarding day length, experiment C confirmed the hypothesis derived from field observations, that *P. delicatissima* and *P. seriata* are adapted to spring and summer light conditions. Both species showed highest biomass yields under the light length that they are exposed to in the field at times of their natural peak densities.

The influence of temperature on *P. seriata* can be compared between all experiments, it seemed to have an effect on DA production. The growth study on Danish *P. seriata* cultures showed that their DA production was enhanced at 4°C compared to 15°C (Lundholm et al. 1994). Those results are comparable with measurements for the Scottish *P. seriata* (this study). DA concentrations in cultures grown at 15°C (0.16-0.23 pg DA · cell⁻¹ on day 25) and a 12:12 L:D cycle (see chapter 3, Fehling et al. 2004a) were lower than those in cultures of the same strain grown at 10°C under a long and a short L:D cycle (1.35-2.36 pg DA · cell⁻¹, day 30) (this study), although higher concentrations were found under similar light and temperature conditions during P and Si-limitation (experiment A, day 19, maximal 5.9 and 14.7 pg DA · cell⁻¹, respectively), indicating that nutrient limitation might influence DA production stronger than temperature or the L:D cycle. The difference in non-nutrient-limited cultures might have been an effect of temperature, or could simply be explained by the different stage of the growth phase of cultures at the time when samples for DA analysis were taken. To investigate the effect of the L:D cycle, the same strain could additionally be grown at 10°C under a 12:12 h L:D cycle.

Bacteria are likely to have a strong impact on *Pseudo-nitzschia* in the field. Within this study only three strains that are potentially associated with *P. seriata* were identified and experiment D can only be seen as a pilot study. However, an extensive study of *Pseudo-nitzschia* associated bacteria would be interesting, as it is likely that they are

playing an essential role in *Pseudo-nitzschia* bloom dynamics and their toxin production.

5 Chapter 5: Summary and general conclusions

5.1 Background

In 1999, an area of 49,000 km² was closed to the shellfish harvesting industry in Western Scottish waters, due to domoic acid accumulation in shellfish (Campbell et al. 2003). The toxin has appeared in shellfish every year since, and has led to more harvesting closures. Before this study the only confirmed toxin producer in these waters was *P. australis*. Other potentially toxic species had been observed in a few samples (Gallacher et al. 2001), but no cells were isolated or reliably identified. Hence, there was a need to clarify which *Pseudo-nitzschia* species contributed to ASP in Scottish waters, to investigate their ecology, toxin production and growth dynamics.

5.2 This study

The study consisted of three parts, including

- field investigations (chapter 2)
- cultivation/identification of *Pseudo-nitzschia* species (chapter 3)
- laboratory experiments (chapter 4).

5.2.1 Major results

The study was based around a phytoplankton monitoring programme at station LY1 (chapter 2). Samples were taken fortnightly from November until March and weekly during the rest of the year for a period of 33 months. This provided information about seasonality of phytoplankton taxa, including *Pseudo-nitzschia* species. Environmental factors that influenced the phytoplankton composition were monitored and patterns of phytoplankton blooms in Scottish waters were evaluated. The field study gave insight

into the diversity of *Pseudo-nitzschia* species and furthermore provided material for culturing *Pseudo-nitzschia* species. The cultures were used to analyse the toxin production of different strains, to identify strains with classical morphologic and genetic methods and to conduct laboratory experiments investigating *Pseudo-nitzschia* physiology. The major results are detailed below:

Temporal distribution

- From the temporal monitoring it emerged that the presence of *Pseudo-nitzschia* species in the phytoplankton assemblage followed the same seasonal pattern in 2001, 2002 and 2003.
- In each year, *P. delicatissima* was a dominant part of the spring bloom, but also showed elevated cell densities during the summer. Diatoms belonging to the *P. seriata* group were most abundant from June to October. Electron microscopy of field samples showed that blooms of the *P. seriata* group always consisted of several species, including *P. australis*, *P. seriata*, *P. fraudulenta*, *P. pungens* and *P. cf. subpacifica*. Periods of high cell densities, that could have led to DA accumulation in shellfish, lasted from 1 week to 8 weeks. A phytoplankton monitoring with a lower frequency than weekly would have missed some blooms or maximal cell densities.
- Many other phytoplankters were observed to follow a seasonal pattern and the timing of their occurrence was very similar in each year. For example *Skeletonema costatum* occurred every year in spring and was observed together with diatoms of the *P. delicatissima* group, while *Chaetoceros* species were highly abundant in late spring and early summer. Every summer red clouds consisting of the ciliate *Mesodinium rubrum* were observed in the water column. Those blooms coincided

with high cell densities of diatoms belonging to the *P. seriata* group and seemed to irritate the local farmed salmon.

- Nutrient concentrations in the summer were low and it is possible that some macronutrients, such as DIP and DSi were limiting phytoplankton growth. However, several phytoplankton taxa, amongst them diatoms of the *P. seriata* group, occurred in high cell densities at that time, suggesting a nutrient supply that was immediately taken up by the algae and hence not measured. While nitrate concentrations were low, ammonium concentrations were elevated and hence potentially available as an alternative nitrogen source for phytoplankton.
- Species *P. cf. subpacificica* and *P. cf. delicatissima* were observed in the samples. Their morphology, and in case of *P. cf. subpacificica* their rDNA sequences, did not match type descriptions in the keys or databases. Hence they might be intermediate forms (e.g. between *P. subpacificica* and *P. heimii*) or new species.

Spatial distribution

- Studying the spatial distribution of *Pseudo-nitzschia* spp. and other phytoplankton along the Ellett Line in early autumn showed that the taxa were associated with distinct water masses: the Scottish coastal current and Atlantic water.
- At that time of the year diatoms belonging to the *P. seriata* group, including the toxic species *P. seriata* and *P. australis*, were still highly abundant in coastal waters, but not in the open ocean.
- In contrast, species belonging to the *P. delicatissima* group dominated the phytoplankton assemblage at the open ocean stations.
- Statistical analysis of the spatial study showed that salinity (as an indicator of the water mass) was the only significant factor influencing the spatial phytoplankton distribution.

Toxin monitoring in culture

- From the temporal and spatial study 59 *Pseudo-nitzschia* cultures were established and identified with classical morphologic and genetic methods. Seven species were identified from the cultures, including *P. australis*, *P. calliantha*, *P. delicatissima*, *P. fraudulenta*, *P. pungens*, *P. seriata* and *P. subpacific*. From field samples *P. americana*, *P. pseudodelicatissima* and *P. cf. delicatissima* were identified.
- *P. calliantha*, *P. cf. subpacific* and *P. americana* were for the first time reported from Scottish waters.
- Of the cultured species only *P. australis* and *P. seriata* produced domoic acid. These results confirmed *P. australis* as a toxin producer and showed that *P. seriata* was a second toxic diatom species in Scottish waters. As it was found in high numbers together with *P. australis*, it is likely to contribute to ASP in Scottish waters.
- Other species that had previously been named as potential toxin producers in Scottish waters (Gallacher et al. 2001), such as *P. delicatissima*, *P. fraudulenta* and *P. pungens* were shown to be non-toxic in these waters.

Phylogeny

- Phylogenetic relationships between the Scottish species showed the close relationship between the toxin producers *P. australis* and *P. seriata*, and confirmed relationships between species with similar morphological features (e.g. possession of a central interspace).
- Sequencing the ITS region showed its potential as a template for molecular probes to distinguish between closely related species such as *P. australis* and *P. seriata*. However, to distinguish between strains belonging to one species, genes with higher variability in their nucleotide sequences have to be used. Being able to distinguish

between strains might help to understand differences in the toxicity of strains, as for example observed in the two *P. australis* strains (PLY1St.54B and PLY1St.19A).

Laboratory studies

- The laboratory experiment on *P. seriata* investigating its growth and toxin production dynamics under phosphate and silicate limitation was the first of its kind with that species.
- The results showed that this species follows the same toxin production pattern as *P. multiseriata*, with toxin production starting in late exponential phase, after the limiting nutrient had become exhausted.
- Toxicity of *P. seriata* was enhanced in DSi limited cultures compared to DIP limited cultures.
- Silicate limitation would enhance the toxin production of a *P. seriata* bloom, and due to the ability of this species to take up silicate in low concentrations, the bloom would persist for a long time.
- *P. seriata* readily utilised NH_4 as a nitrogen source. During summer in the field, when NO_3 was exhausted, the algae might have utilised NH_4 that was available and would also have extended the duration of a toxic diatom bloom.
- Enhanced biomass yield was found for *P. delicatissima* under short light conditions, while *P. seriata* reached highest biomass yields under long light conditions. This confirmed field observations, which showed that *P. delicatissima* species were most abundant in early spring, when day length in these latitudes was short. In contrast, diatoms belonging to the *P. seriata* group bloomed during summer, when day length was maximal.

- Toxin production in *P. seriata* was elevated under long light conditions, which might be a consequence of enhanced availability of energy. This excess energy, might have potentially been channelled into more toxin production.

5.3 Recommendations for future work

- A continuation of the phytoplankton monitoring at LY1 would eventually provide insights in long term changes in physical factors and phytoplankton ecology. This was indicated by the comparison with 25 year old temperature data from the same station, which showed significantly lower temperatures in past winters.
- The availability of a plankton time series in conjunction with this physical dataset will allow study, potentially involving modeling, of spring bloom development and species succession.
- In regard to *Pseudo-nitzschia* species it would be useful to isolate fresh cultures for further laboratory experiments investigating toxin production under other nutrient (e.g. comparison of DA production in NO_3 and NH_4 based media), light (DA production of *P. seriata* under different irradiances) and temperature conditions.
- Further investigation is needed to identify changes in the toxin production of strains after being kept in culture for years.
- For *P. australis* and *P. seriata* study of effects of temperature on the morphology of cells and their toxin production would be interesting. There are indications of a reduction in the number of rows of poroids in *P. seriata* when grown in higher temperatures. However, this has not been investigated for *P. australis*. Furthermore, the temperature and the duration of exposure to that temperature at which changes in the fine structure are observed are unknown.

- To complete the picture of toxin production of *Pseudo-nitzschia* species in western Scottish waters, other potentially toxic species, such as *P. calliantha* and *P. pseudodelicatissima* should be isolated, cultured and tested for DA production.
- Interaction between *Pseudo-nitzschia* species and bacteria are evident (e.g. Bates et al. 2004). Experiment D (chapter 4) demonstrated the enhanced growth of *P. seriata* in presence of bacteria. However, further investigations are needed to elucidate the exact implications of interactions between the algae and their associated bacteria.

5.4 General conclusions

This study has shown that at least two domoic acid producing *Pseudo-nitzschia* species (*P. australis* and *P. seriata*) regularly occur in a repeatable pattern in Scottish waters, in numbers exceeding 10^5 cells · L⁻¹, the threshold density thought to cause ASP. Blooms persisting from one to several week are likely to be the cause of DA contamination of shellfish. A phytoplankton monitoring programme in a high temporal resolution, together with shellfish testing is hence required in Western Scottish waters to detect ASP.

While light microscopy can separate diatoms belonging to the *P. delicatissima* group from those belonging to the *P. seriata* group, it is necessary to identify the species within these groups, as both contain toxic species, or in case of the *P. delicatissima* group potentially toxic species. The main difference between *Pseudo-nitzschia* species of the *P. delicatissima* group and the *P. seriata* group was, that the former occurred in highest cell densities in spring and the latter in summer. However, it is suggested that each species within each group has its own ecological preferences. Some species belonging to the *P. delicatissima* group might be occurring in summer rather than in spring. The preferences of each species in the *P. seriata* group are not clear. This study suggested that blooms of the *P. seriata* group were multispecies blooms. While the

experiments focussed on *P. seriata*, it is not known if the other species exhibit similar ecological preferences. This study showed the importance of cultivation of *Pseudonitzschia* species for toxin testing, together with reliable identification of species by TEM and genetic methods. This is necessary to sufficiently assess which species can be a threat to marine wildlife and humans.

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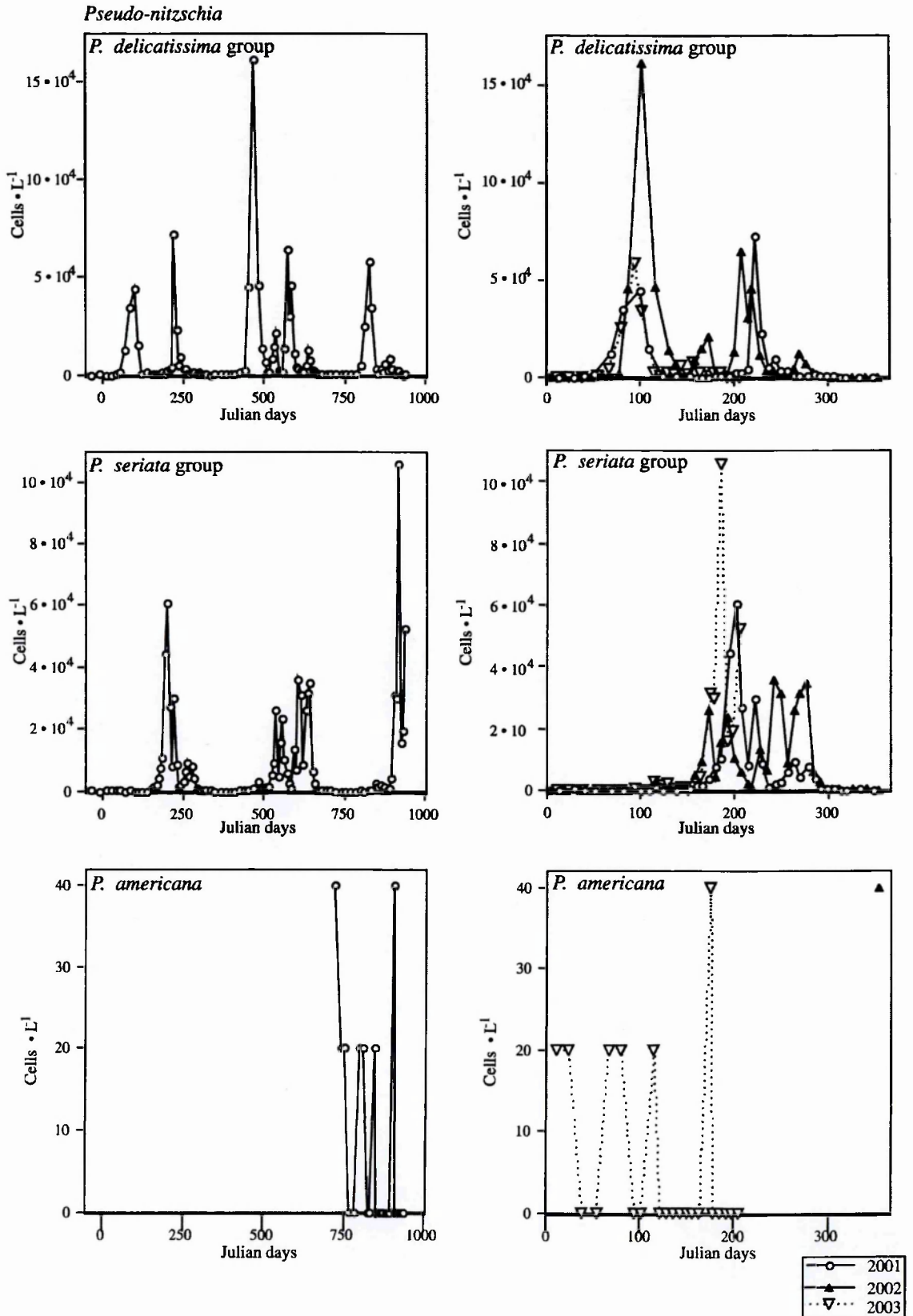
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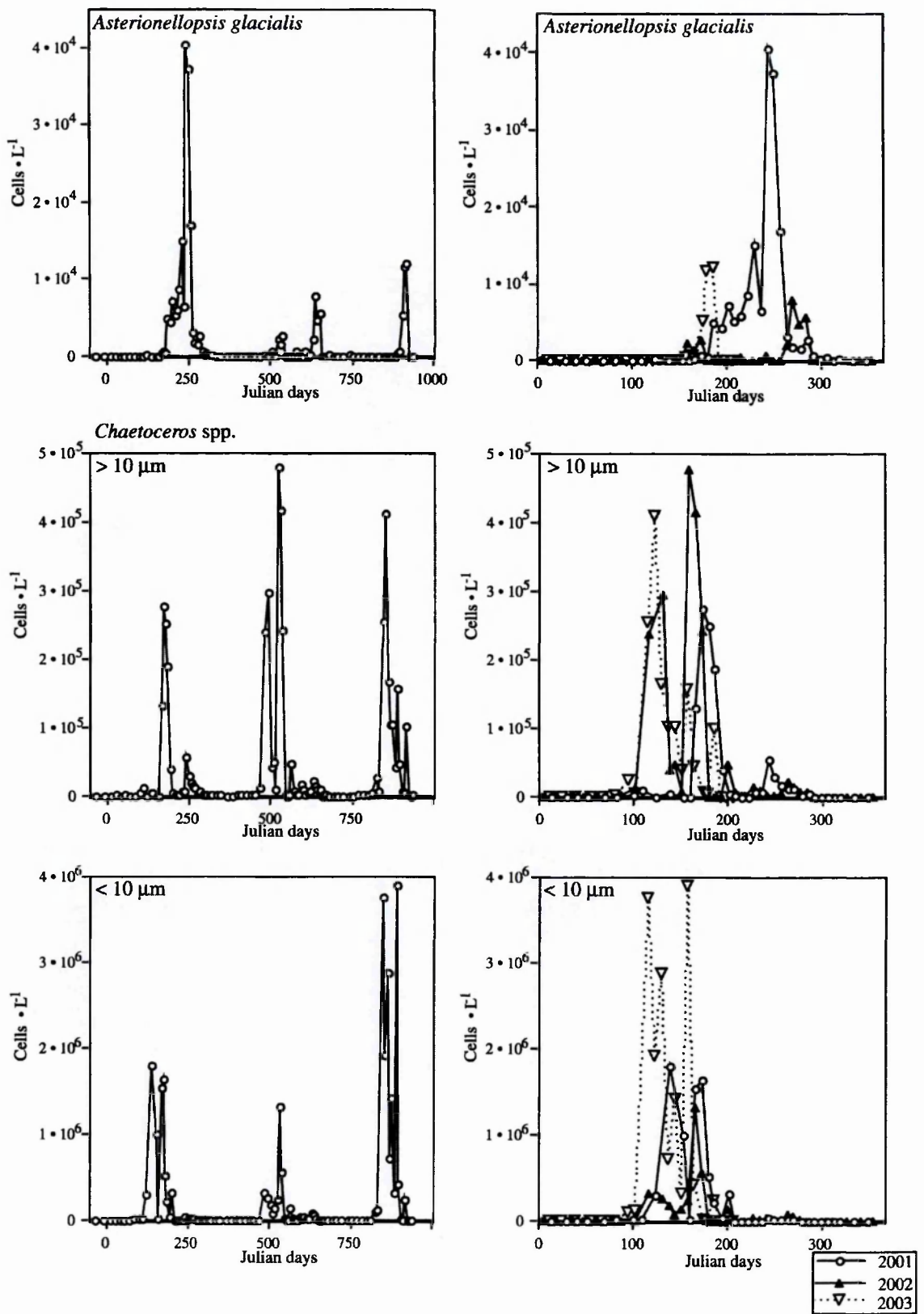
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Appendix 1: Phytoplankton counts at LY1

