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Seasonality of *Pseudo-nitzschia* spp. (Bacillariophyceae) in western Scottish waters

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ABSTRACT: Some diatoms belonging to the genus *Pseudo-nitzschia* produce the neurotoxin domoic acid (DA), which causes amnesic shellfish poisoning (ASP). In Europe, accumulation of DA in shellfish has led to shellfish harvesting closures in western Scottish waters since 1999. However, little is yet known of the temporal appearance and succession of *Pseudo-nitzschia* species and how this may relate to environmental forcing. A phytoplankton monitoring programme was established for almost 3 yr at a coastal station in western Scottish waters to study the diversity and seasonal variation of *Pseudo-nitzschia* species in relation to physical and chemical parameters of the water column. Samples were collected with a high temporal resolution. Annually repeatable seasonal patterns of *Pseudo-nitzschia* abundance were evident. *Pseudo-nitzschia* cells were categorised by shape and size into 2 groups, the 'delicatissima-group' and the 'seriata-group'. Cluster analysis demonstrated the different dynamics of the 2 *Pseudo-nitzschia* groups. The delicatissima-group was dominated in spring by non-toxic *P. delicatissima*, while the seriata-group occurred mainly during the summer months. Summer to autumn blooms of both groups were composed of several species, including toxic *P. australis* and *P. seriata* and potentially toxic *P. pseudo-delicatissima*. Redundancy analysis indicated that 31% of the variance in *Pseudo-nitzschia* spp. abundance could be explained by the measured environmental variables. The repeatable annual nature of the blooms suggests that deterministic forecasting of the timing of future toxic *Pseudo-nitzschia* spp. events may be an achievable goal.

KEY WORDS: Amnesic Shellfish Poisoning · *Pseudo-nitzschia* spp. · Inorganic nutrients · Phytoplankton monitoring · Seasonal patterns

INTRODUCTION

Some species of diatoms of the genus *Pseudo-nitzschia* (Hasle, 1994) produce the neurotoxin domoic acid (DA). When concentrated within shellfish tissue, this toxin may reach sufficiently high concentrations to result in amnesic shellfish poisoning (ASP) of animal or human consumers (Bates et al. 1989). In recent years in UK waters, *Pseudo-nitzschia* spp. blooms have resulted in frequent shellfish harvesting closures along the Scottish west coast and Islands (Campbell et al. 2001, Gallacher et al. 2001). Studies of phytoplankton ecology in the region have been reviewed by Tett (1992). However, few studies have been conducted since the early 1980s and, in particular, no long term phytoplankton monitoring has been conducted in Scottish waters.

Currently, we have only limited knowledge of the toxic and non-toxic *Pseudo-nitzschia* species present in Scottish waters (Gallacher et al. 2001, Fehling et al. 2004b) and little or no knowledge of their abundance and temporal changes thereof both within and between years. Hence we do not know whether the presence of *Pseudo-nitzschia* spp. and/or their production of DA in these waters is a recent or long term phenomenon, whether blooms occur repeatedly at the same time of year on an annual basis, nor how environmental conditions influence the succession of toxic and non-toxic species within this genus.

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Studies conducted in 1979 and 1980 (Tett & Edwards 2002) reported *Nitzschia seriata* to be present in Loch Creran 56° 31.0 N, 5° 23.0 W (see Fig. 1), a western Scottish sealoch. However, the taxonomy at the time was not sufficiently developed for us to confirm that these are indeed the same organisms now being reported in these waters. No toxin analysis was possible (nor indeed thought necessary) at that time. Sampling then was quite frequent (at weekly intervals), but the only other variables measured were chlorophyll *a*, temperature and salinity, and the samples were taken from a pier at a depth of 1 to 2 m and so can be considered only representative of the superficial layer of the inner part of the loch.

While knowledge of the presence of other phytoplankton species and environmental conditions is now marginally more advanced, this knowledge has been gained from piecemeal studies often with other objectives. Many studies in the area have concentrated on hydrographic and bulk biochemical parameters (such as chlorophyll *a*) without collecting or analysing phytoplankton samples (Tett & Wallis 1978, Tett et al. 1981, Watts et al. 1998); therefore detailed understanding of the temporal phytoplankton dynamics and species succession is lacking.