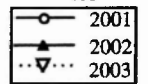
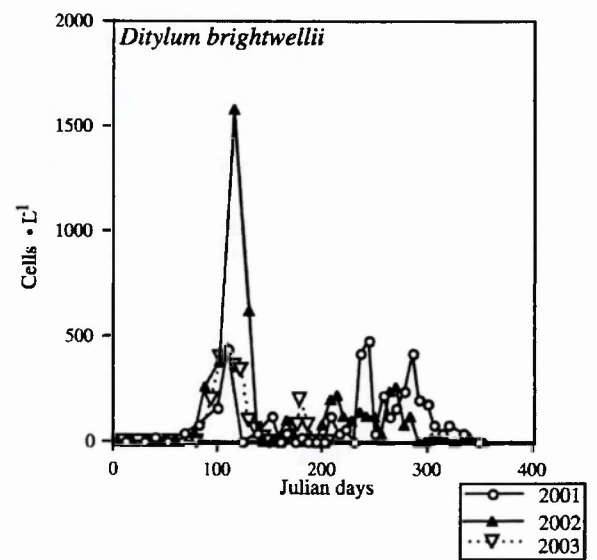
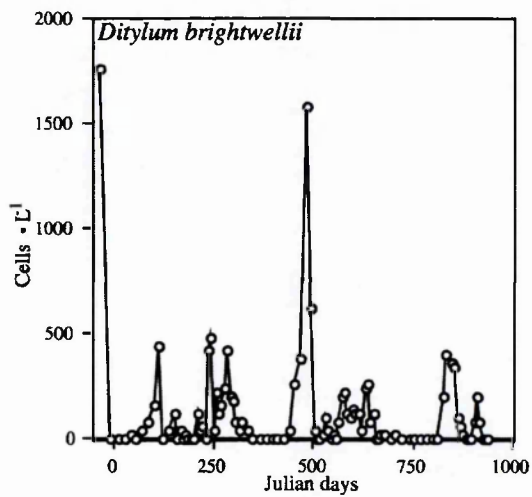
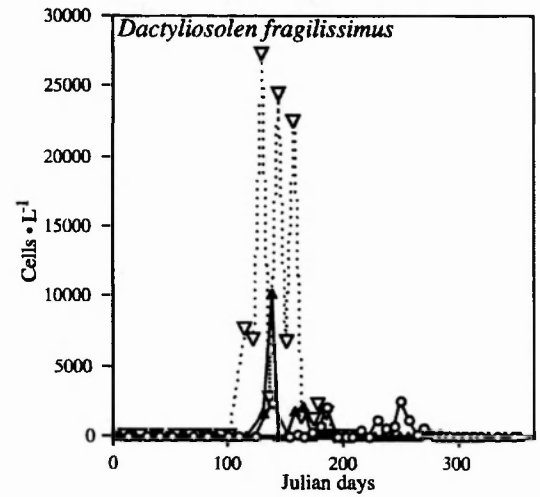
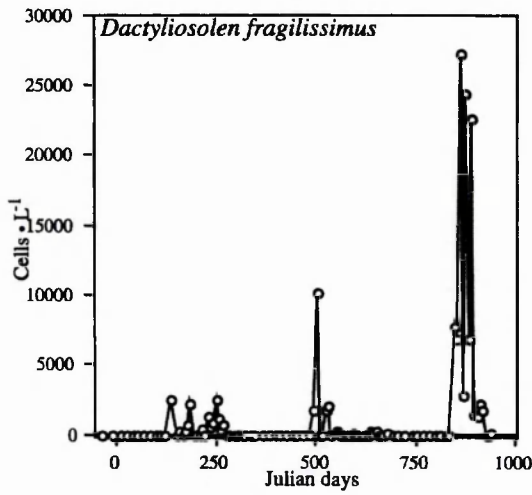
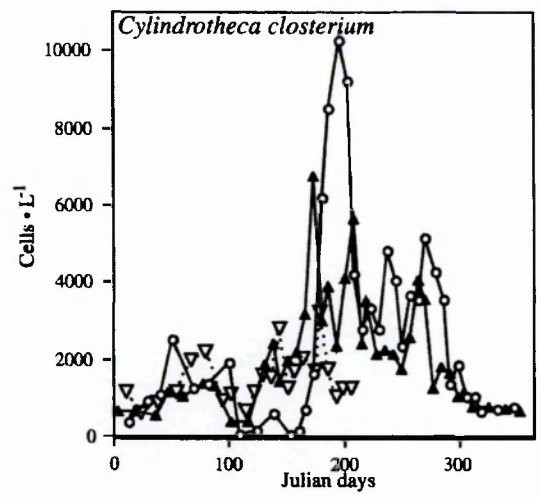
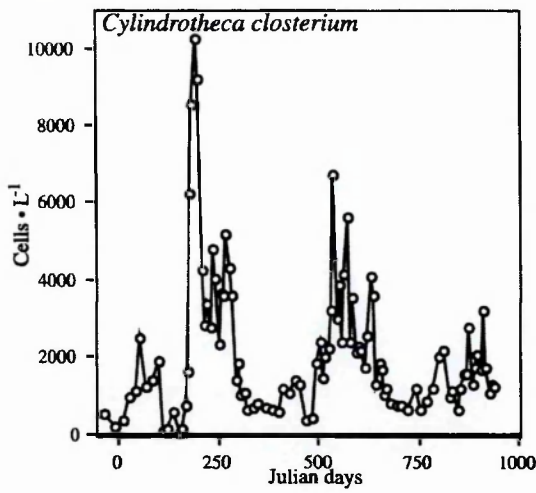
Species abundance [cells · L⁻¹] at LY1 stations. For each species cell densities are presented in one graph over the sampled period (Nov 2000 to July 2003) and as single, overlaid plots for the years 2001, 2002 and 2003.



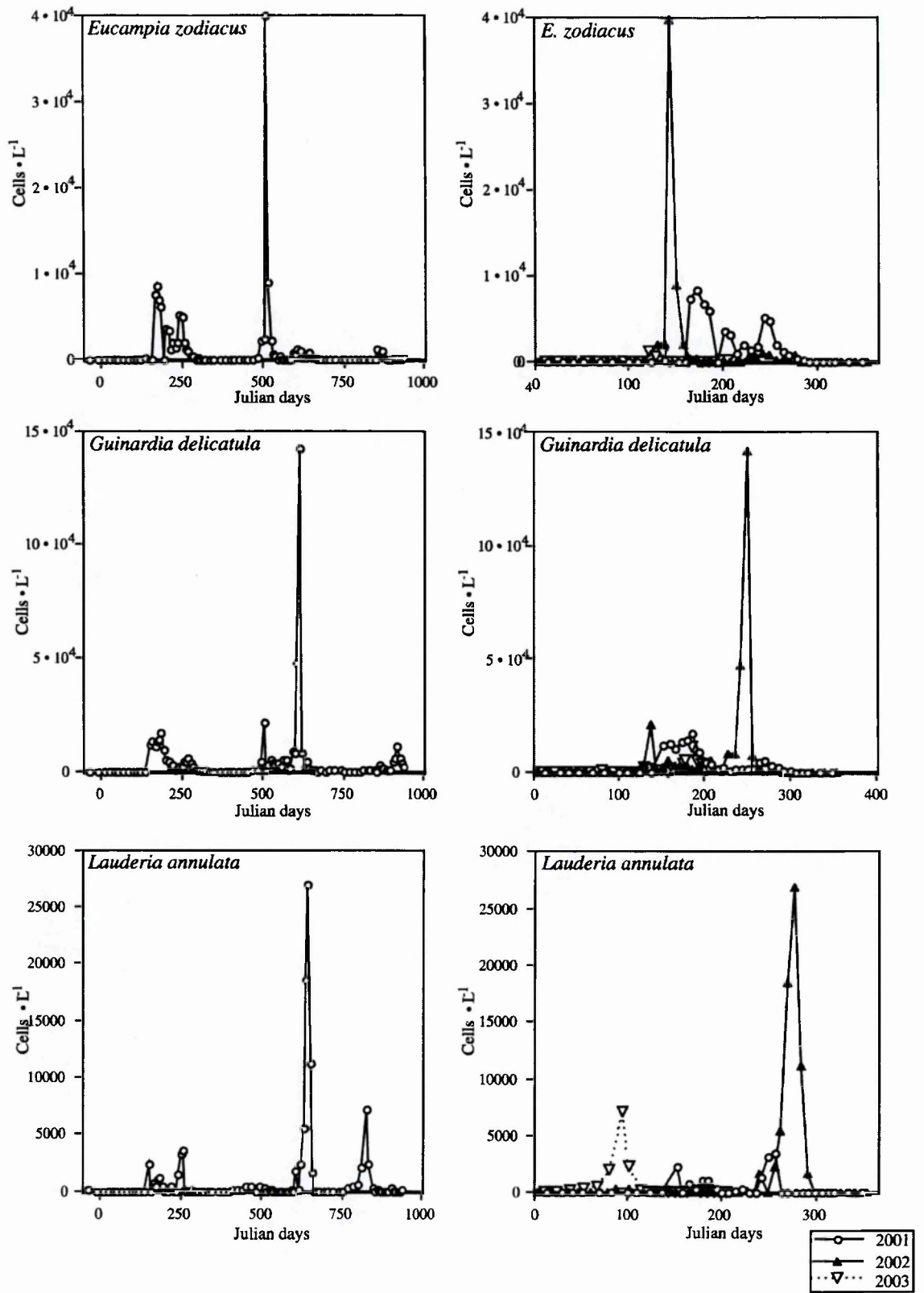
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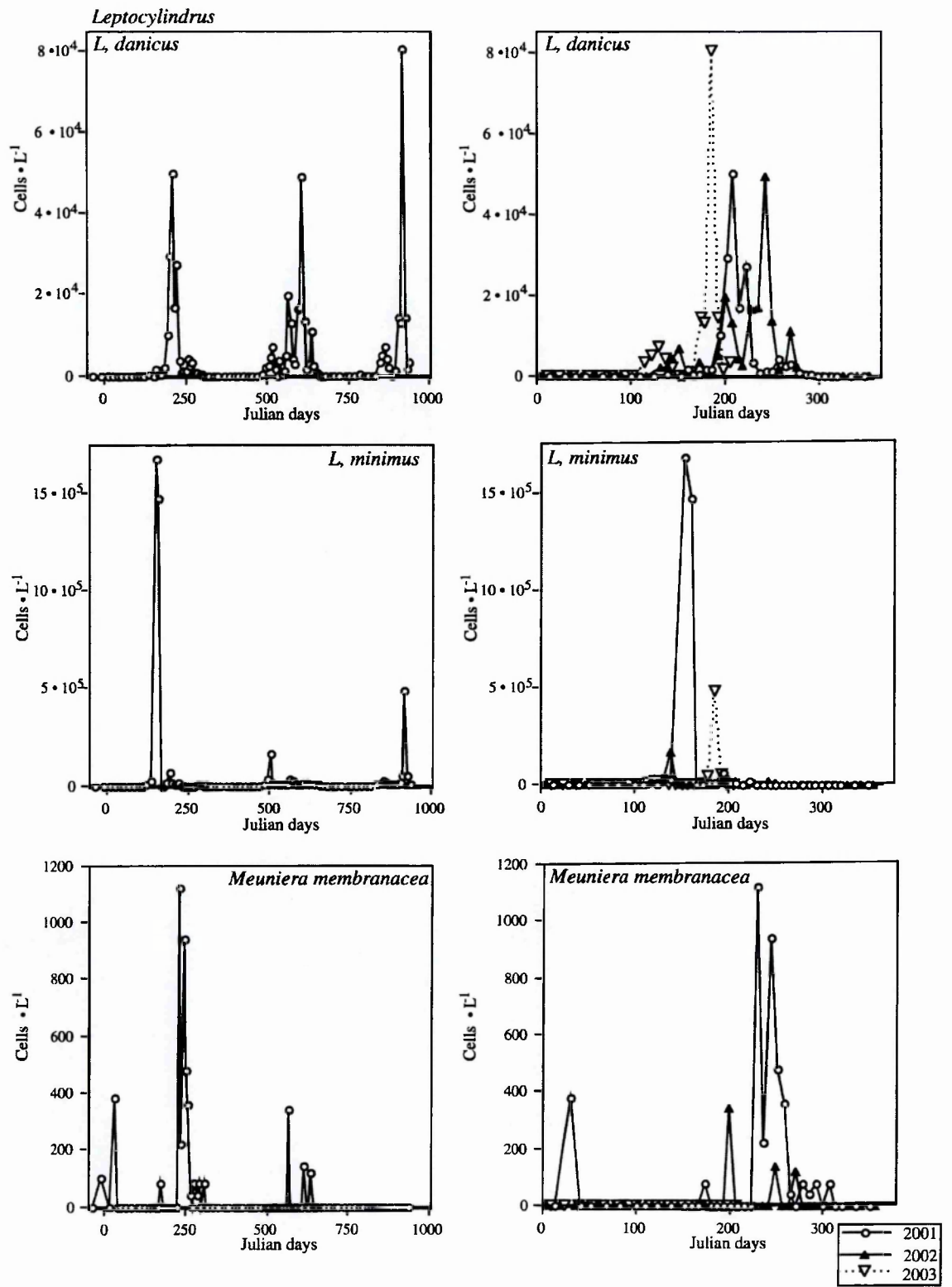
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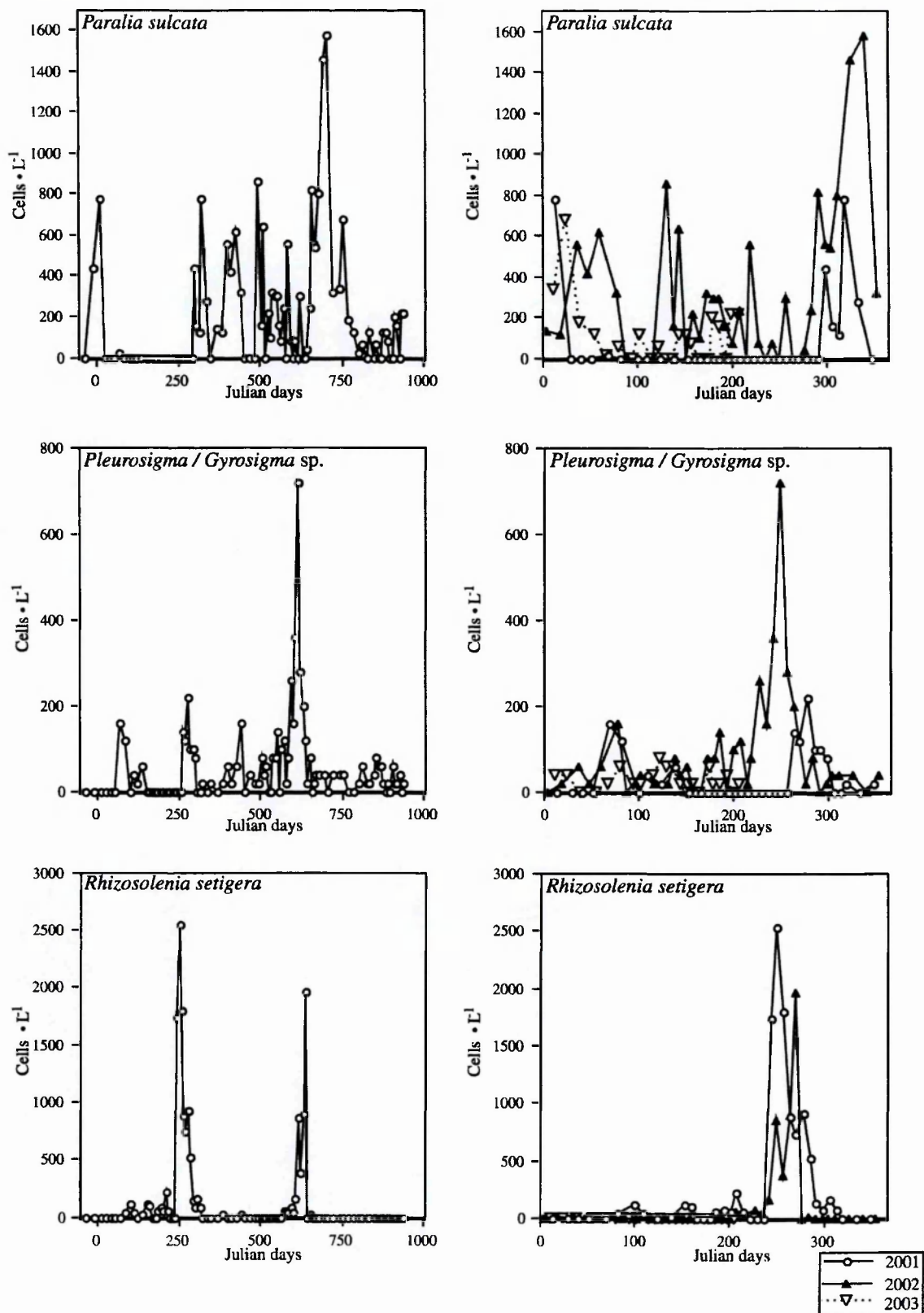
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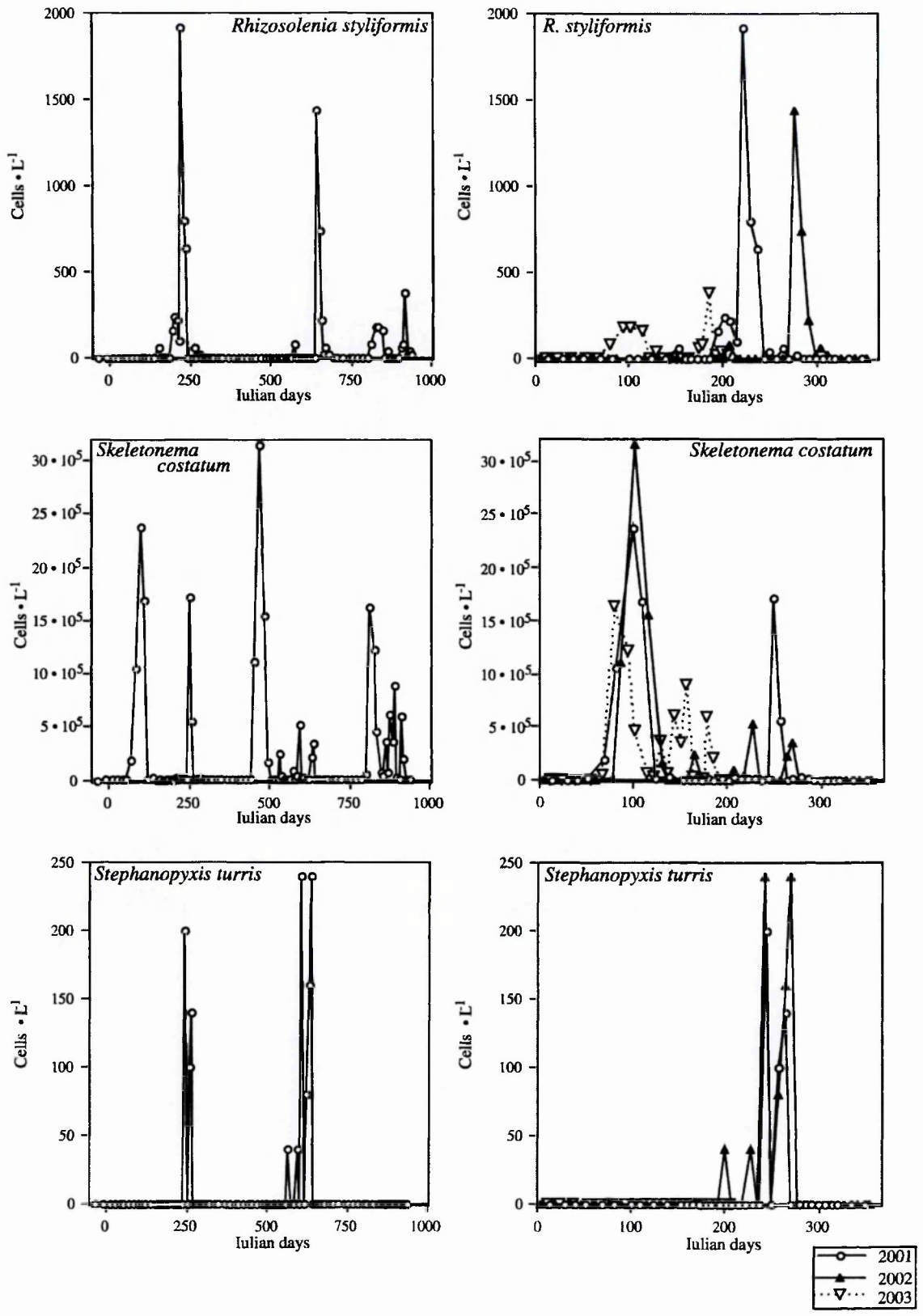
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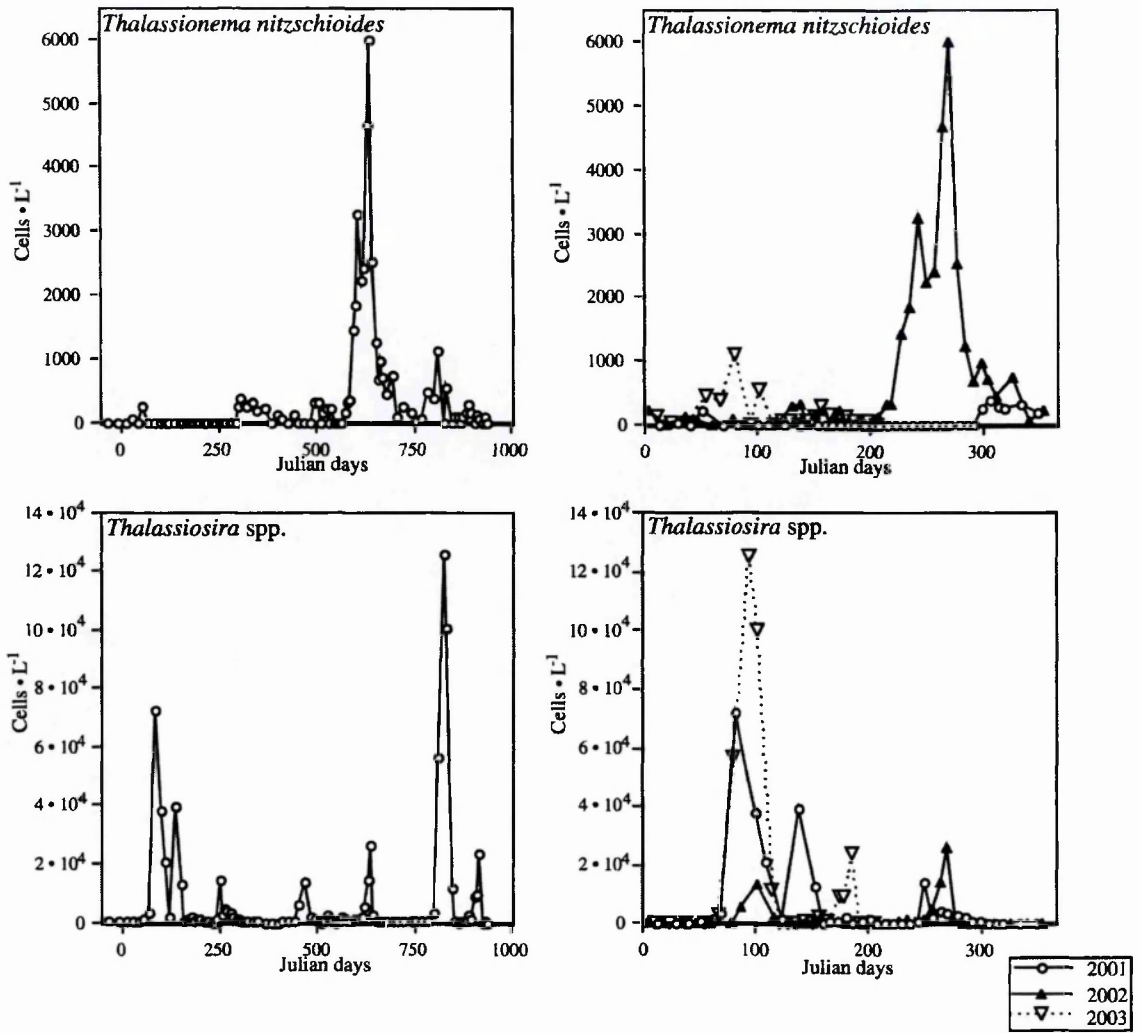
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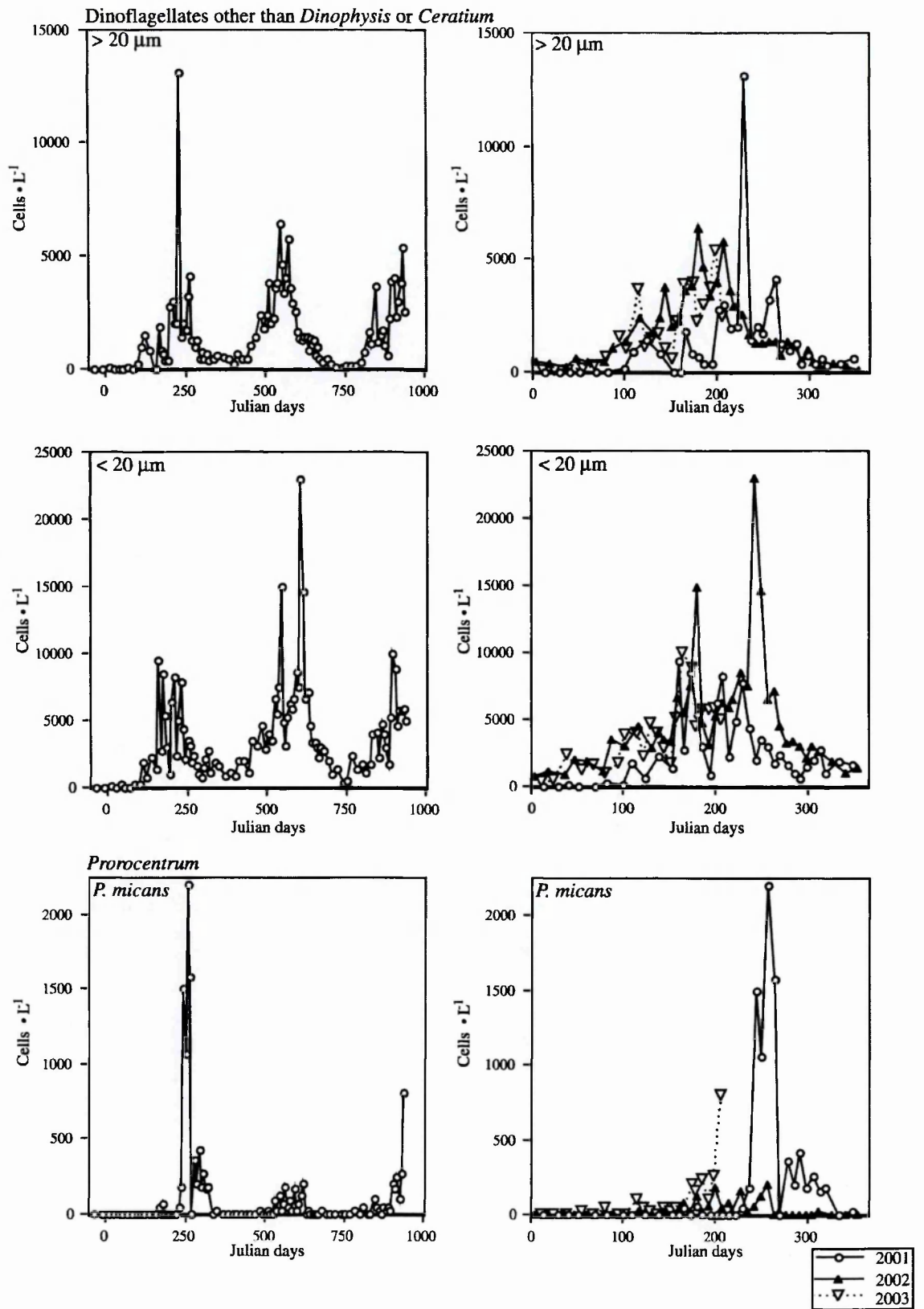
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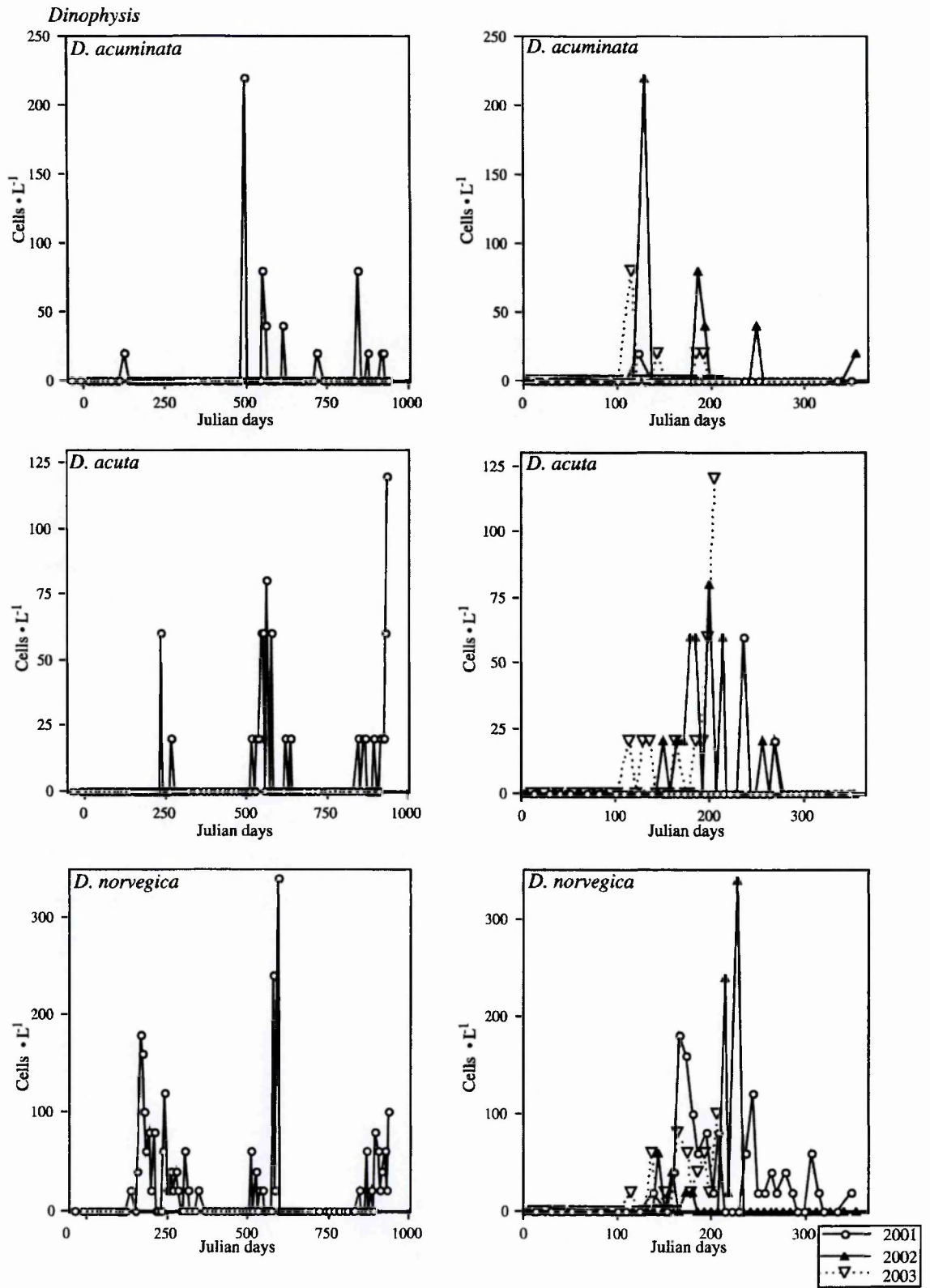
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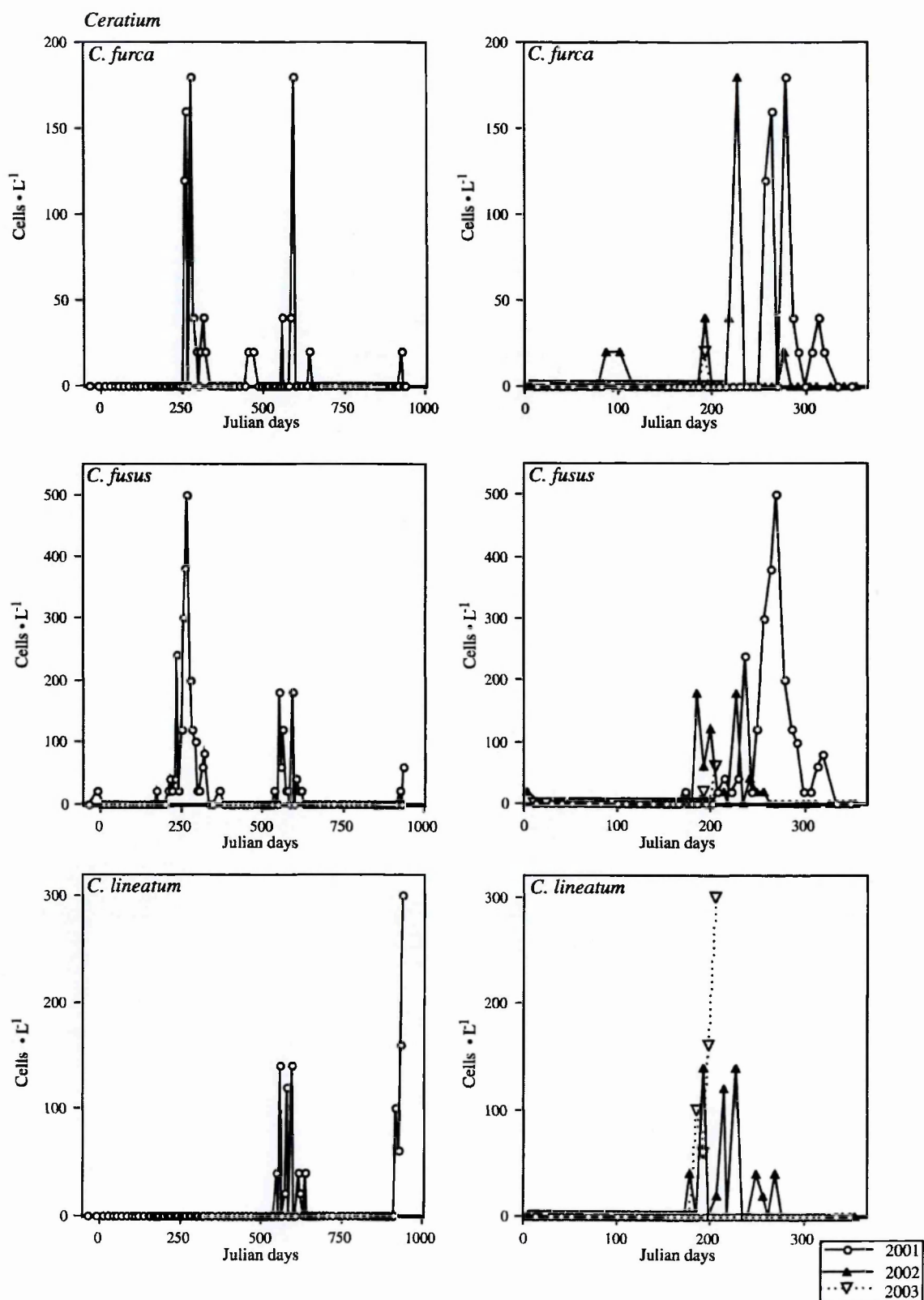
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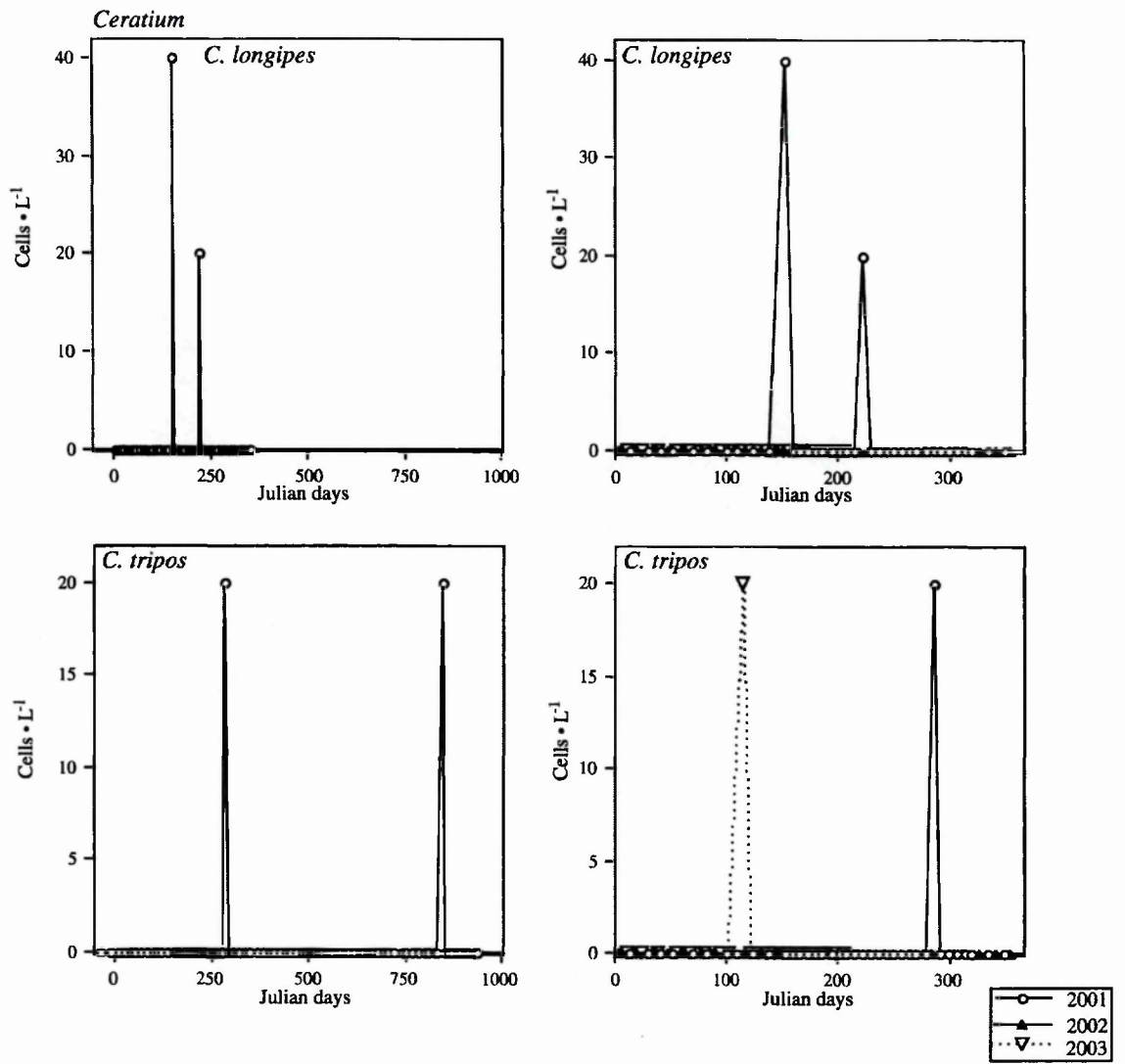
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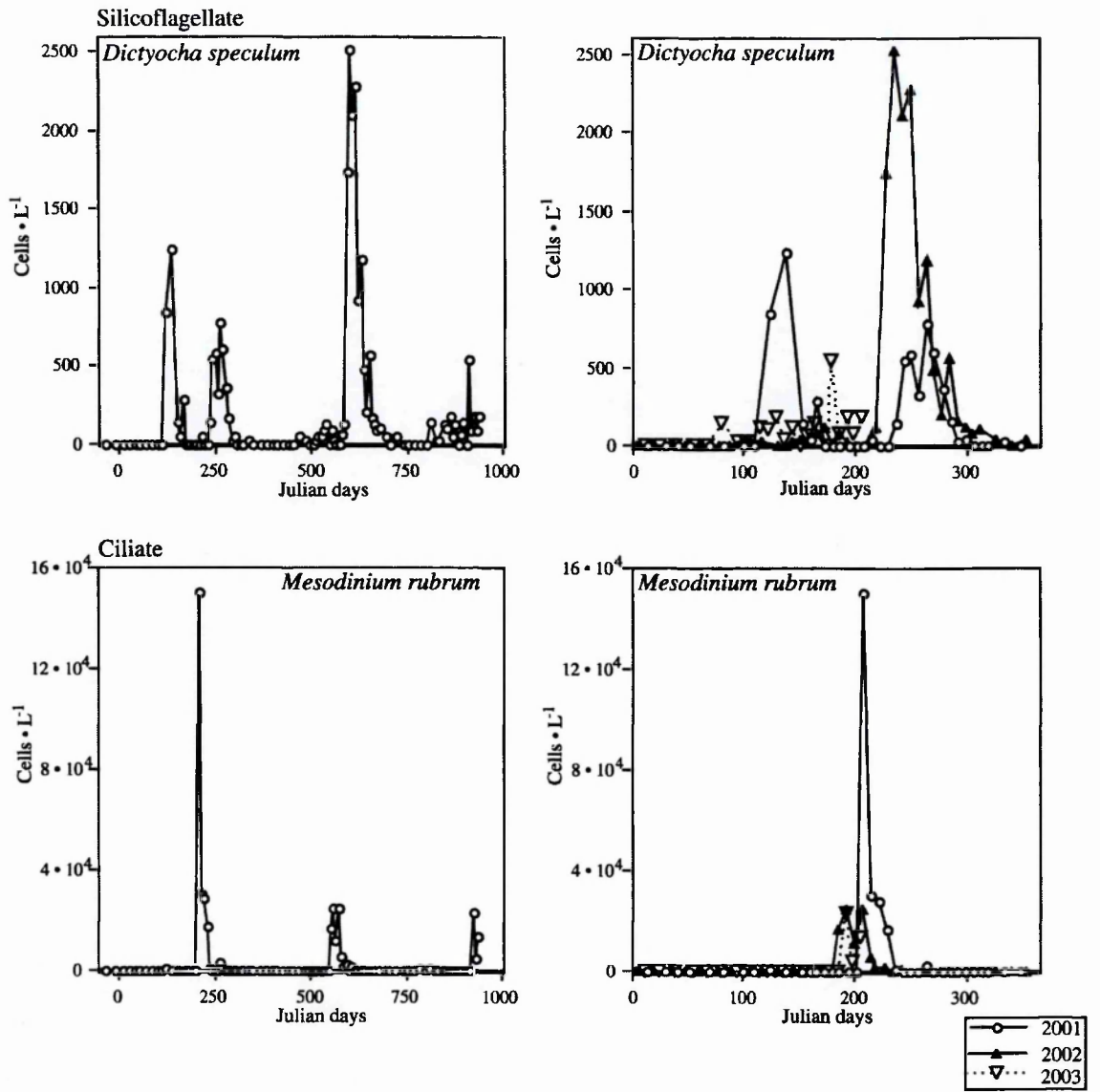
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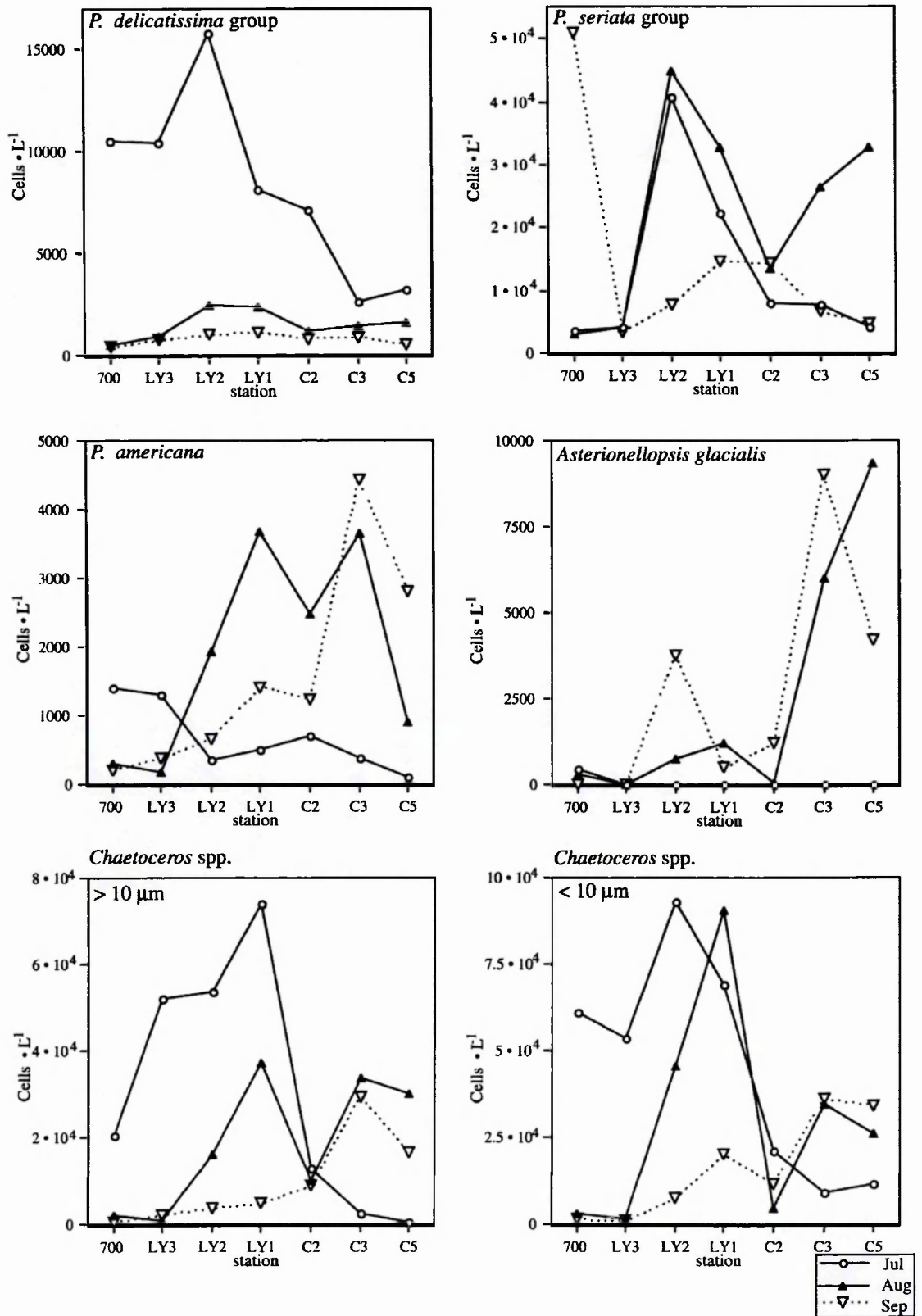