The present paper is a result of a study, begun in 2000 and reported in a thesis by Fehling (2004), aimed at redressing the lack of information. We present results from almost 3 yr of sampling for *Pseudo-nitzschia* spp. and relevant environmental variables at a single site in the Firth of Lorne on the west coast of Scotland. As will be discussed, the site and main sampling depth were chosen as being typical of western Scottish coastal waters and yet largely free of the influence of fish farms. The aims of the study were: (1) to report, and attempt to explain, seasonal variation in 2 morphologically distinct groups of *Pseudo-nitzschia* spp., and (2) to characterise these groups by the species identified within them, using modern taxonomic methods.

MATERIALS AND METHODS

Sampling site. Site LY1 (56° 28.9 N, 5° 30.1 W, ~52 m depth, Firth of Lorne; Fig. 1), was chosen as a monitoring station that broadly represents western Scottish coastal waters, that receives waters from the open sea, and also outflow from the fjordic sea lochs Creran, Etive and Linnhe (Fig. 1). As will be discussed below, and has been reported in more detail in Fehling (2004), a transect of 7 stations (Fig. 1), including LY1 and covering a horizontal distance of about 35 km from Loch Spelve in the south to Loch Creran in the north, was sampled in July, August and September 2001 and confirmed that LY1 represents the region as a whole in terms of hydrographic and biotic parameters. The sampling site LY1 was easily accessible (20 min on the steamer RV 'Seol Mara') and had previously been studied for short periods during the 1970s and 1980s, providing some background data for comparison.

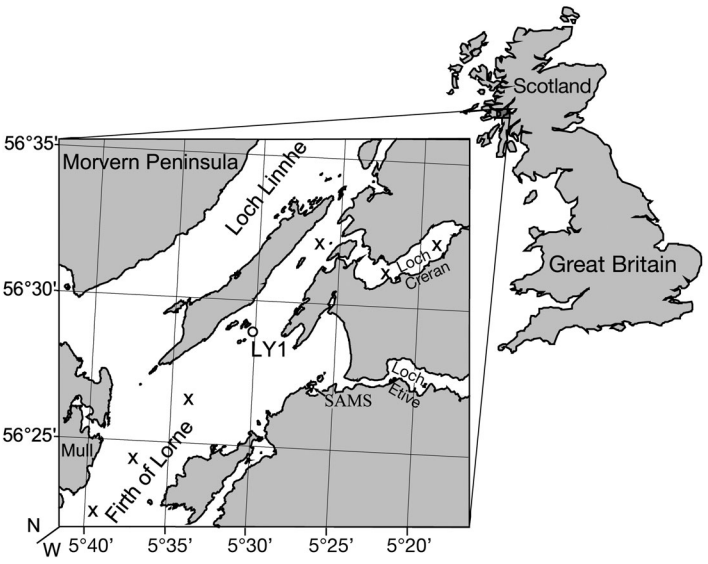

Fig. 1. Location of phytoplankton monitoring site (LY1, O) and stations along Spelve–Creran transect (×) in Firth of Lorne, UK. SAMS: Scottish Association for Marine Science

Sampling and *Pseudo-nitzschia* identification. Site LY1 was sampled from November 2000 to July 2003, weekly between April and November, and fortnightly during the remaining months. The temperature (data not shown) and salinity (see Fig. 2) profiles of the upper 15 m of the water column were recorded with a CTD probe (Seabird SBE 19, Sea-Bird Electronics). Based on a preliminary study, we chose to collect (using a water sampler attached to a winch) samples for cell enumeration and inorganic nutrient concentrations from 10 m depth. This sampling depth was chosen to avoid sampling the superficial layer, which contained outflow from Lochs Creran, Etive and Linnhe. Integrated net samples for transmission electron microscopy (TEM) identification, isolation and cultivation of *Pseudo-nitzschia* spp. were taken from 0 to 20 m depth with a 20 µm mesh, handheld, plankton net (Hydro-Bios). For cell counts, 200 ml of the water sample and 50 ml of the plankton net sample for TEM inspection (as described in Fehling et al. 2004b) of *Pseudo-nitzschia* rich samples were immediately preserved with Lugol's iodine (1% final concentration).

Inorganic nutrient concentrations. For the determination of inorganic nutrient concentrations, samples were filtered under low vacuum through glass-fibre filters (Type A/E, Pall Corporation) to exclude cells and debris, and the filtrate was analysed with a Quick

Chem 8000 autoanalyser (LACHAT Instruments), using flow injection after standard autoanalyser methods. Blank samples were found to be low or zero during the analysis.

Cell counts. Cells were counted with an inverted Zeiss Axiovert 100 light microscope (Jena) at 200× magnification, using Utermöhl's (1931) method. Prior to counting, the Lugol's iodine fixed sample was settled in a 50 ml chamber (Hydrobios) for 24 h. Inspecting the whole chamber area fixed the limit of detection at 20 individuals l^{-1}. *Pseudo-nitzschia* spp. cells were allocated to 1 of 2 *Pseudo-nitzschia* groups (delicatissima-group, width <3 µm, linear shape; seriata-group, width >3 µm, lanceolate shape of cells: after Hasle 1965), as light microscopy does not allow exact specific delineation (Skov et al. 1999). Some *P. pungens* cells with a width of <3 µm were found; these were easily distinguished from the delicatissima-group and allocated to the seriata-group by their shape. *P.* cf. *americana*, easily distinguishable from other *Pseudo-nitzschia* cells due to its small size and different shape, was counted separately from the 2 *Pseudo-nitzschia* groups, but was only recognised in samples after December 2002, although it is likely that it was present before.

Biomass. To determine their cell carbon biomass, length, width and height, at least 30 cells per taxon were measured, using the image analysis software axiovision 4.1 (Zeiss). From these measurements, cell volumes for *Pseudo-nitzschia* spp. and other phytoplankton were calculated after Hillebrand et al. (1999). Mean cell volumes were then converted into carbon, applying the formulae of Menden-Deuer & Lessard (2000).

Isolation, cultivation and identification. From non-preserved net samples single *Pseudo-nitzschia* spp. cells or chains were isolated by micropipette, grown into clonal culture at 15°C in Si-enriched f/2 medium (Guillard 1975). Cultured strains (see Table 1) were named after the sampling site (LY1) and the number of sampling and isolate (e.g. PLY1St.16B *Pseudo-nitzschia* sample taken at LY1, Sampling Event 16, Isolate B).

Cultures of *Pseudo-nitzschia* spp. isolates and cells from net samples containing high *Pseudo-nitzschia* numbers were identified using TEM (Tables 1 & 2),

Table 1. *Pseudo-nitzschia* spp. Fine-structure measurements of some cultures from Site LY1, identified by TEM. +: present; –: absent. Minus values in parentheses indicate reduction of poroids

Strain	Isolation date (dd.mm.yy)	Central interspace	Fibulae 10 µm^{-1}	Interstriae 10 µm^{-1}	Rows of poroids	Poroids 1 µm^{-1}	Width (µm)
P. australis							
PLY1St.24D	17.08.01	–	15	16	2	4–5	?
PLY1St.27E	07.09.01	–	15	15	2	4–5	6.6
PLY1St.37A	15.11.01	–	17	17	2	4–7	4.8–5.4
PLY1St.37B	15.11.01	–	14	14–17	2	4–6	5.1–6.5
PLY1St.54C	14.06.02	–	14–16	14–16	2	4–5	4.9–6.8
PLY1St.54D	14.06.02	–	14–15	15	2	5	6.3–7.2
P. seriata f. *seriata*							
PLY1St.16B	02.06.01	–	14–20	14–20	2 (–1)	6–8	5–5.2
PLY1St.52B	31.05.02	–	18–19	16–20	2 (–1)	7–8	4.6–6
P. pungens							
PLY1St.33C	19.10.01	–	12	12	2	3–4	3.1
P. fraudulenta							
PLY1St.12C	17.05.01	+	24	25	2–3	5–6	4.3
PLY1St.36A	09.11.01	+	24	24–26	2–3	5–6	4.7
PLY1St.85E	04.04.03	+	20	22	2–3	5–6	5.1
P. delicatissima							
PLY1St.42A	04.02.02	+	20	39	2	9	1.85
PLY1St.43D	15.02.02	+	21	34–40	2	9–10	2.2–2.5
PLY1St.43F	15.02.02	+	22	40	2	9–10	2.0
PLY1St.45E	18.03.02	+	20	38	2	9–11	2.0
PLY1St.46A	28.03.02	+	22–24	35–40	2	10–14	1.7–1.8
PLY1St.46C	28.03.02	+	22	40	2	8–10	2.1–2.2
PLY1St.48A	26.04.02	+	23–27	35–37	2	9–12	2.4
PLY1St.48B	26.04.02	+	23	35–39	2	8–11	2.3–2.8

Table 2. *Pseudo-nitzschia* spp. Fine-structure measurements of seriata-group and *P. americana* from some plankton-net field samples. +: present; –: absent. Values in parentheses indicate additional single poroids