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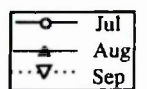
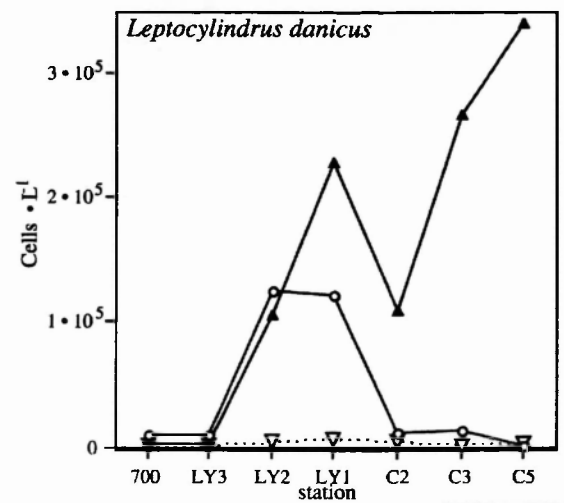
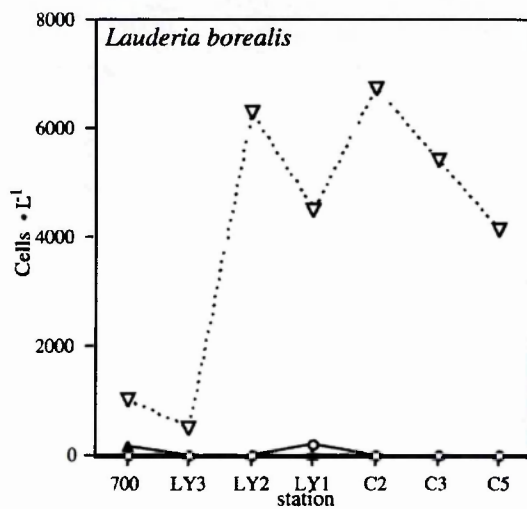
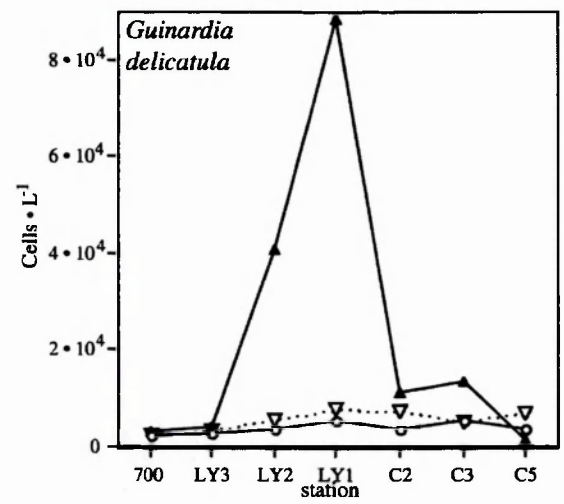
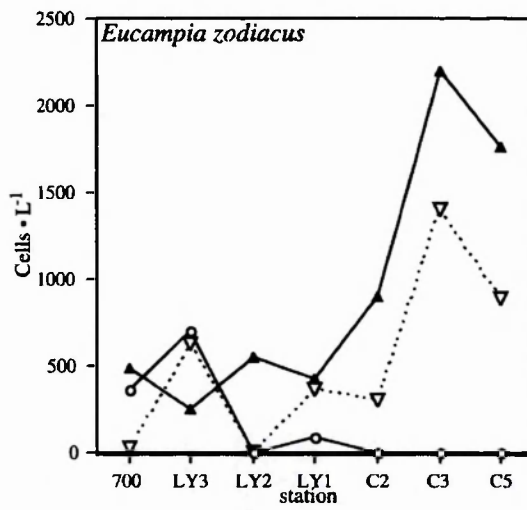
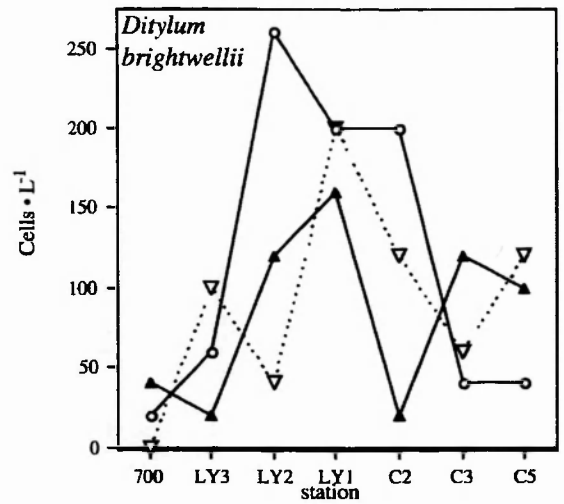
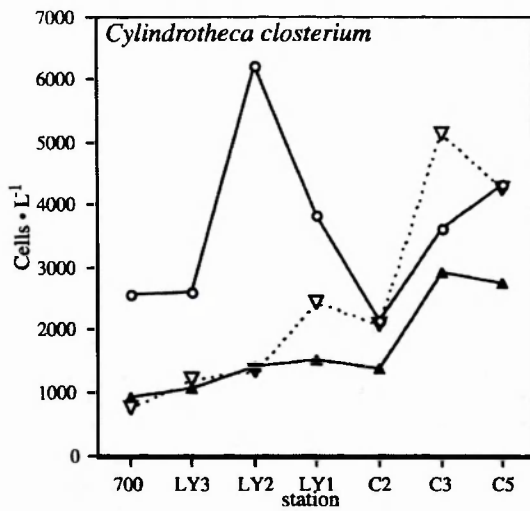


Appendix 2: Phytoplankton counts along the Spelve-Creran transect

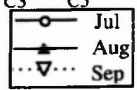
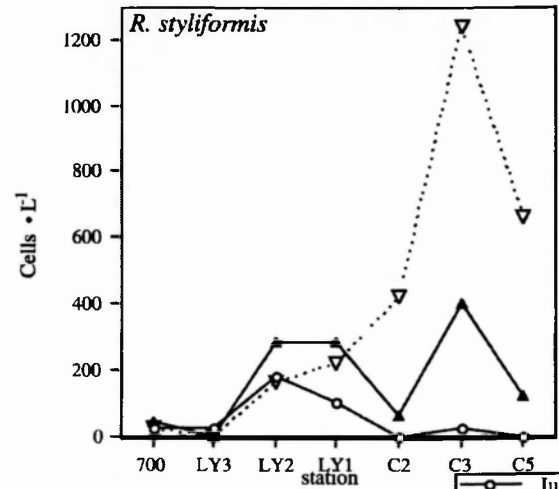
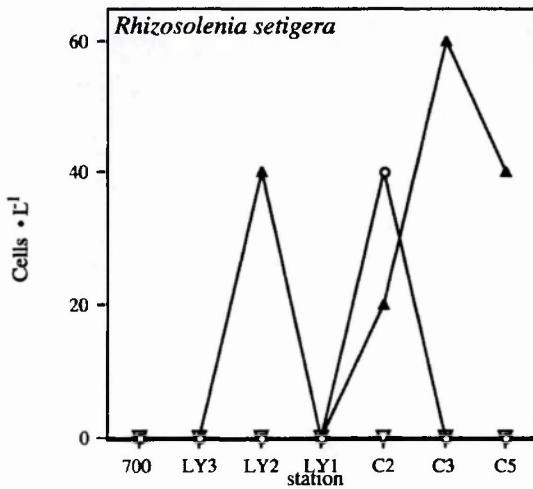
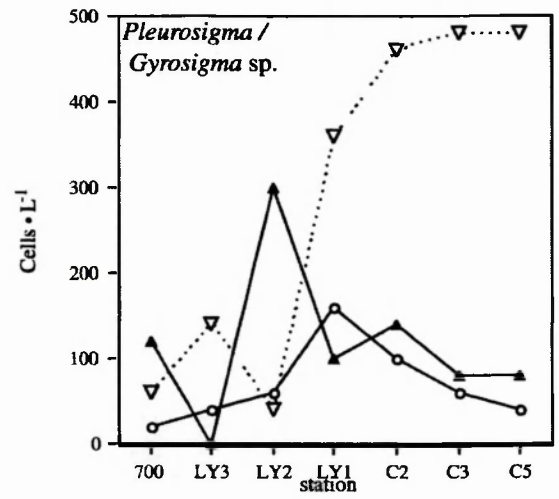
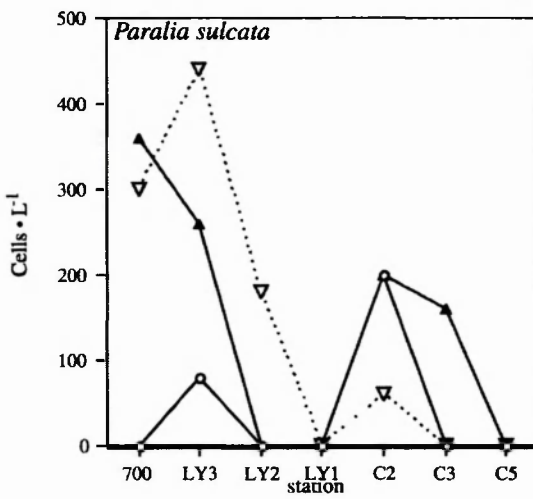
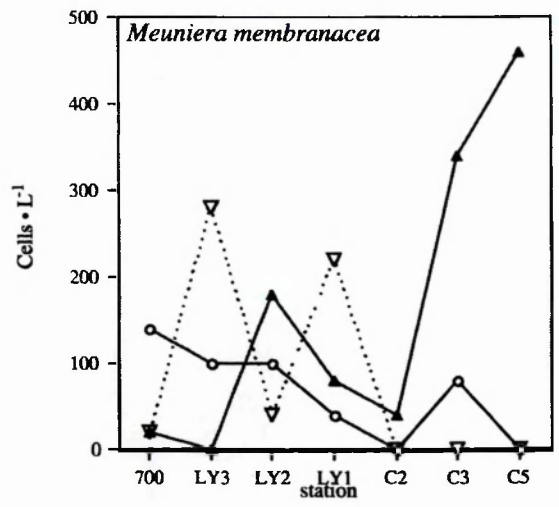
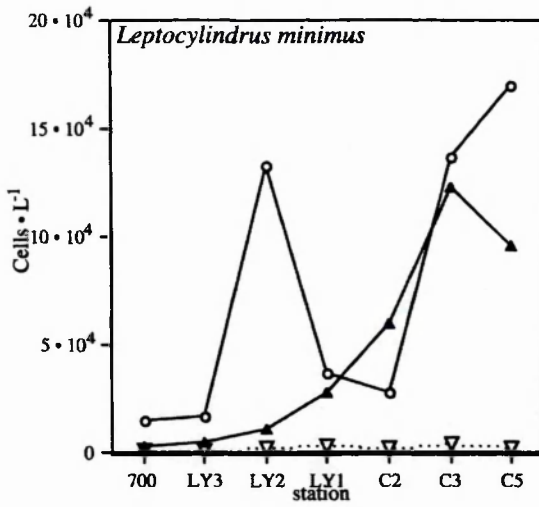
Species abundance [cells · L⁻¹] along the Spelve-Creran transect in July (circles) August (closed triangles) and September (open triangles) 2002.