Strain	Sampling date (dd.mm.yy)	Central interspace	Fibulae 10 µm^{-1}	Interstriae 10 µm^{-1}	Rows of poroids	Poroids 1 µm^{-1}	Width (µm)
P. australis							
LY1St.20	20.07.01	–	14–15	14–15	2	4–5	7–7.4
LY1St.55	21.06.02	–	15–16	15–16	2 (+1)	5–6	8.0
LY1St.58	12.07.02	–	15–16	15–16	2	4–5	7.2–7.6
			13	13	2 (+1)	4–5	7.2–7.6
LY1St.61	02.08.02	–	15	16	2	5–6	?
LY1St.70	04.10.02	–	16	16	2	5–6	5.6–6.4
P. seriata f. *seriata*							
LY1St.55	21.06.02	–	18	18	2 (+2)	7–8	5.4
LY1St.70	04.10.02	–	16	16	2 (+1)	6–7	5.6–6.4
P. pungens							
LY1St.60	26.07.02	–	11	12	2	3–4	2.7
LY1St.70	04.10.02	–	10	10	2	3	2.2
P. fraudulenta							
LY1St.20	20.07.01	+	24	22–24	2–3	6–7	5.1
LY1St.65	29.08.02						
LY1St.70	04.10.02						
P. cf. *subpacifica*							
LY1St.70	04.10.02	+	17–18	26–28	2	8–9	5
P. americana							
LY1St.61	04.10.02	–	16	25	2 (+1)	8	1.8
P. delicatissima							
LY1St.55	20.07.01	+	30	40	2	9–10	1.5
LY1St.60	21.06.02						
LY1St.61	02.08.02	+	28	40	2	10	1.5
P. pseudodelicatissima /							
LY1St.62	07.08.02	+	24	40–42	1	6–7 / 2[a]	1.1
P. cf. *pseudodelicatissima*							
LY1St.70	04.10.02	+	24	40	1	6–7 / 4–6[a]	1.6

[a]Sectors of poroid in *P. pseudo-delicatissima*

as described in Fehling et al. (2004b). Molecular identification of cultures followed the methods described in detail by Fehling et al. (2004b), which are briefly described here. DNA was extracted using a phenol/chloroform method (Bolch et al. 1998) and the Internal Transcribed Spacer (ITS) Regions I and II, the 5.8S and the partial large subunit (LSU) were amplified by PCR using the primers of Adachi et al. (1996) and Scholin et al. (1994) respectively. PCR was carried out in 50 µl reactions, using ABgene *Taq* polymerase and ABgene PCR Buffer IV (ABGene) and the PCR conditions described in Fehling et al. (2004b). Successful PCR products were purified through microcon PCR filter devices and cycle sequenced using ABI Big-Dye terminator chemistry with the respective forward and reverse amplification primers. Sequences were corrected manually by inspection of electropherograms, and consensus sequences were aligned and compared to determine the rDNA genotypes of each strain.

Cluster and redundancy analysis. The distribution of the *Pseudo-nitzschia* spp. population over the annual cycle and the benefit of separating them into 2 groups were investigated by cluster analysis using the statistical software PRIMER (Clark & Warwick 1994). Data were square root transformed prior to analysis.

To investigate the relationship between *Pseudo-nitzschia* spp. appearance and environmental variables, we applied redundancy analysis (RDA) using the programme CANOCO (ter Braak & Smilauer 1998). Redundancy analysis allows the estimation of the strengths of relationships between species composition and different environmental variables. Forward selec-

tion was applied within the RDA to test the significance of each of the environmental variables. This was achieved using an unrestricted Monte Carlo permutation test. Data were square root transformed prior to analysis.

RESULTS

Temperature, salinity and inorganic nutrients

The salinity contours in Fig. 2 show that the low salinities that occurred episodically in the superficial layer did not influence the 10 m phytoplankton sampling depth. A strong seasonal signal was observed in the 15 m temperature profile of the water column at LY1 with, in general, increasing water temperatures from spring towards autumn and decreasing temperatures in winter. At 10 m depth, coldest temperatures were regularly found in March (6.6°C) and warmest in September (14°C) (Fig. 3). Some interannual variability was evident, with a lower minimum temperature in winter 2001 than in the other years, and a highest annual water temperature of 15°C in August 2003 (at the surface, data not shown), after sampling for this study had been completed.