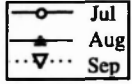
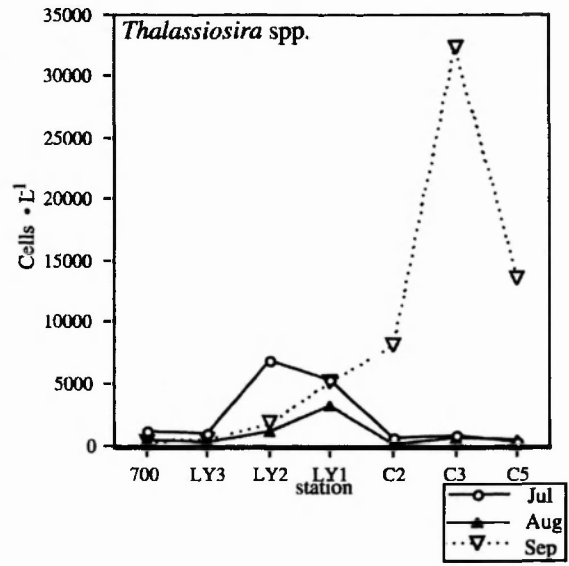
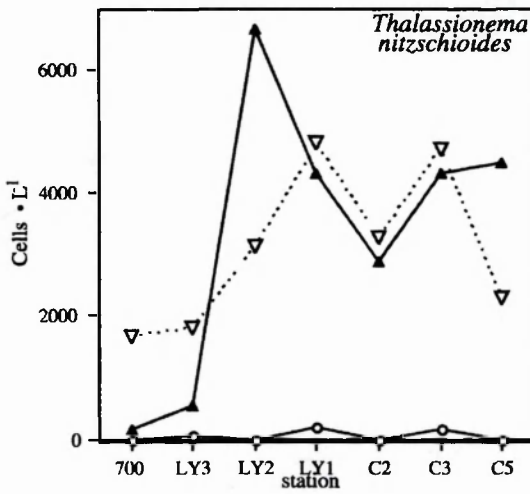
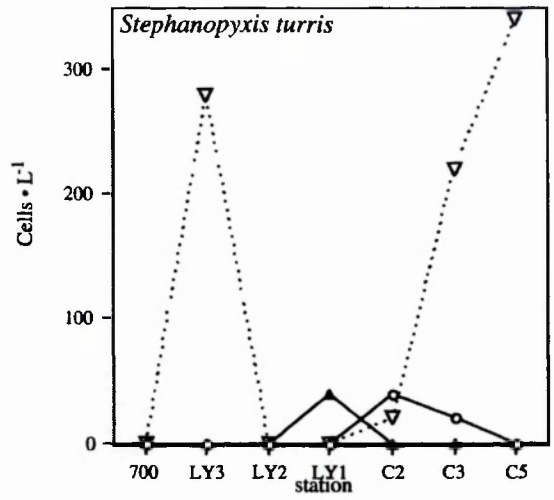
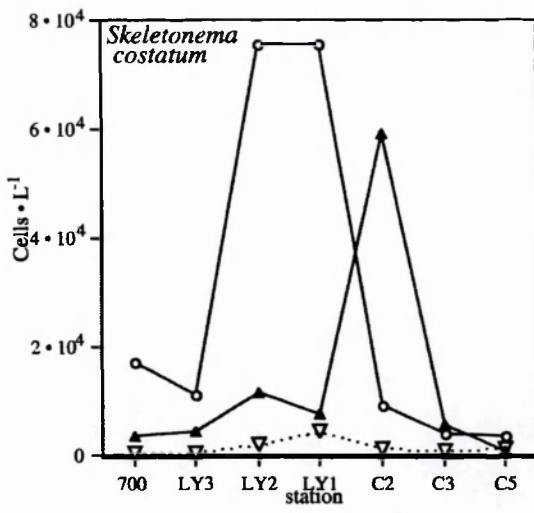
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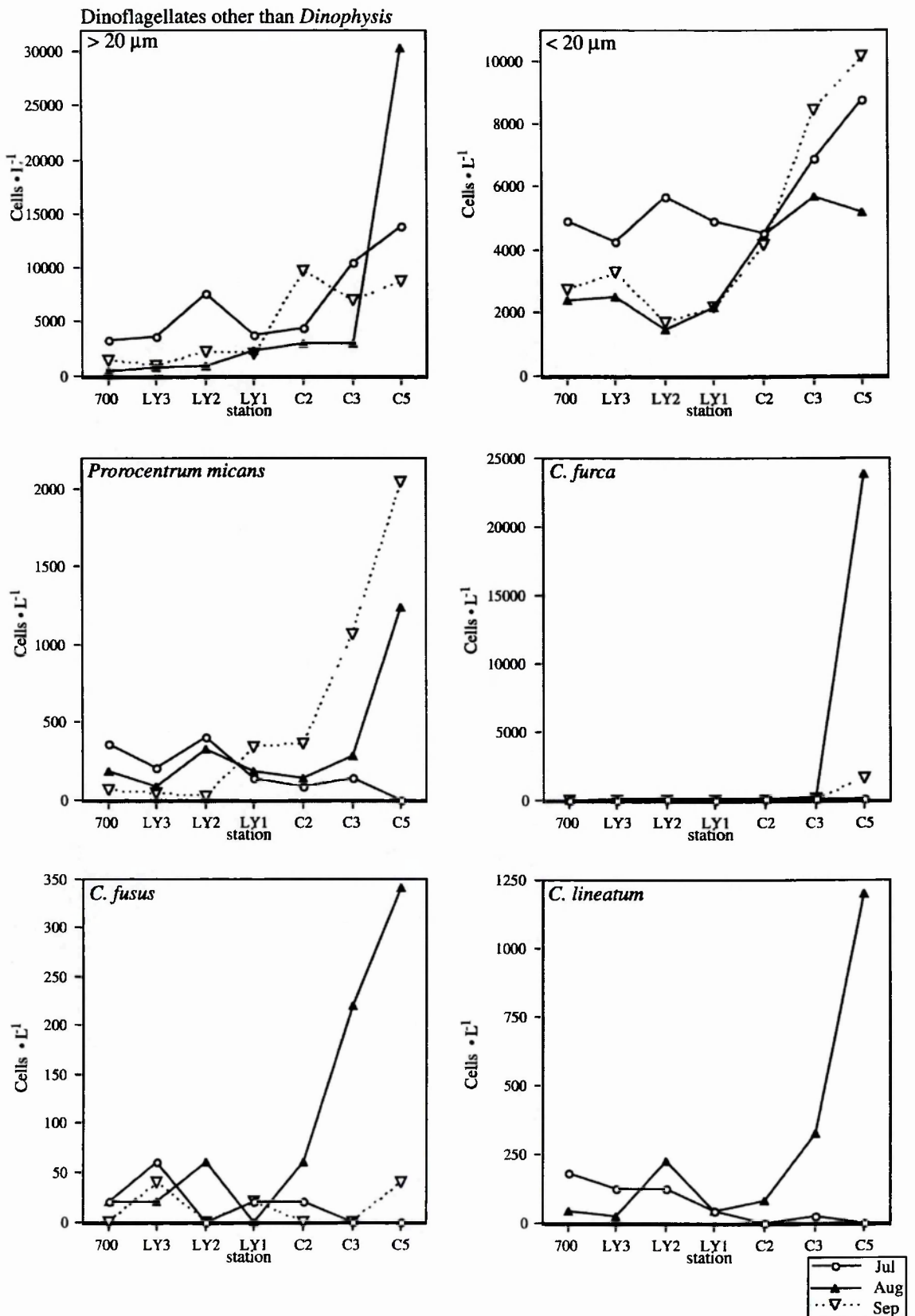
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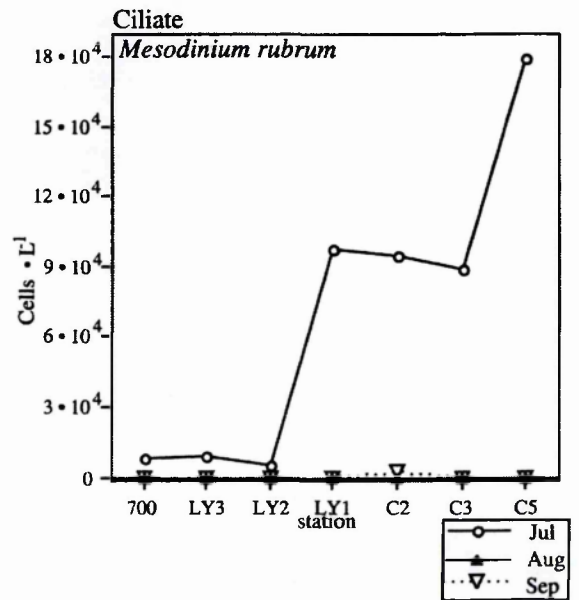
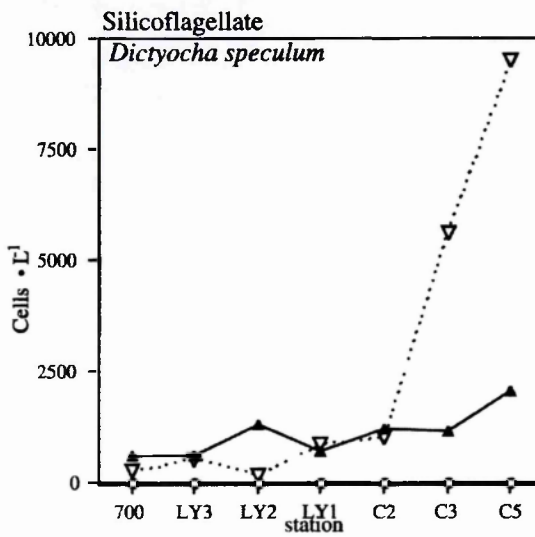
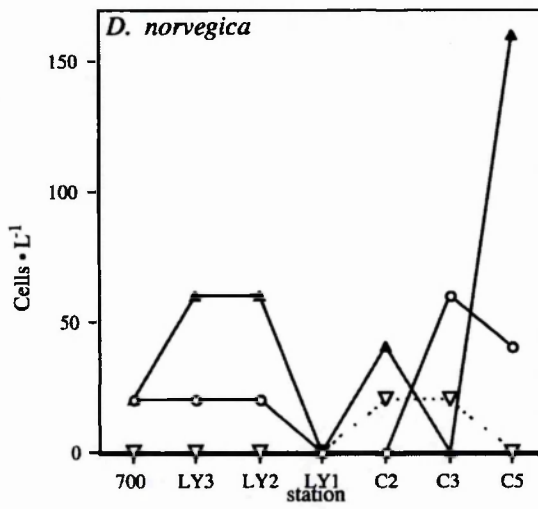
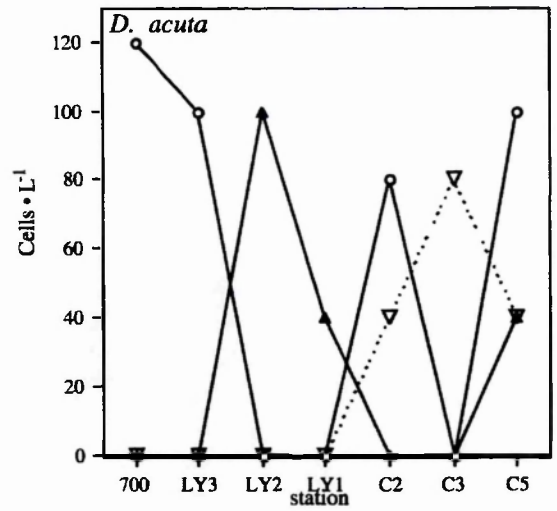
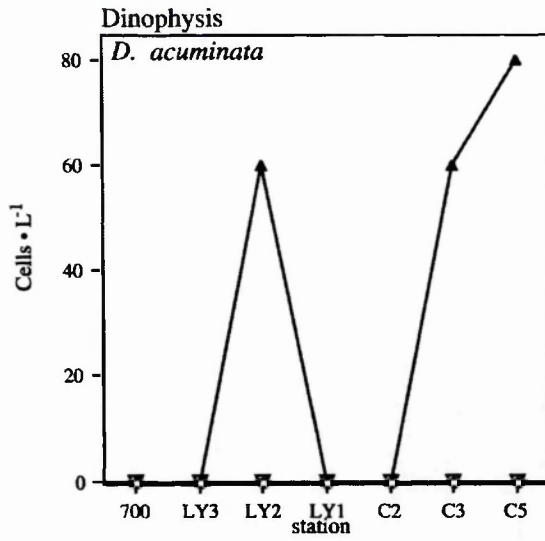
Appendix 2



Appendix 2

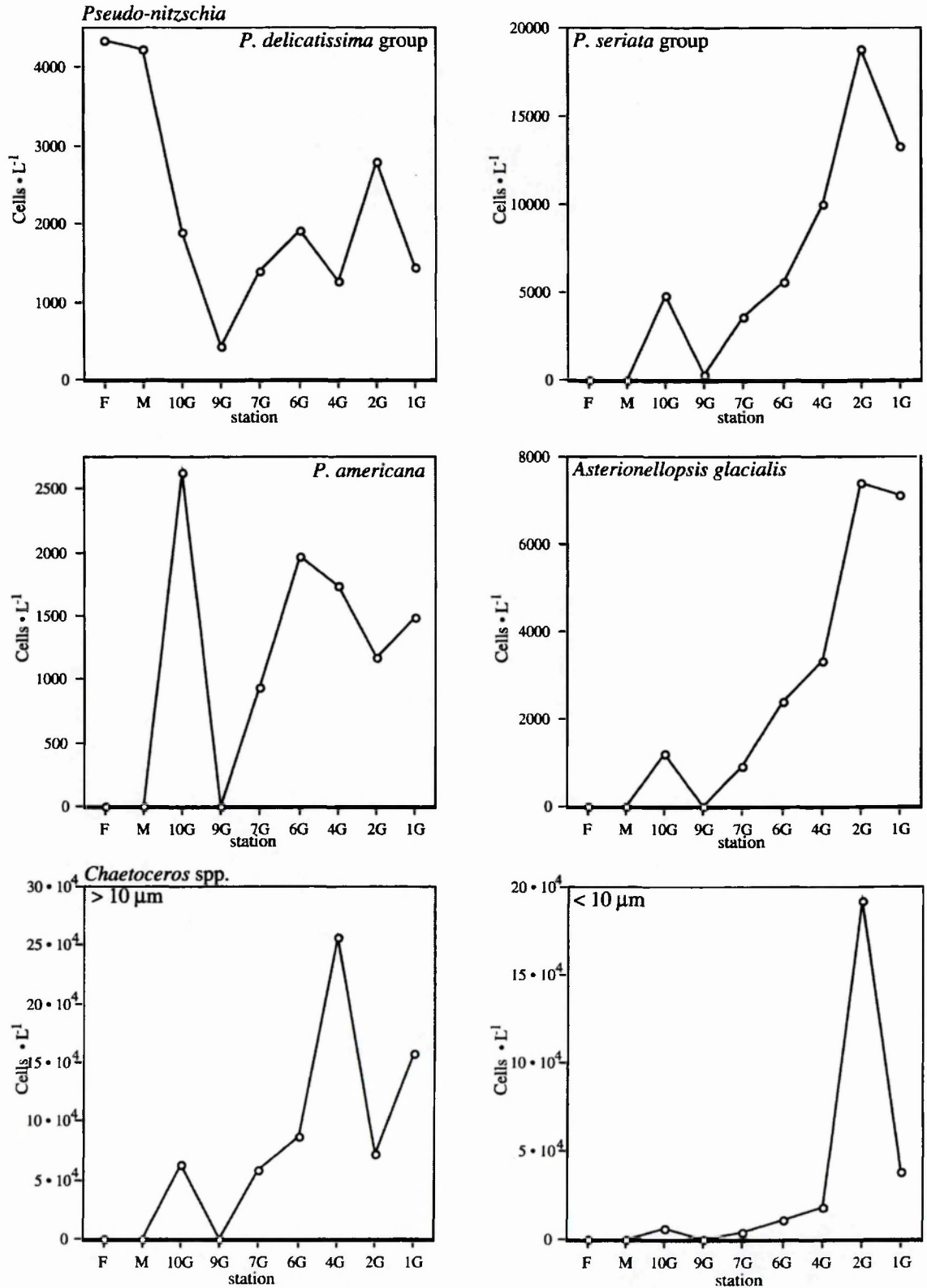


Appendix 2

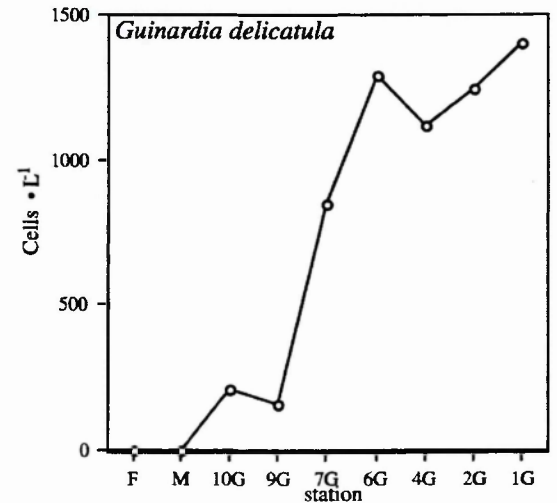
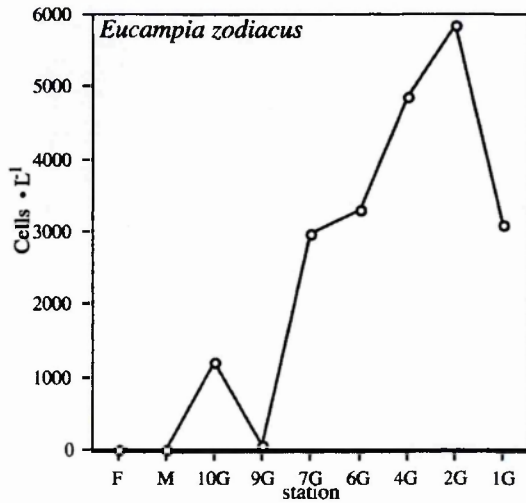
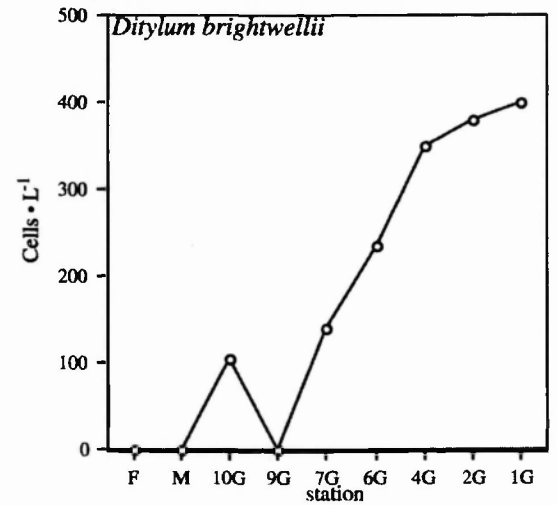
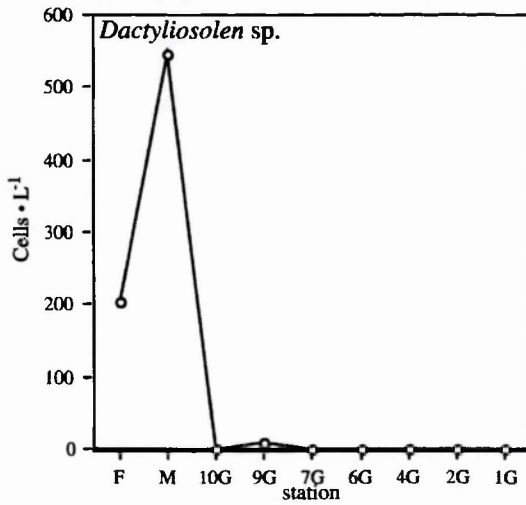
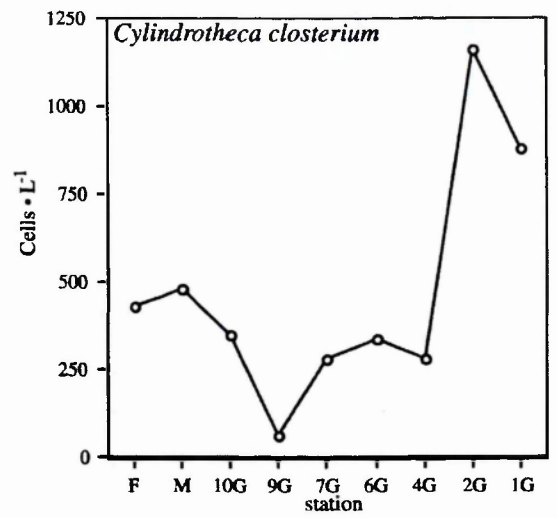
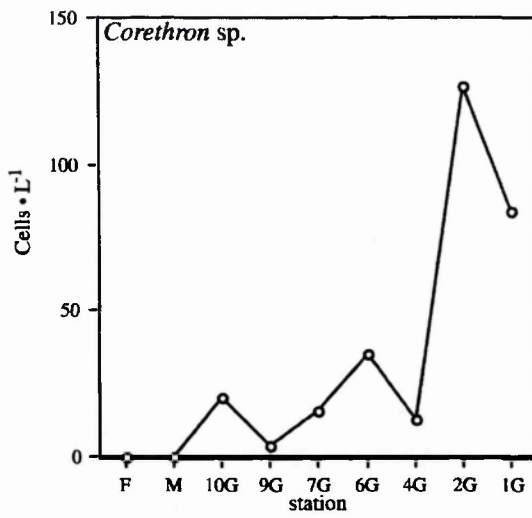


Appendix 3: Phytoplankton counts along the Ellett Line

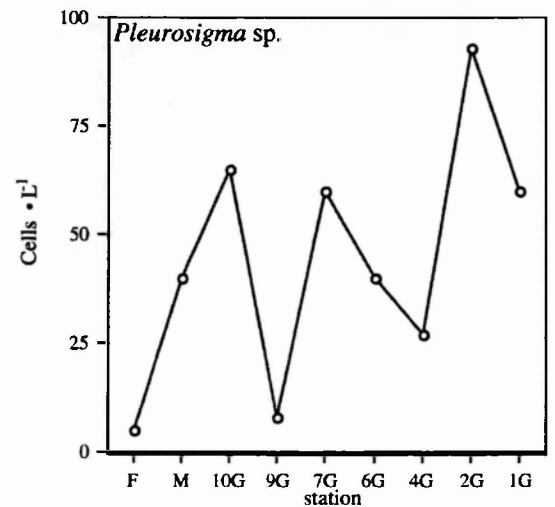
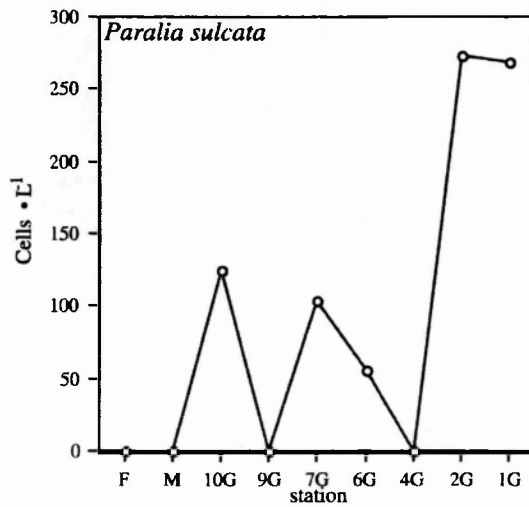
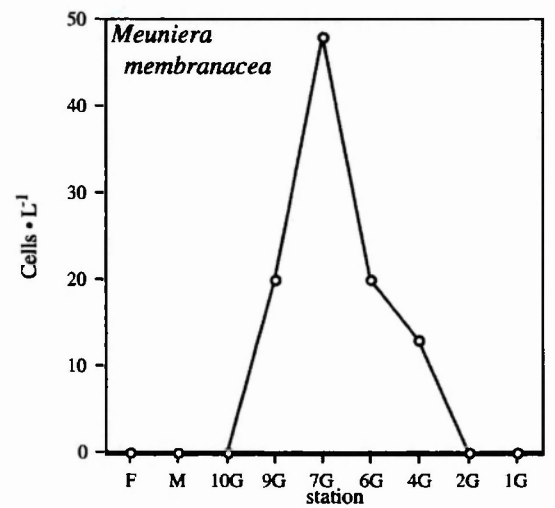
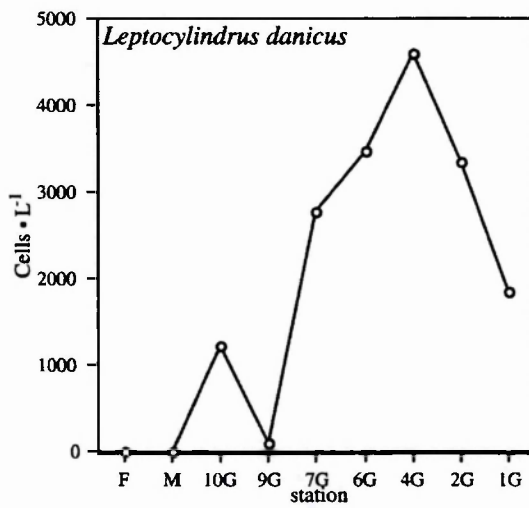
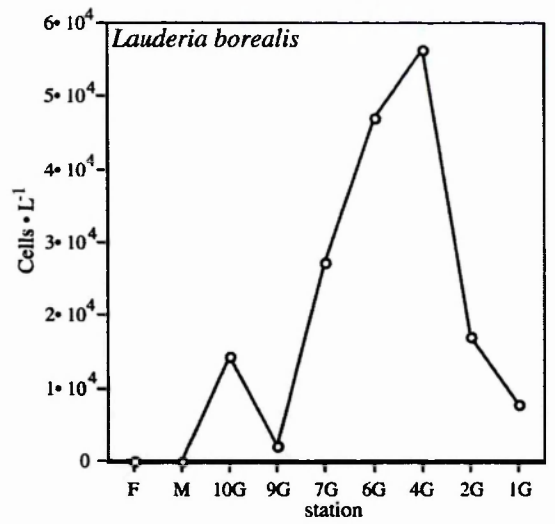
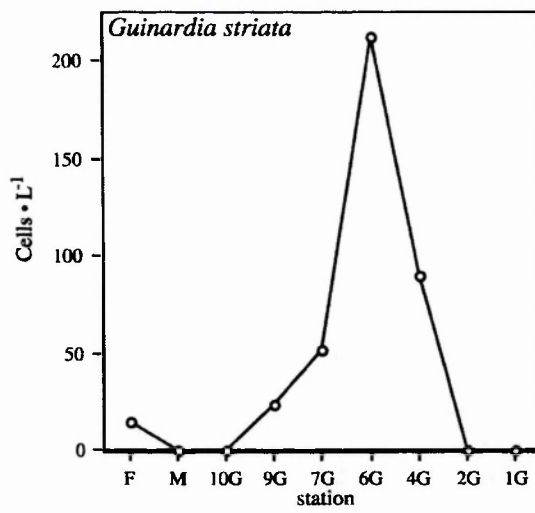
Species abundance [cells · L⁻¹] at Ellett Line stations during cruise D257 in autumn 2001. Mean of cell densities in samples of the top 100 m water column are plotted.



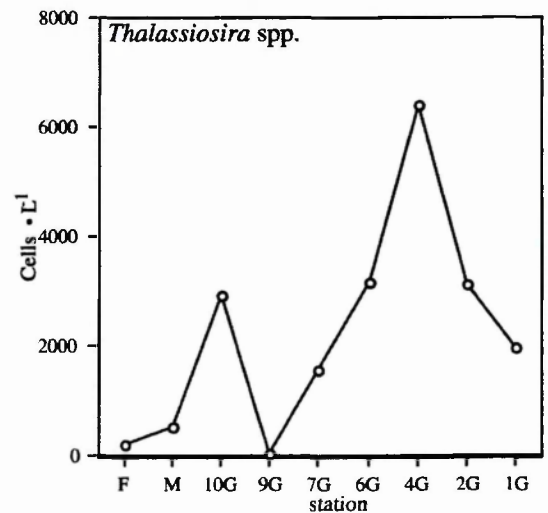
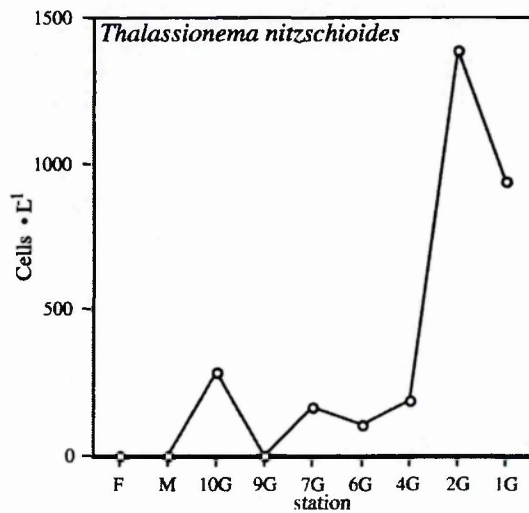
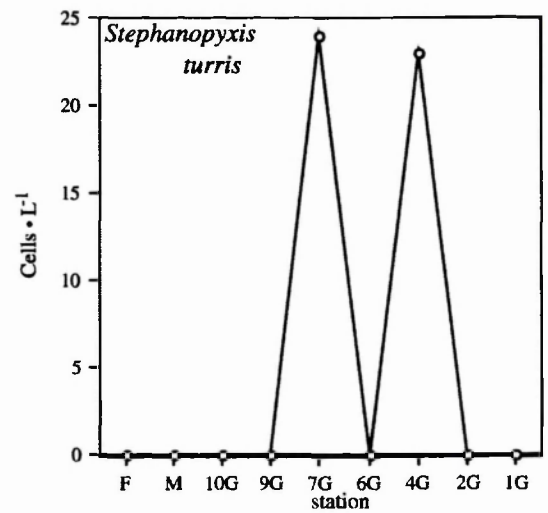
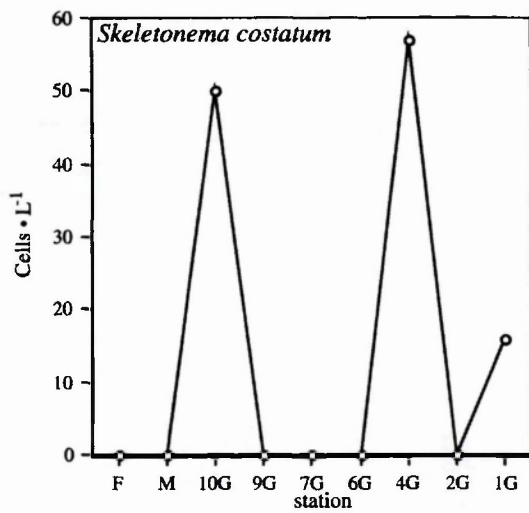
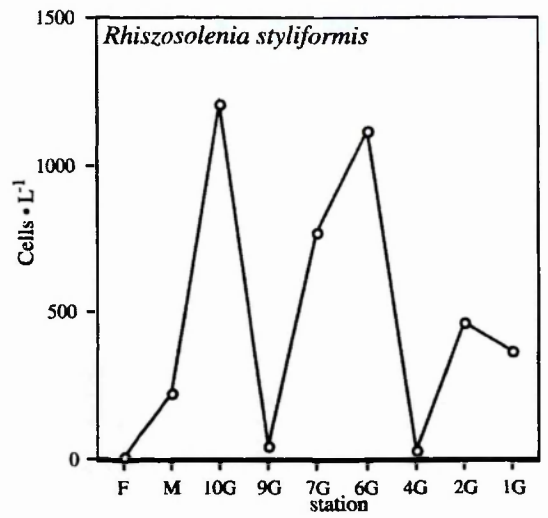
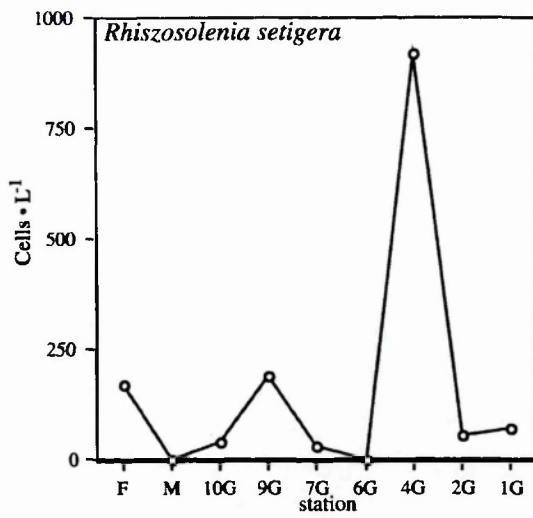
Appendix 3



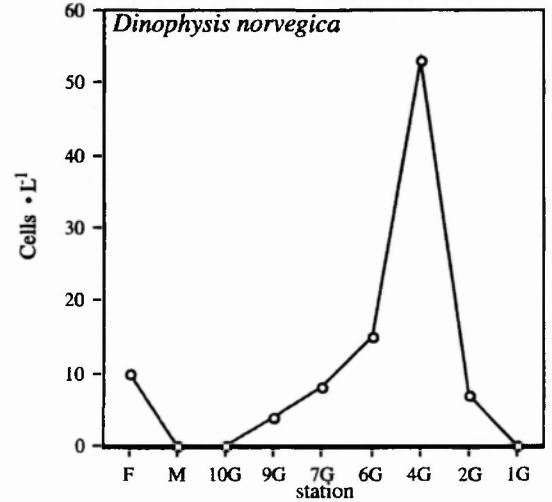
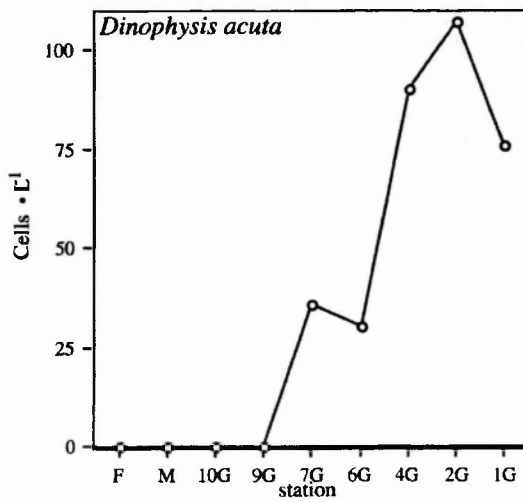
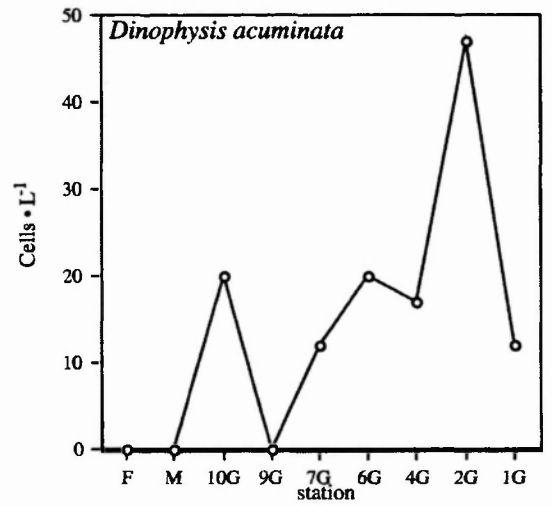
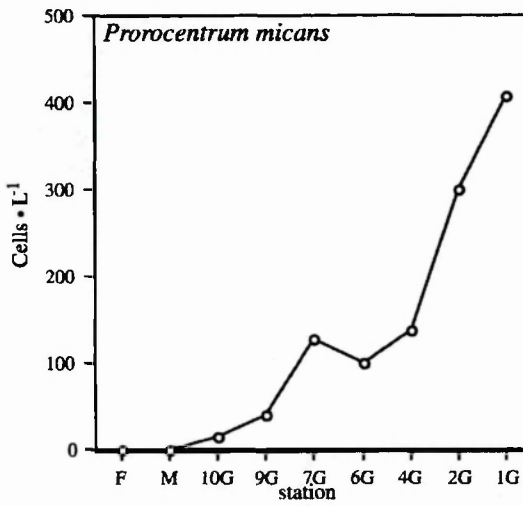
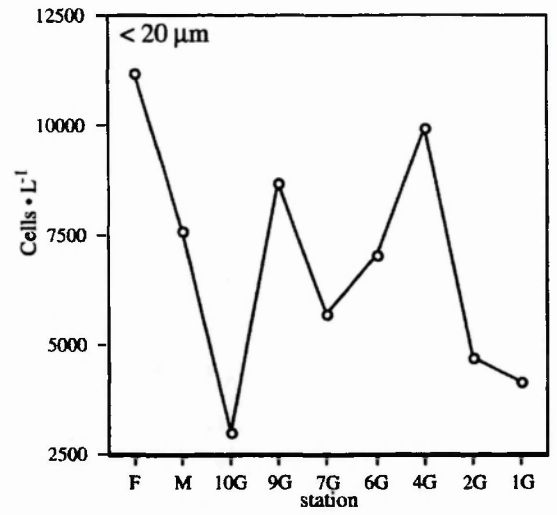
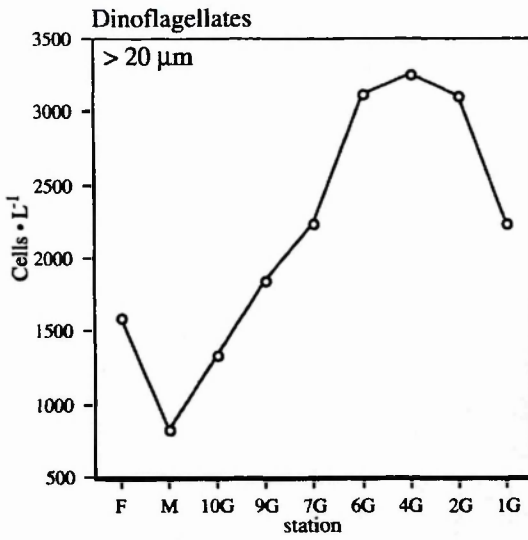
Appendix 3



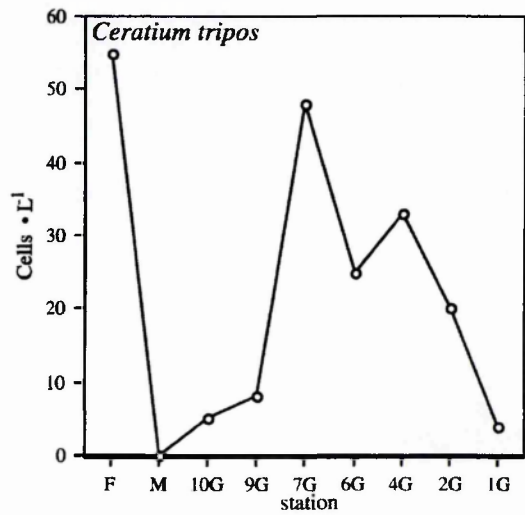
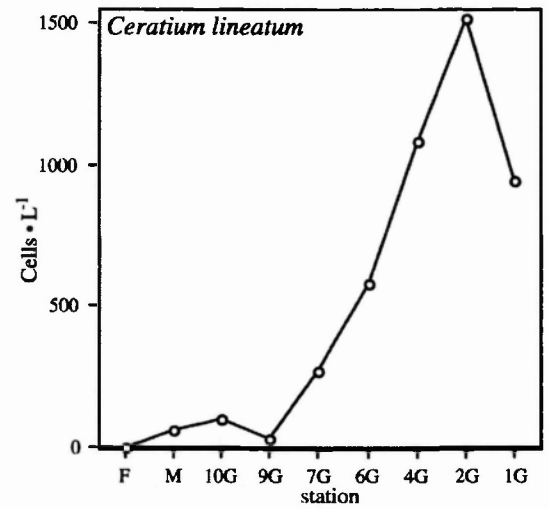
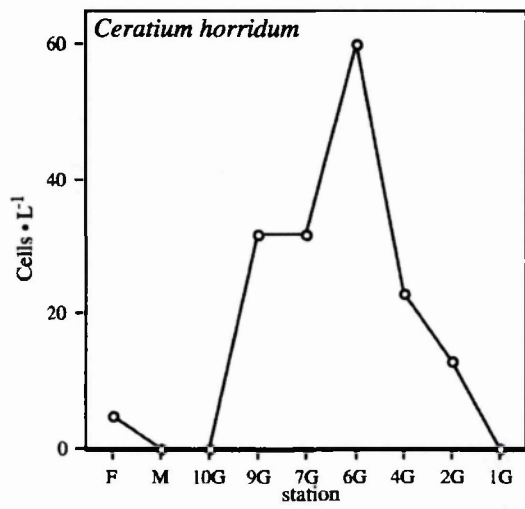
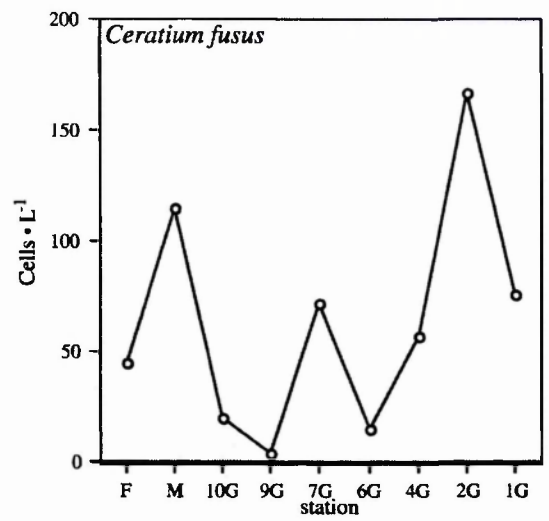
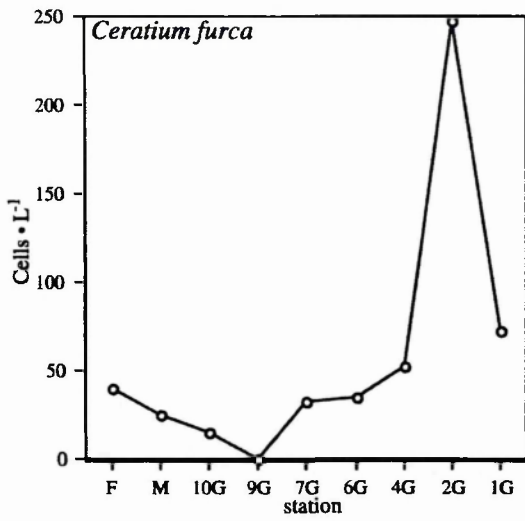
Appendix 3



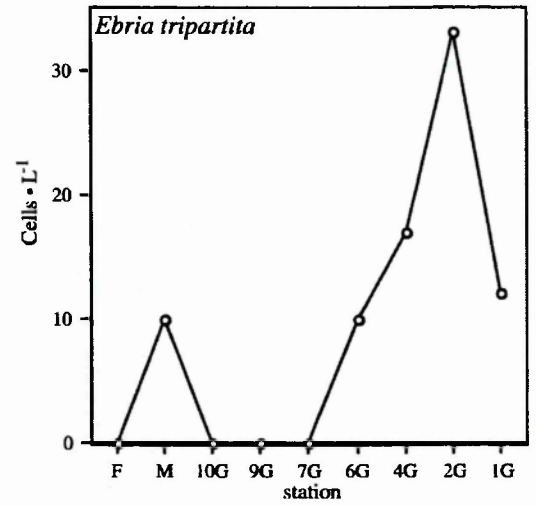
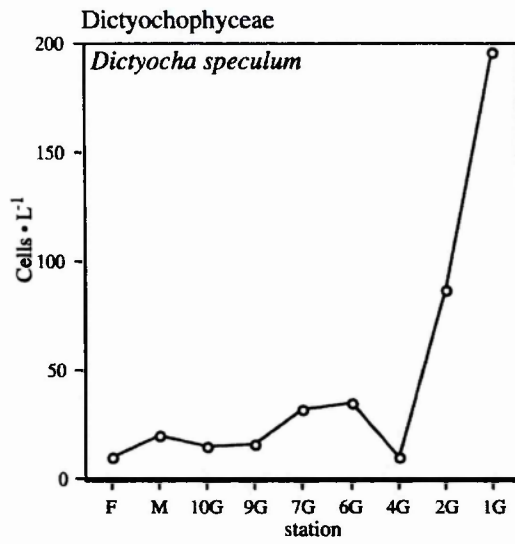
Appendix 3