Dissolved inorganic phosphate, nitrate and silicate concentrations reflected a seasonal pattern, with highest values in winter and lowest values in summer (Fig. 4). Some interannual variability was noted, with generally increasing concentrations of phosphate and silicate between November 2000 and July 2003. Phosphate concentrations ranged from 0.05 µM (May 2001) to 0.72 µM (February 2003), silicate concentrations from 0.26 µM (May 2001) to 8.43 µM (February 2003). Lowest nitrate concentrations were found in July 2002 (0 µM) and highest in February 2002 (7.59 µM). Ammonium concentrations (data not shown) did not show any interannual trends. In general, elevated concentrations up to 1.5 µM were observed between May and September and low concentrations during autumn and winter.

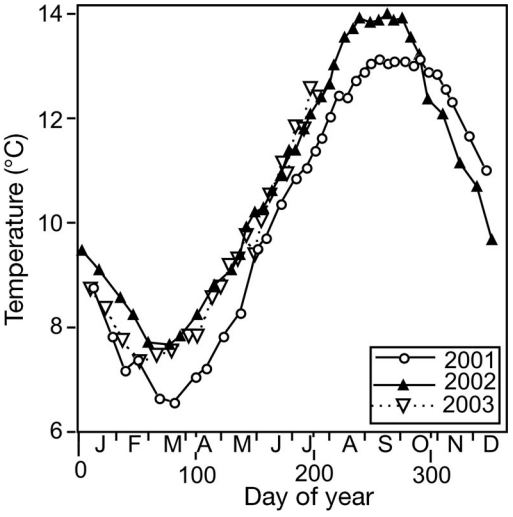

Fig. 3. Water temperature at Site LY1, 10 m depth, in 2001, 2002 and 2003

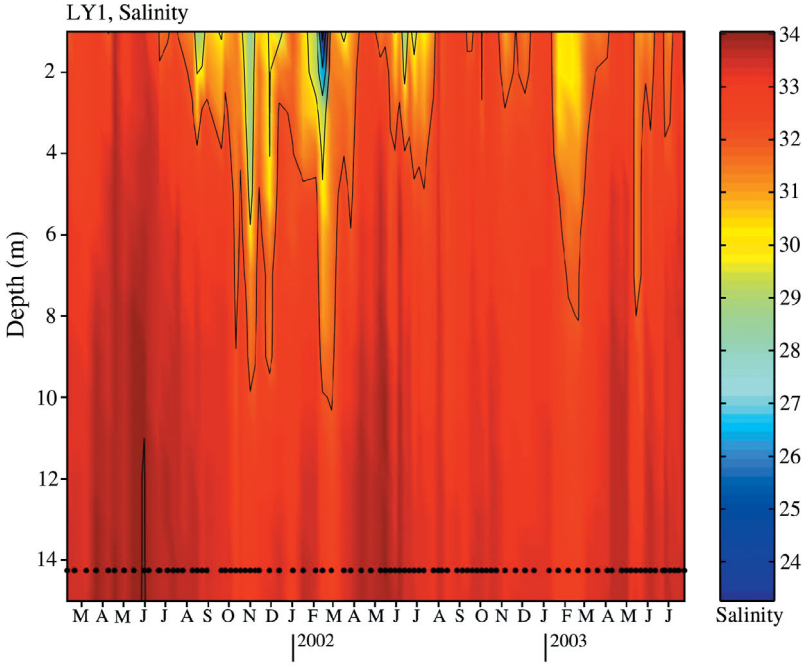

Fig. 2. Salinity profile of 0 to 15 m water column at Site LY1 from February 2001 to 25 July 2003. (•) Sampling events for which salinity data are included

The delicatissima-group

Fig. 5 shows the seasonal cycle of cells within both groups between November 2000 and July 2003. Cells belonging to the delicatissima group occurred in 95% of all samples, with highest numbers in a single sample reaching 1.6×10^5 cells l^{-1} (April 2002). In all years, blooms with a density of $>10^4$ cells l^{-1} persisted for up to 6 wk in spring (March, April, also May), and again in June, July and August. TEM examinations of field samples taken in spring were monospecific with respect to *Pseudo-nitzschia* indicating that, at that time, *P. delicatissima* was most probably the only species within the delicatissima-group. In spring 2001 and 2002, *P. delicatissima* was the most abundant taxon after *Skeletonema* sp.,

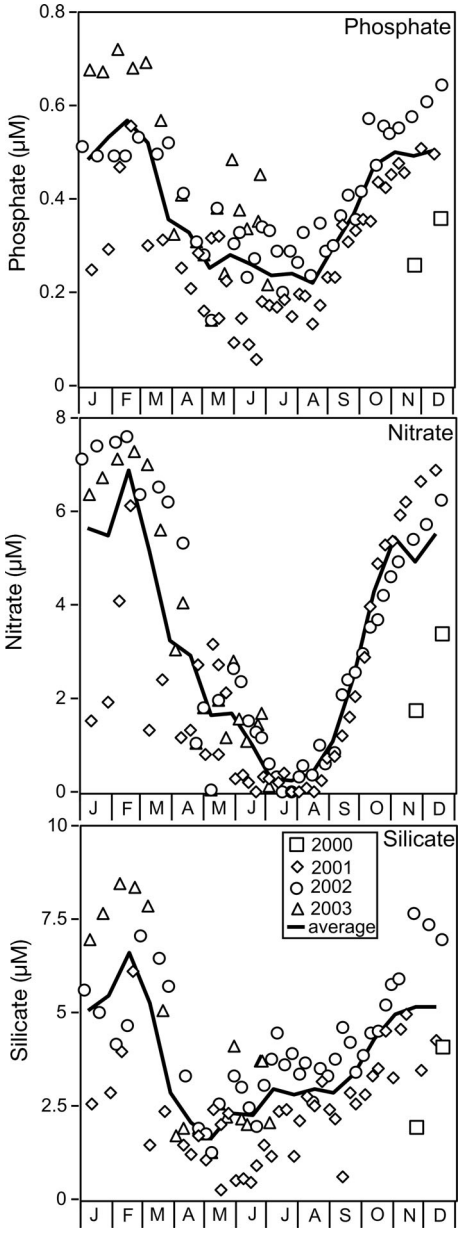

Fig. 4. Phosphate, nitrate and silicate concentrations at Site LY1, 10 m depth, between November 2000 and July 2003

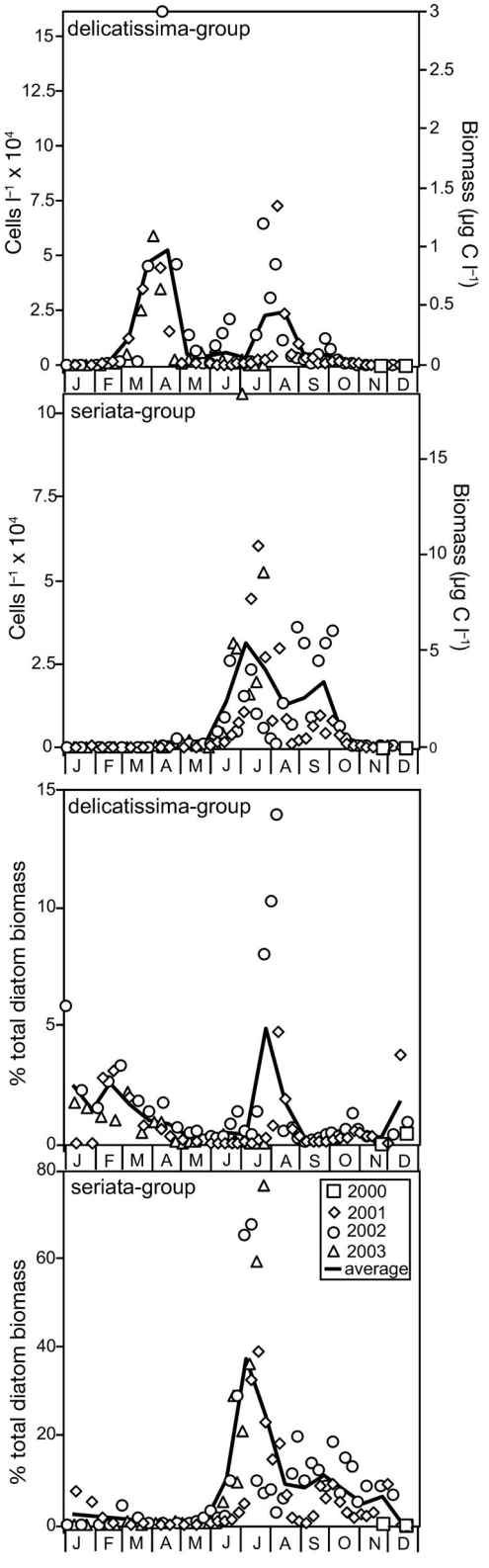

Fig. 5. *Pseudo-nitzschia* spp. Cell numbers and biomass of delicatissima- and seriata-groups, and biomass of both groups as percentage of total diatom biomass at Site LY1, 10 m depth, between November 2000 and July 2003

and in spring 2003 the third most abundant taxon (after *Skeletonema* sp. and *Thalassiosira* spp.). During summer the delicatissima-group was more diverse, being composed of *P. delicatissima*, *P. pseudodelicatissima* and *P. calliantha.* Fig. 6 shows TEM pictures of species belonging to the delicatissima-group that were found at LY1.