Appendix 3



Appendix 3



Appendix 4: Alignment of *Pseudo-nitzschia* sequences (including the outgroup *Cylindrotheca closterium*) for phylogenetic analysis

Species	Abbreviation	represented by sequence of strain
<i>Cylindrotheca closterium</i>	cyl	AF289049
<i>P. seriata</i>	ser	PLY1St.52B
<i>P. australis</i>	aust	PLY1St.19A
<i>P. fraudulenta</i>	fraud	PLY1St.15A
<i>P. delicatissima</i>	deli	P orkney9
<i>P. cf. subpacifica</i>	subpac	D257F
<i>P. pungens</i>	pung	Ppung6

Matrix:

	10	20	30	40	50	60
cyl	---TAACAAGGTTCCG	TAGTGAACCTGCGGA	AAGGATCATTACCAC	ACCGATCCAAGATCT		
ser	-----	GAACCTGCGGA	AAGGATCATTACCAC	ACCGATCCAAGATCA		
aust	-----	GAACCTGCGGA	AAGGATCATTACCAC	ACCGATCCAAGATCA		
fraud	-----	GAACCTGCGGA	AAGGATCATTACCAC	ACCGATCCAAGATCA		
deli	-----	GAACCTGCGGA	AAGGATCATTACCAC	ACCGATCCAAGATCA		
subpac	-----	GAACCTGCGGA	AAGGATCATTACCAC	ACCGATCCAAGATCT		
pung	AACAAGGTTCCG	GTAAGGTTGAACCTG	GCGGAAGGATCATTACCAC	ACCGATCCAAGATCA		
	70	80	90	100	110	120
cyl	-TCTTTATTGTGAA	----ATGTTGG	-----TTGTTTC	-----T	---CTCGGGAGA	
ser	GTCTTCATTGTGAAT	CTGATTTTCAGAGGC	-----GGCTT	-----	GCTTTTAAAGC	
aust	GTCTTCATTGTGAAT	CTGATTTTCAGAGGC	-----GGCTT	-----	GCTTTTAAAGC	
fraud	GTCTTCATTGTGAAT	TTGATTTGCGTGGC	ACTCTGGCTTCGG	CCCGTTTGCCT	CAAAAGT	
deli	GTCTTCATTGTGAAT	CTGATTTTCAGAGGC	AGCCTCGCT	-----GTT	---CTCTGTCGT	
subpac	GTCTTCATTGTGAAT	CTGATTTTCGTTG	CAGAGTC	---CTTCAGT	GGGCTGTACTT	CGTCGT
pung	GTCTTCATTGTGAAT	CTGATTGCACCGG	-----	-----	-----TCTAGT	
	130	140	150	160	170	180
cyl	CAACC	-----	GTGCTATTCTA	-----GCAAC	-CACTCAG	---AGT
ser	-----	-----	TCTCCGTCGCTG	TCGTC	-----AACTAT	TTTTTCAGTGAT
aust	-----	-----	TCTCCGTCCTCT	GTCGTC	-----AACTTTTT	-AGTGAT
fraud	CAACTTG	TATACAGGTGGC	CTTGTCCCCC	TCGCGCACAGTAT	--ACCTGTG	TTTCGGCGC
deli	CAACTT	TAAC	-----	CCTGGTG	CTATCCAGCC	CGTGCAAT--ACCTGTG
subpac	CAACAT	TATTGGGATATGC	--TGTCATTG	CCCACTGTGGT	GATGGTGGT	TGTCCCGTGAT
pung	AT	-----	TTTTACTAA	ACTGCTGCCG	TCA--AACTTAA	ACTTGCAAC
	190	200	210	220	230	240
cyl	ACTCTTGTA	--G	TATTCTGTTTT	-----	ACC	---AAATTGACC
ser	ATGCTATTT	-ACTAGTGT	-ATCA	-----	CAACCCCC	ATTCTTAACGATTGGTAA
aust	GCGCTAGCT	-ACTAGCGT	-ATTA	-----	CAACCCCC	ATTCTTAACGATTGGTAA
fraud	GCGACATCG	CACAGTGGC	ATTAAGTGAAAT	G	TCCACACCCC	ATCTTTTACGATCGGTAA
deli	GGGTGGTT	--GGTGT	CACTTT	-----	ACCCCC	CATATTCTAT-ATGGA-AA
subpac	CTGCCA	TATCAGCCTTGTG	CTGTAGCGGATCG	CAAACCCCC	ATTCTATAT	----GGTAA
pung	GCGTTGATT	--AATTCCG	-CGTT	-----	GCTGCC	ATTCTTTACGATTGGTAA
	250	260	270	280	290	300
cyl	CTTGATA	AATAC	----TGATCTAA	AGCAAAGAG	--TGCTGCC	-----TGAAC
ser	CTGGAA	AGAACCAAATGAC	CTAAAGCTAAAA	---	TGCAGTGGTCTGGT	--TTGCCG
aust	CTGGAA	AGAACCAAATGAC	CTAAAGCTAAAA	---	TGCAGTGGTCTGGT	--TTGCCG
fraud	CTTGAT	AGAACCAAATGAC	CTAAAGCTTAAAG	--	TGCAGTGGTCCGGCA	-ACTGAGC
deli	CTGGAA	AGAACCAAATGAC	CTAAAGCT-AAAG	--	TGCAGTGGTCTGGT	-GTTGCCG
subpac	CCGAA	AGAACCAAATGAC	CTAAAGTA-AAATG	-TGCAGATGGT	CTGGT	-GTTGTGC
pung	CTGGAA	AGAACCAAATGAC	CTAAAGTAGAAATGAT	G	CAGTGTTCGGAG	CGCTGAGCGG

Appendix 4

		310	320	330	340	350	360
cyl	---	CTAG	-----	TGTTTGG	-----	GCCCTCGA	--AAATAT--
ser	---	CTCG	-----	TGCCAACA	---CC-----	GCCCTGTACATTATATC	
aust	---	CTCG	-----	TGCCAACA	---CC-----	GCCCTGTACATTATACC	
fraud	---	CTTG	-----	TGCCAGGAG	---CC-----	GCCCTGTACATAAAACC	
deli	---	CTTG	-----	TGCCAACA	---CC-----	GCCCTGTACAC-AAACC	
subpac	---	TTAG	-----	TGCCAACA	---CCG-----	ATCCTGTACACAAAACC	
pung	CGTCTTAAGTATTAGATGCATACCAAACCGACTCTATTGCTGGCACTGCACATTACACCC						
		370	380	390	400	410	420
cyl	-AATCTAGTACAAC	TTT	CAGCGGTGGATGTCTAGGTTCC	CACAACGATGAAGAACGCAGC			
ser	-AATTCATTACAAC	TTT	CAGCGGTGGATGTCTAGGTTCC	CACAACGATGAAGAACGCAGC			
aust	AATTCA-TTACAAC	TTT	CAGCGGTGGATGTCTAGGTTCC	CACAACGATGAAGAACGCAGC			
fraud	TAATGACTTACAAC	TTT	CAGCGGTGGATGTCTAGGTTCC	CACAACGATGAAGAACGCAGC			
deli	TAATGACTTACAAC	TTT	CAGCGGTGGATGTCTAGGTTCC	CACAACGATGAAGAACGCAGC			
subpac	TAATGACTTACAAC	TTT	CAGCGGTGGATGTCTAGGTTCC	CACAACGATGAAGAACGCAGC			
pung	TAATACATTACAAC	TTT	CAGCGGTGGATGTCTAGGTTCC	CACAACGATGAAGAACGCAGC			
		430	440	450	460	470	480
cyl	GAAATGCGATACGTAATGCGAATTGCAAGACCTCGTGAATCATTAAAGATTTTGAACGCAC						
ser	GAAATGCGATACGTAATGCGAATTGCAAGACCTCGTGAATCATTAAAGATTTTGAACGCAC						
aust	GAAATGCGATACGTAATGCGAATTGCAAGACCTCGTGAATCATTAAAGATTTTGAACGCAC						
fraud	GAAATGCGATACGTAATGCGAATTGCAAGACCTCGTGAATCATTAAAGATTTTGAACGCAC						
deli	GAAATGCGATACGTAATGCGAATTGCAAGACCTCGTGAATCATTAAAGATTTTGAACGCAC						
subpac	GAAATGCGATACGTAATGCGAATTGCAAGACCTCGTGAATCATTAAATTTTGAACGCAC						
pung	GAAATGCGATACGTAATGCGAATTGCAAGACCTCGTGAATCATCAAGATTTTGAACGCAC						
		490	500	510	520	530	540
cyl	ATTGCGCTTTCCGGATTTTCCCGGTAGCATGCTTGTCTGAGTGTCTGTGAACCCACTCA						
ser	ATTGCGCTTTCCGGATTTTCCCGGTAGCATGCTTGTCTGAGTGTCTGTGGATCCCACTCA						
aust	ATTGCGCTTTCCGGATTTTCCCGGTAGCATGCTTGTCTGAGTGTCTGTGGATCCCACTCA						
fraud	ATTGCGCTTTCCGGATTTTCCCGGTAGCATGCTTGTCTGAGTGTCTGTGGATCCCACTCA						
deli	ATTGCGCTTTCCGGATTTTCCCGGTAGCATGCTTGTCTGAGTGTCTGTGGATCCCACTCA						
subpac	ATTGCGCTTTCCGGATTTTCCCGGGAGCATGCTTGTCTGAGTGTCTGTGGATCCCACTCA						
pung	ATTGCGCTTTCCGG--TATTTCCCGGTAGCATGCTTGTCTGAGTGTCTGTGGATCCCACTCA						
		550	560	570	580	590	600
cyl	GCGTTGATGTATTTCAACAGCT--GGATTGTGTGAGTGTCTATCTTTTGGATGGACTCTT						
ser	GCGCTGGTTTA-----GGCCGGTCGCTGGTTGTTTTGGCCTTGACAGCTACTA						
aust	GCGCTGGTTTA-----GGCCAGTCGCTGGTTGTTTTGGCCTTGACAGCTACTA						
fraud	GCGCTGGTCTACT----CTGTAGGCCAGTTGCTGGTTGCTATGGCTTGACCCTCTA						
deli	GCACTGGTCTATT----CTGTA--GGCTAGTCGCTGGTTGCTTTGGCCTTGACTGGCTCTG						
subpac	GCGCTGGTCTGCCCTTTGGGT--GGATTGGTAGCTGGTTACTTTGGCTTTGATGGATTCTG						
pung	GCGCTGGCTTC-----GCGCTGGCTGCTGGTC--TTTTGGCCGTGACTGAA--CTT						
		610	620	630	640	650	660
cyl	A---CGAAATGTTTCGTAGACGTTTGTCTGA--GCAA---GCATTGTTTCTAGTTTTT--CAC						
ser	GTAGCTGTCTCTGCTTAAAGTTCTACGGTATCGTAC--GTGCATAGAT--TTAGTTACCTCAA						
aust	GTAGCTGTCTCTGCTTAAAGTTCTACGGTATCGTAC--GTGCATAGAT--TTAGAGACCTCAA						
fraud	GTAGCGGTCTCTGCTTAAATTTCTACAACATTGTACCGTGCATAGAT--CTAGAAAAGTC--						
deli	G---CTGTCTCTGCTTAAATTTCTACAACA--CGTAC--GTGCATAGAT--CTAGAAACGTCTC						
subpac	T---CCTTCTTTGCTTAAATTTCTACATACATGTAC--GCGAATAGAT--CTAGAGGAGTC--						
pung	GTT--TAGTCTCGGCTTAAAGTTTACGTCA--AGTAC--GTGCATAGAT--CTAGAAAGCTCTG						
		670	680	690	700	710	720
cyl	ACAGTGT--TTG---TGTCGAGAGCCGAACATATCGTCTATCTCCGGTAACAC--TTTACG						
ser	CCTGTCT--TGTC---AAGACGGCGTTGACACTCTTGTCTATCTCTGGTAGCTTGTATTTA						
aust	CCTGTCT--TGTC---AAGACGGCGTTGACATTCTTGTCTATCTCTGGTAGCTTGTATTTA						
fraud	--TTGTGT--TGTCTGAATGACGGCGTTGACGCTCTTGTCTATCTCTGGTAGCTTGTATGAA						
deli	AGCCCGT--TCTA---ACTACGGCGTTGACATTCTCATCTATCTCTGGTAGCTTTTGT---						
subpac	--TTGTGTCTGTCTAAGGACGGCATTGGCAATCTCATCTATCTCTGGTAGAGTTAAT---						
pung	CCCCTCT--AGTTAAAAACACGGCGCTGACACTCTTGTCTATCTCTGGTAGGTTTATTCAA						

Appendix 4

	730	740	750	760	770	780
cyl						
ser	-----TGTCGAGTGAGTTTTAGCGCGTAGTTTTAGTTTTTGAT---GCTTTTTAACACGGTA					
aust	TTACAAATATCTGGAGTTTGT-----TAAGATTGTCATGTTTCAGCTGTTTGA-AACTT					
fraud	TTACAAATATCTGGAGTTTGT-----TAAGATTGTCATGTTTCAGCTGTTTGA-AACTT					
deli	AATGCATTCTCTGGAGTTTAAAC-----TAGGGGTGTC-TATT-AGCTGTCTGCCAACT					
subpac	-----CTCTGGAGTTCAA-----TTGAGTTTGTATC-AGCTGTTTGAAGGACT					
pung	-----TCTCTGGAGTTTGA-----ACGAGTTTGCCTTTAGCTGTTTTGAGTTCA					
	-----CTGGAGTTTGC-----TAGGAGTCGTCTGT--AGCTGTTTTGAAACTA					
	790	800	810	820	830	840
cyl						
ser	AATTAGACTC--AGTGTTT-TCTGGGCA---AATATCGCGTGTATTAATTTACACGTGAT					
aust	TTCGATTGAGTGTCACCTAGATCTAGCTAGCTATACTTTCCTGTATTAG----CTAGCTAT					
fraud	TTCGATTGAGCGTCACTAGATCTAGCTAGCTATACTTTCCTAGTACTAG----CTAGCTAT					
deli	ATCGAGTTTT--TCTCTGGGTCTGGCCA-CGCAGCTGTGTAAAAGCAGTCTGTCTGGCTAT					
subpac	ACTGAGTCTG--ACACTA-----AGTCTAGCTTGCAGTTTTCTGTCTCGCTAT					
pung	ACCAAAGCTT--TTTTAG-----T-GAAGGGCCAGTAGCAGT--AATGCCTAC					
	ATGGAATGAGCGGTTTTAGATCT-----AACTAGCTGAAACAGT----TAGTTAT					
	850	860	870	880	890	900
cyl						
ser	---ATAATTCATTCCGGATCTCA-AATCAAGC-AAGAAGACCC-GCCGAATTTAAGCATA					
aust	---CCAA----TTCCGGATCTCA-GATCAAGC-AAGAGGACCC-GCTGAACTTAAGCATA					
fraud	---CCAA----TTCCGGATCTCA-GATCAAGC-AAGAGGACCC-GCTGAACTTAAGCAT-					
deli	T---CCAA----TTCCGGATCTCAAGATCAAGCCAAGAGGACCCCGCTGAACTTAAGCATT					
subpac	---CTAA----TTCCGGATCTCA-GATCAAGC-AAGATGACCC-GCTGAACTTAAGCATA					
pung	TGACCAACTAATTCGGAT-----					
	---ACAA----TTCCGGATCTCA-GATCAAGC-AAGAGGACCCGCTTGAACCTTAAGCAT-					
	910	920	930	940	950	960
cyl						
ser	TAATTAAGCGGAGGAAAAGAACTAACCAGGATTCCTCAGTAAGGGCGACTGAAGCGGG					
aust	TAATTAAGCGGAGGAAAAGAACTAACTAGGATTCCTCAGTAACGGCGAGTGAAGCGGG					
fraud	-----GAAAAGAACTAACTAGGATTCCTCAGTAACGGCGAGTGAAGCGGG					
deli	T-----ACTAAGGATTCCTCAGTAACGGCGAGTGAAGCGGG					
subpac	T-----ACTAAGGATTCCTCAGTAACGGCGAGTGAAGCGGG					
pung	-----AAAAGAACTAACTAGGATTCCTCAGTAACGGCGAGTGAAGCGGG					
	-----ACTAAGGATTCCTCAGTAACGGCGAGTGAAGCGGG					
	970	980	990	1000	1010	1020
cyl						
ser	AAGTGCTCAGGATGTGAATCTGCGC---TAT-GCGCCGAATTGTGGTCTGTAGACTGTGA					
aust	ACTAGCTCAGGATGTGAATCTGCGCTTTTATGGCGCCGAATTGTGGTCTGTAGACTTTGA					
fraud	ACTAGCTCAGGATGTGAATCTGCGCTTTTATGGCGCCGAATTGTGGTCTGTAGACTTTGA					
deli	ACTAGCTCAGGATGTGAATCTGCGCTTTTATGGCGCCGAATTGTGGTCTATAGACTTTGA					
subpac	ACTAGCTCAGGATGTAAAATCTGCACTTT-ATGGTGCCGAATTGTGGTCTGAAGACCTTGA					
pung	ACTAGCTCAGGATGTGAATCTGCGCTTTTATGGCGCCGAATTGTGGTCTGTAGACTTTGA					
	1030	1040	1050	1060	1070	1080
cyl						
ser	CATTATTGGCCGGCCAAGTCCCTTGAAAAGGGCAGCAGAGAGGGTGAGACTCCCGTCC					
aust	CATTATCTGCCGGCCAAGTTCCTTGAAAAGGACAGCTGAGAGGGTGAGACTCCCGTCC					
fraud	CATTATCTGCCGGCCAAGTTCCTTGAAAAGGACAGCTGAGAGGGTGAGACTCCCGTCC					
deli	CGTTATCTGCCGGCCAAGTTCCTTGAAAAGGACAGCTGAGAGGGTGAACTCCCGTCC					
subpac	CGTTATCTGCCGGCCAAGTTCCTTGAAAAGGACAGCTGAGAGGGTGAGACTCCCGTCC					
pung	TGTTATCTGCCGGCCAAGTTCCTTGAAAAGGACAGCTGAGAGGGTGAGACTCCCGTCC					
	CATTATTTGCCGGCCAAGTTCCTTGAAAAGGACAGCTGAGAGGGTGACACTCCCGTCC					
	1090	1100	1110	1120	1130	1140
cyl						
ser	GCCTGGCCGAGTGAGTCGCTAGTCAACGAGTCGAGTTGTTTGGGATTGCAGCTCTAAGTG					
aust	GCCTGGTAGAGTGAGTCATTTGTCAACGAGTCGAGTTGTTTGGGATTGCAGCTCTAATTT					
fraud	GCCTGGTAGAATGAGTC-TTTGTCAACGAGTCGAGTTGTTTGGGATTGCAGCTCTAATTT					
deli	GCCTGGTAGAATGAGTC-TTTGTCAACGAGTCGAGTTGTTTGGGATTGCAGCTCTAATTT					
subpac	GTCTGGTAGAATGAATCAGGTGTCAACGAGTCGAGTTGTTTGGGATTGCAGCTCTAATTT					
pung	GCCTGGTAAAGTGAGTCATTTGTCAACGAGTCGAGTTGTTTGGGATTGCAGCTCTAATTT					