We established 13 clonal cultures of *Pseudo-nitzschia delicatissima* by isolating cells from samples taken at LY1 between 2001 and 2003. All cultures isolated during spring were identified by TEM and/or molecular methods (Fehling et al. 2004b) as *P. deli-*

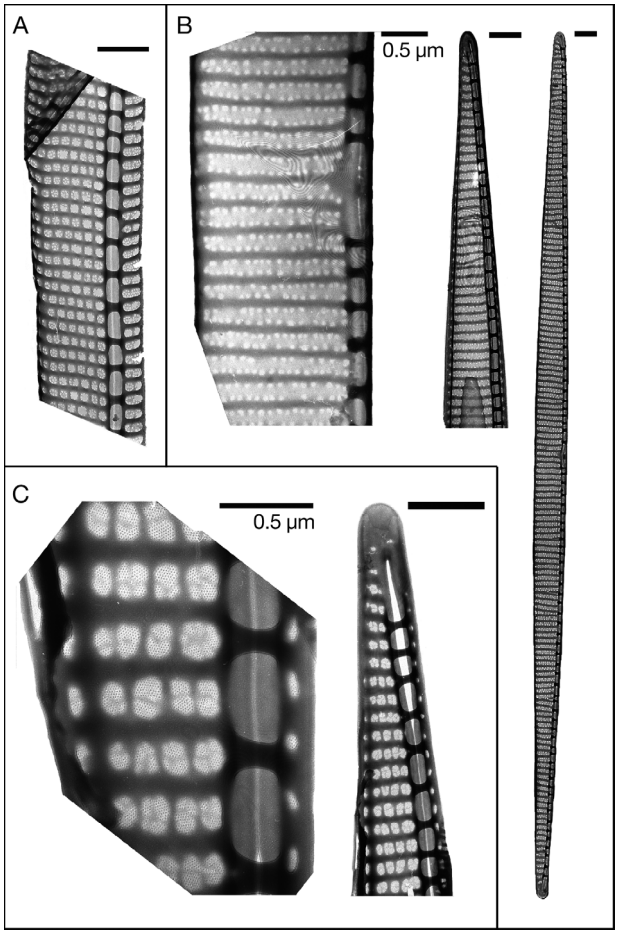

Fig. 6. *Pseudo-nitzschia* spp. TEM micrographs of frustules of Scottish strains (A and C from field samples, B from culture) belonging to delicatissima-group. (A) *P. calliantha*, (B) *P. delicatissima*, (C) *P. pseudodelicatissima*. Scale bars represent 1 µm where not labelled otherwise

Table 3. *Pseudo-nitzschia* spp. Molecular identification of cultured strains, showing GenBank accession numbers (Acc. No.)

Strain	Isolation date	ITS1, 5.8S, ITS2 (Acc. No.)	LSU (Acc. No.)
P. australis			
PLY1St.19A	13.07.01	AY452527	AY452529
PLY1St.20A	20.07.01	–	AM118053
PLY1St.24D	17.08.01	AM118029	AM118054
PLY1St.27E	07.09.01	AM118030	AM118055
PLY1St.33A	19.10.01	AM118031	AM118056
PLY1St.37A	15.11.01	AM118032	AM118057
PLY1St.37B	15.11.01	AM118033	AM118058
PLY1St.54B	14.06.02	AY452528	AY452530
PLY1St.54C	14.06.02	AM118034	AM118059
PLY1St.56B	26.06.02	AM118035	AM118060
PLY1St.68C	20.09.02	AM118036	AM118061
P. seriata			
PLY1St.16B	22.06.01	AY452523	AY452525
PLY1St.52B	31.05.02	AY452524	AY452526
P. fraudulenta			
PLY1St.11A	03.05.01	–	AM118062
PLY1St.11C	03.05.01	AM118037	AM118063
PLY1St.11D	03.05.01	AM118038	AM118064
PLY1St.12A	17.05.01	AM118039	AM118065
PLY1St.15A	15.06.01	AM118040	AM118066
PLY1St.36A	09.11.01	AM118041	AM118067
PLY1St.85E	04.04.03	AM118042	AM118068
P. delicatissima			
PLY1St.5	09.02.01	AM118043	AM118069
PLY1St.42A	04.02.02	AM118044	AM118070
PLY1St.43C	15.02.02	AM118045	AM118071
PLY1St.45E	18.03.02	AM118046	AM118072
PLY1St.46A	28.03.02	AM118047	AM118073
PLY1St.48A	26.04.02	AM118048	AM118074
PLY1St.77C	06.12.02	AM118049	AM118075
PLY1St.85A	04.04.03	AM118050	AM118076
PLY1St.85B	04.04.03	AM118051	AM118077
PLY1St.85Z	04.04.03	AM118052	–