Appendix 4

	1150	1160	1170	1180	1190	1200
cyl	GGTGGTAAATTC	ATCTAAAGCTAA	AATATTGGTGGG	GAGACCGGATAG	CGGTACAAGTACC	CGT
ser	GGTGGTAAATTC	ATCTAAAGCTAA	AATATTGGTGGG	GAGACCGGATAG	CGGTACAAGTACC	CGT
aust	GGTGGTAAATTC	ATCTAAAGCTAA	AATATTGGTGGG	GAGACCGGATAG	CGGTACAAGTACC	CGT
fraud	GGTGGTAAATTC	ATCTAAAGCTAA	AATATTGGTGGG	GAGACCGGATAG	CGGTACAAGTACC	CGT
deli	GGTGGTAAATTC	ATCTAAAGCTAA	AATATTGGTGGG	GAGACCGGATAG	CGGTACAAGTACC	CGT
subpac	GGTGGTAAATTC	ATCTAAAGCTAA	AATATTGGTGGG	GAGACCGGATAG	CGGTACAAGTACC	CGT
pung	GGTGGTAAATTC	ATCTAAAGCTAA	AATATTGGTGGG	GAGACCGGATAG	CGGTACAAGTACC	CGT
	1210	1220	1230	1240	1250	1260
cyl	GAGGGAAAGATG	CAAAGAAC	TTTGAAAAGAG	AGTTAAAGAGT	ACCTGAAATTG	GCTGAAAG
ser	GAGGGAAAGATG	CAAAGAAC	TTTGAAAAGAG	AGTTAAAGAGT	ACCTGAAACTG	GCTGAAAC
aust	GAGGGAAAGATG	CAAAGAAC	TTTGAAAAGAG	AGTTAAAGAGT	ACCTGAAACTG	GCTGAAAC
fraud	GAGGGAAAGATG	CAAAGAAC	TTTGAAAAGAG	AGTTAAAGAGT	ACCTGAAATTG	GCTGAAAC
deli	GAGGGAAAGATG	CAAAGAAC	TTTGAAAAGAG	AGTTAAAGAGT	ACCTGAAATTG	GCTGAAAC
subpac	GAGGGAAAGATG	CAAAGAAC	TTTGAAAAGAG	AGTTAAAGAGT	ACCTGAAATTG	GCTGAAAC
pung	GAGGGAAAGATG	CAAAGAAC	TTTGAAAAGAG	AGTTAAAGAGT	ACCTGAAACTG	GCTGAAAC
	1270	1280	1290	1300	1310	1320
cyl	GGAAGCGAAGG	AAACCAGTGT	TGAATGTGTCA	TACTTCTTGAT	CCGCTTGCCG	GATTGGGC
ser	GGAAGCGAAGG	AAACCAGTGT	TGTTGGTTCAT	ATTTCCCGGCC	ACTTGTGGTT	TGGGC
aust	GGAAGCGAAGG	AAACCAGTGT	TGTTGGTTCAT	ATTTCCCGGCC	ACTTGTGGTT	TGGGC
fraud	GGAAGCGAAGG	AAACCAGTGT	TGTTGGTTCAT	ATTTCCCTGGC	CACTTGTGGTT	TGGGC
deli	GGAAGCGAAGG	AAACCAGTGT	TGTTGGTTCAT	ATTTCCCTGGC	CACTTGTGGTT	TGGGC
subpac	GGAAGCGAAGG	AAACCAGTGT	TGTTGGTTCAT	ATTTCCCTGGC	CACTTGTGGT	TGGGC
pung	GGAAGCGAAGG	AAACCAGTGT	TGTTGGTTCAT	ATTTCCCTGGC	CACTTGTGGT	TGGGC
	1330	1340	1350	1360	1370	1380
cyl	GCTGTGTCATG	TTTGGGTTG	TCTTGGTTGG	TGCGGATGGA	AGAGCGCTAA	AGGAGTTG
ser	GCTGTGAGCTT	GCGTGAGTT	TGGGTTGGTT	GAAATCCTT	GGAAGAGCGC	CAGTCAAGTTG
aust	GCTGTGAGCTT	GCGTGAGTT	TGGGTTGGTT	GAAATCCTT	GGAAGAGCGC	CAGTCAAGTTG
fraud	GCTGTGAGCTT	GCGTGAGTT	TGGGTTGGTT	GATCCCTT	GGAAGAGCGC	CAGTCAAGTTG
deli	GCTGTGAGCTT	GCGTGAGTT	TGGGTTGGTT	GATCCCTT	GGAAGAGCGC	CAGACAGAGTTG
subpac	GCTGTGGATAT	GCGTGGGTT	TGATTTGGTT	GATCCCTT	GGAAGAGCGC	CAGTCAAGTTG
pung	GCTGTGGACTT	GCGTGAGTT	TGGGTTGGTT	GATCCCTT	GGAAGAGCGC	CAGTCAAGTTG
	1390	1400	1410	1420	1430	1440
cyl	ACTTTGGTTG	CTAGCATTG	CTTCTGACTG	AGGAGGACG	AAATGGTTTT	TCTTTACCCCGTC
ser	ATGTCTGTTG	CTAGCACTG	GATTTGACTG	ATGCAGACG	AAATGGTTTT	TCTTTACCCCGTC
aust	ATGTCTGTTG	CTAGCACTG	GATTTGACTG	ATGCAGACG	AAATGGTTTT	TCTTTACCCCGTC
fraud	ATGTCTGTTG	CTAGCACTG	GGGTTTACTG	ATGCAGACG	AAATGGTTTT	TCTTTACCCCGTC
deli	ATGTCTGTTG	CTAGCACTG	GGGTTTACTA	ATGCAGACG	AAATGGTTTT	TCTTTACCCCGTC
subpac	ATGTCTGTTG	CTAGCACTG	GGGTTTACTA	ATGCAGACG	AAATGGTTTT	TCTTTACCCCGTC
pung	ATGTCTGTTG	CTAGCACTG	GGGTTTACTG	ATGCAGACG	AAATGGTTTT	TCTTTACCCCGTC
	1450	1460	1470	1480	1490	1500
cyl	TTGAAACACGG	ACCAAGGAGT	CTAACATATG	TGCGAGTATAG	GAGTGTCAA	ACTCCCATG
ser	TTGAAACACGG	ACCAAGGAGT	CTAACATATG	TGCGAGTACAG	GGGTGTCAA	ACCCCGGTG
aust	TTGAAACACGG	ACCAAGGAGT	CTAACATATG	TGCGAGTACAG	GGGTGTCAA	ACCCCGGTG
fraud	TTGAAACACGG	ACCAAGGAGT	CTAACATATG	TGCGAGTACAG	GGGTGTCAA	ACCCCGGTG
deli	TTGAAACACGG	ACCAAGGAGT	CTAACATATG	TGCGAGTACAG	GGGTGTCAA	ACCCCGGTG
subpac	TTGAAACACGG	ACCAAGGAGT	CTAACATATG	TGCGAGTACAG	GGGTGTCAA	ACCCCGGTG
pung	TTGAAACACGG	ACCAAGGAGT	CTAACATATG	TGCGAGTACAG	GGGTGTCAA	ACCCCGGTG
	1510	1520	1530	1540	1550	1560
cyl	CGCAATGAAAG	TGACAGTGG	TGGAC-CTTT	GGGCACAAT	CCGCCGGCCT	CAATCCTTCG
ser	CGTAATGAAAG	TGACAGTGG	TGGAA--AT	TTTG-CACA	ATCCGCCGGC	CCTCAATCCTTCG
aust	CGTAATGAAAG	TGACAGTGG	TGGAA--AT	TTTG-CACA	ATCCGCCGGC	CCTCAATCCTTCG
fraud	CGTAATGAAAG	TGACAGTGG	TGGAT-AT	TTTG-CACA	ATCCGCCGGC	CCTCAATCCTTCG
deli	CGTAATGAAAG	TGACAGTGG	TGGAT-AT	TTTG-CACA	ATCCGCCGGC	CCTCAATCCTTCG
subpac	CGTAATGAAAG	TGACAGTGG	TGGACAAT	TTTTGC	ACAATCCGCC	GGCCTCAATCCTTCG
pung	CGCAATGAAAG	TGACAGTGG	TGGAA-T	TTTTG-	CACAATCCGCC	GGCCTCAATCCTTCG

Appendix 4

	1570	1580	1590	1600	1610	1620
cyl	GGAGAACGGTCTGAGTGTGAGCATA	CATGTTGGGACCCGAAAGATGGTGA	ACTATGCCTG			
ser	GGAGAACGGTCTGAGTGTGAGCATA	CATGTTGGGACCCGAAAGATGGTGA	ACTATGCCTG			
aust	GGAGAACGGTCTGAGTGTGAGCATA	CATGTTGGGACCCGAAAGATGGTGA	ACTATGCCTG			
fraud	GGAGAACGGTCTGAGTGTGAGCATA	CATGTTGGGACCCGAAAGATGGTGA	ACTATGCCTG			
deli	GGAGAACGGTCTGAGTGTGAGCATA	CATGTTGGGACCCGAAAGATGGTGA	ACTATGCCTG			
subpac	GGAGAACGGTCTGAGTGTGAGCACAC	ATGTTGGGACCCGAAAGATGGTGA	ACTATGCCTG			
pung	GGAGAACGGTCTGAGTGTGAGCATA	CATGTTGGGACCCGAAAGATGGTGA	ACTATGCCTG			
	1630	1640	1650	1660	1670	1680
cyl	AATAGGGTGAAGCCAGGGGAAACTCT	GGTGGAGGCTCGTAGCGATTCTGAC	GTGCAAATC			
ser	AATAGGGTGAAGCCAGGGGAAACTCT	GGTGGAGGCTCGTAGCGATTCTGAC	GTGCAAATC			
aust	AATAGGGTGAAGCCAGGGGAAACTCT	GGTGGAGGCTCGTAGCGATTCTGAC	GTGCAAATC			
fraud	AATAGGGTGAAGCCAGGGGAAACTCT	GGTGGAGGCTCGTAGCGATTCTGAC	GTGCAAATC			
deli	AATAGGGTGAAGCCAGGGGAAACTCT	GGTGGAGGCTCGTAACGATTCTGAC	GTGCAAATC			
subpac	AATAGGGTGAAGCCAGGGGAAACTCT	GGTGGAGGCTCGTAGCGATTCTGAC	GTGCAAATC			
pung	AATAGGGTGAAGCCAGGGGAAACTCT	GGTGGAGGCTCGTAGCGATTCTGAC	GTGCAAATC			
	1690	1700	1710	1720	1730	1740
cyl	GATCGTCAAATTTGGGTATAGGGGCG	AAAGACTAATCGAACC-ATCTAGTAG	CTGGTTCC			
ser	GATCGTCAAATTTGGGTATAGGGGCG	AAAGACTAATCGAACC-ATCTAGTAG	CTGGTTCC			
aust	GATCGTCAAATTTGGGTATAGGGGCG	AAAG-----				
fraud	GATCGTCAAATTTGGGTATAGGGGCG	AAAGACTAATCGAACCATCTAGTAG	CTGGTTCC			
deli	GATCGTCAAATTTGGGTATAGGGGCG	-----				
subpac	GATCGTCAAATTTGGGTATAGGGGCG	AAAGACTAATCGAACCATCTAGTAG	CTGG----			
pung	GATCGTCAAATTTGGGTATAGGGGCG	AAAGACTAATCGAACC-----				
	1742					
cyl	C-					
ser	C-					
aust	--					
fraud	CC					
77C	--					
subpac	--					
pung	--					

Appendix 5: FASTA alignments of *P. subpacifica* (AY257859), *P. heimei* (AF440777) and *P. cf. subpacifica* (D257F)

LSU

P. subpacifica (AY257859, clone Limens 8) = P.sub
P. heimei (AF440777, clone Zhenbo7BL) = P.hei
 and D257F = D257F

- = gap; * = match; . = mismatch

```
P.sub -----GAATTTAAGCATATAATTAAGCGGAGGAAAAGAACT
P.hei GCCCTTACCCGCTGAATTTAAGCATATAATTAAGCGGAGGAAAAGAACT
D257F -----AAAAGAACT
*****
```

```
P.sub AACTAGGATTCCTCCAGTAACGGCGAGTGAAGCGGGACTAGCTCAGGATG
P.hei AACTAGGATTCCTCCAGTAACGGCGAGTGAAGCGGGACTAGCTCAGGATG
D257F AACTAGGATTCCTCCAGTAACGGCGAGTGAAGCGGGACTAGCTCAGGATG
*****
```

```
P.sub TAAATCTGCACTTTATGGTGCCGAATTGTGGTCTGAAGACCTTGATGTTA
P.hei TAAATCTGCACTTTATGGTGCCGAATTGTGGTCTGAAGACCTTGATGTTA
D257F TAAATCTGCACTTTATGGTGCCGAATTGTGGTCTGAAGACCTTGATGTTA
*****
```

```
P.sub TCTGCCGGACCAAGTTCCTTGGAAAAGGACAGCTGAGAGGGTGAGACTCC
P.hei TCTGCCGGGCAAGTTCCTTGGAAAAGGACAGCTGAGAGGGTGAGACTCC
D257F TCTGCCGGGCAAGTTCCTTGGAAAAGGACAGCTGAGAGGGTGAGACTCC
*****
```

```
P.sub CGTCCGTCTGGTAGAATGAGTCAGGTGTCAACGAGTCGAGTTGTTTGGGA
P.hei CGTCCGTCTGGTAGAATGAGTCAGGTGTCAACGAGTCGAGTTGTTTGGGA
D257F CGTCCGTCTGGTAGAATGAATCAGGTGTCAACGAGTCGAGTTGTTTGGGA
*****
```

```
P.sub TTGCAGCTCTAATTTGGTGGTAAATTCATCTAAAGCTAAATATTGGTGG
P.hei TTGCAGCTCTAATTTGGTGGTAAATTCATCTAAAGCTAAATATTGGTGG
D257F TTGCAGCTCTAATTTGGTGGTAAATTCATCTAAAGCTAAATATTGGTGG
*****
```

```
P.sub GAGACCGATAGCGTACAAGTACCGTGAGGGAAAGATGCAAAGAACTTTGA
P.hei GAGACCGATAGCGTACAAGTACCGTGAGGGAAAGATGCAAAGAACTTTGA
D257F GAGACCGATAGCGTACAAGTACCGTGAGGGAAAGATGCAAAGAACTTTGA
*****
```

```
P.sub AAAGAGAGTTAAAGAGTACCTGAAATTGCTGAAACGGAAGCGAAGGAAAC
P.hei AAAGAGAGTTAAAGAGTACCTGAAATTGCTGAAACGGAAGCGAAGGAAAC
D257F AAAGAGAGTTAAAGAGTACCTGAAATTGCTGAAACGGAAGCGAAGGAAAC
*****
```

```
P.sub CAGTGTTTGTATGTTTCATATTTCCCTTGCCACTTGTGGTGTGGGCGCTGT
P.hei CAGTGTTTGTATGTTTCATATTTCCCTTGCCACTTGTGGTGTGGGCGCTGT
D257F CAGTGTTTGTATGTTTCATATTTCCCTTGCCACTTGTGGTGTGGGCGCTGT
*****
```

Appendix 5

P.sub GGATATGCGTGGGTTTGATTGGTTGATCCCTTTGGAAGAGCGCAGTCAG
P.hei GGATATGCGTGGGTTTGATTGGTTGATCCCTTTGGAAGAGCGCAGTCAG
D257F GGATATGCGTGGGTTTGATTGGTTGATCCCTTTGGAAGAGCGCAGTCAG

P.sub AGTTGATGTCTGTTGCTAGCACTGGGTTTACTCAATCAGACGAAATGGT
P.hei AGTTGATGTCTGTTGCTAGCACTGGGTTTACTCAATCAGACGAAATGGT
D257F AGTTGATGTCTGTTGCTAGCACTGGGTTTACTCAATCAGACGAAATGGT

P.sub TTTCTTTACCCCGTCTTGAAACACGGACCAAGGAGTCTAACATATGTGCG
P.hei TTTCTTTACCCCGTCTTGAAACACGGACCAAGGAGTCTAACATATGTGCG
D257F TTTCTTTACCCCGTCTTGAAACACGGACCAAGGAGTCTAACATATGTGCG

P.sub AGTACAGGGGTGTCAAACCCCGGTGCGTAATGAAAGTGACAGTGTTGGA
P.hei AGTACAGGGGTGTCAAACCCCGGTGCGTAATGAAAGTGACAGTGTTGGA
D257F AGTACAGGGGTGTCAAACCCCGGTGCGTAATGAAAGTGACAGTGTTGGA

P.sub CAATTTTGCACAATCCGCCGGCCTCAATCCTTCGGGAGAACGGTCTGAG
P.hei CAATTTTGCACAATCCGCCGGCCTCAATCCTTCGGGAGAACGGTCTGAG
D257F CAATTTTGCACAATCCGCCGGCCTCAATCCTTCGGGAGAACGGTCTGAG

P.sub TGTGAGCACACATGTTGGGACCCGAAAGATGGTGA ACTATGCCTGAATAG
P.hei TGTGAGCACACATGTTGGGACCCGAAAGAAGGTGA ACTATGCCTGAATAG
D257F TGTGAGCACACATGTTGGGACCCGAAAGATGGTGA ACTATGCCTGAATAG

P.sub GGTGAAGCCAGGGGAAACTCTGGTGGAGGCTCGTAGCGATTCTGACGTGC
P.hei GGTGAAGCCAGGGGAAACTCTGGTGGAGGCTCGTAGCGATTCTGACGTGC
D257F GGTGAAGCCAGGGGAAACTCTGGTGGAGGCTCGTAGCGATTCTGACGTGC

P.sub AAATCGATCGTCAAATTTGGG
P.hei AAATCGTTCGTAAGGG-----
D257F AAATCGATCGTCAAATTTGGG
***** . **** . * . . *****

ITS

P. subpacific (AY257859, clone Limens 8) = P.sub
and D257F

- = gap; * = match; . = mismatch

P.sub TGAACCTGCGGAAGGATCATTACCACACCGATCCAAGATCTGTCTTCATT
D257F -GAACCTGCGGAAGGATCATTACCACACCGATCCAAGATCTGTCTTCATT

P.sub GTGAATCTGATTTTCGTTGACAGTCCTTCAGTGGGCTGTACTTCGTGTC
D257F GTGAATCTGATTTTCGTTGACAGTCCTTCAGTGGGCTGTACTTCGTGTC

Appendix 5

P.sub ACATTATTGGGATATGCTGCCATTACCCACTGTGGTGATGGTAGTTGTCC
D257F ACATTATTGGGATATGCTGTCATTGCCCACTGTGGTGATGGTGGTTGTCC
*****.****.*****.*****

P.sub CGCGATCTGCCATATCAGCCTTGTGCTGTAGCGGGTCGAAACCCCCCAT
D257F CGTGATCTGCCATATCAGCCTTGTGCTGTAGCGGATCGAAACCCCCCAT
** .*****.*****

P.sub TCTATATGGTAACCGAAAGAACCAAATGACCTAAAGTAAAATGTGCAGA
D257F TCTATATGGTAACCGAAAGAACCAAATGACCTAAAGTAAAATGTGCAGA

P.sub TGGTCTGGTGTGTGCTTAGTGCCAACACCGATCCTGTACACAAAACCTA
D257F TGGTCTGGTGTGTGCTTAGTGCCAACACCGATCCTGTACACAAAACCTA

P.sub ATGACTTACAACCTTTCAGCGGTGGATGTCTAGGTTCCACACACGATGAAG
D257F ATGACTTACAACCTTTCAGCGGTGGATGTCTAGGTTCCACACACGATGAAG

P.sub AACGCAGCGAAATGCGATACGTAATGCGAATTGCAAGACCTCGTGAATCA
D257F AACGCAGCGAAATGCGATACGTAATGCGAATTGCAAGACCTCGTGAATCA

P.sub TTAAAATTTGAACGCACATTGCGCTTTCGGGATCTTCCCGGGAGCATGC
D257F TTAAAATTTGAACGCACATTGCGCTTTCGGGATTTTCCCGGGAGCATGC
*****.*****

P.sub TTGTCTGAGTGTCTGTGGATCCCACTCAGCGCTGGTCTGCCCTTTGGGTG
D257F TTGTCTGAGTGTCTGTGGATCCCACTCAGCGCTGGTCTGCCCTTTGGGTG

P.sub GATTGGTAGCTGGTTACTTTGGCTTTGATGGATTCTATCCTTCTTTGCTT
D257F GATTGGTAGCTGGTTACTTTGGCTTTGATGGATTCTATCCTTCTTTGCTT
*****.*****

P.sub AAATTCTACATACATGTACGCGAATAGATCTGGAGGAGTCTTGTGTCTGT
D257F AAATTCTACATACATGTACGCGAATAGATCTAGAGGAGTCTTGTGTCTGT
*****.*****

P.sub CCTAAGGACGGCATTGGCAATCTCATCTATCTCTGGTAGAGTTAATTCTC
D257F CCTAAGGACGGCATTGGCAATCTCATCTATCTCTGGTAGAGTTAATTCTC

P.sub TGGAGTTTGAACGAGTTTTGCCTTTAGCTGTTTTGAGTTCAACCAAAGCT
D257F TGGAGTTTGAACGAGTTTTGCCTTTAGCTGTTTTGAGTTCAACCAAAGCT

P.sub TTTTCTAGTGAAGGGCCAGTAGCAGTAAATGCCTACTGACCAACTAATTC
D257F TTTTTTAGTGAAGGGCCAGTAGCAGTAA-TGCCTACTGACCAACTAATTC
****.*****.*****

P.sub CGGATC
D257F CGGAT-