catissima; TEM measurements of fine structural features of cultures of this species are listed in Table 1. Strains slightly differed in their numbers of fibulae and interstriae 10 µm^{-1}, poroids 1 µm^{-1} and widths. However, while this morphological variability of *P. delicatissima* might point to further hidden species, our molecular data (ITS, 5.8S and partial LSU sequences; Table 3) for all *P. delicatissim*a strains were identical, and were identified in BLAST searches (Altschul et al. 1997) as *P. delicatissima*. Fine-structure measurements from field samples showed only a slight difference in the number of fibulae between cells collected at different times (Table 2). For *P. delicatissima*, numbers of fibulae were higher in cells from field samples than in cultured cells, which might have been due to the age of the cultures. In diatoms, frequent cell division leads to a reduction in cell length. This might have led to a reduction in the number of fibulae in cultured *P. delicatissima* frustules.

We tested 1 *Pseudo-nitzschia delicatissima* culture (PLY1St.42A) isolated during this study and 3 other cultured strains that had been isolated previously but showed identical molecular ITS, 5.8S and LSU sequences to all *P. delicatissima* strains isolated here for DA production. None of these strains were found to be toxic.

The seriata-group

The seasonal cycle of cells within the seriata-group is presented in Fig. 5, and can be seen to differ markedly from that of the delicatissima-group. The seriata-group cells occurred in 83% of all samples, with a maximum

cell density of 1.1×10^5 cells l^{-1} (July 2003). The seriata-group reached highest densities between June and October, with the greatest cell density being recorded regularly in July. Bloom events with cells densities of $>10^4$ cells l^{-1} would sometimes persist for short periods of <2 wk. In summer, this was the most abundant taxon together with the ciliate *Myrionecta rubra* (all years), the diatom *Leptocylindrus danicus* (2001) or dinoflagellates (2002).

TEM examination of cultured isolates and field samples from that time indicated that this group was diverse, with *Pseudo-nitzschia australis*, *P. seriata*, *P. fraudulenta*, *P. pungens* and *P.* cf. *subpacifica* present (Fig. 7). *P. multiseries* was only seen rarely. TEM-inspected field samples showed that blooms of the seriata-group were often mixed blooms, consisting of several species. While species identification was possible using TEM, within species morphological fine structure was not uniform, with variations being found in number of fibulae and interstriae 10 μm^{-1}, and in poroids 1 μm^{-1} (Table 2). Within *P. australis* and *P. seriata*, the number of rows of poroids varied, in some individuals additional rows were observed. Only *P. fraudulenta* displayed unambiguous morphology that did not vary between individuals examined.

As for the field samples, fine-structure features from cultured cells showed slight differences in the number of fibulae and stria 10 μm^{-1}, poroids 1 μm^{-1} (*Pseudo-nitzschia australis* and *P. seriata*) and width (*P. australis*, *P. seriata* and *P. fraudulenta*) between strains belonging to a single species (Table 1); this kind of variation might be expected. However, ITS and large subunit rRNA gene data (Table 3) indicated that all strains were identical and showed no or only a slight difference from sequences for *Pseudo-nitzschia* strains of each species from other parts of the world.

Within the seriata-group, 2 DA producers were identified amongst our samples — *Pseudo-nitzschia seriata* and *P. australis.* Their toxicity was confirmed from the study of uni-algal isolates (Fehling et al. 2004a,b). Both *P. fraudulenta* and *P. pungens* were also successfully isolated from LY1 net samples and maintained in culture, but were not found to produce detectable levels of DA.

Pseudo-nitzschia americana densities were low; cell numbers did not exceed 40 cells l^{-1} in a single sample and, when present, were mostly epiphytic on *Chaetoceros* spp. cells. *P. americana* is not a known toxin producer (Villac et al. 1993). This species is therefore not significant in terms of numbers, biomass or toxin production, but has been included for completeness.

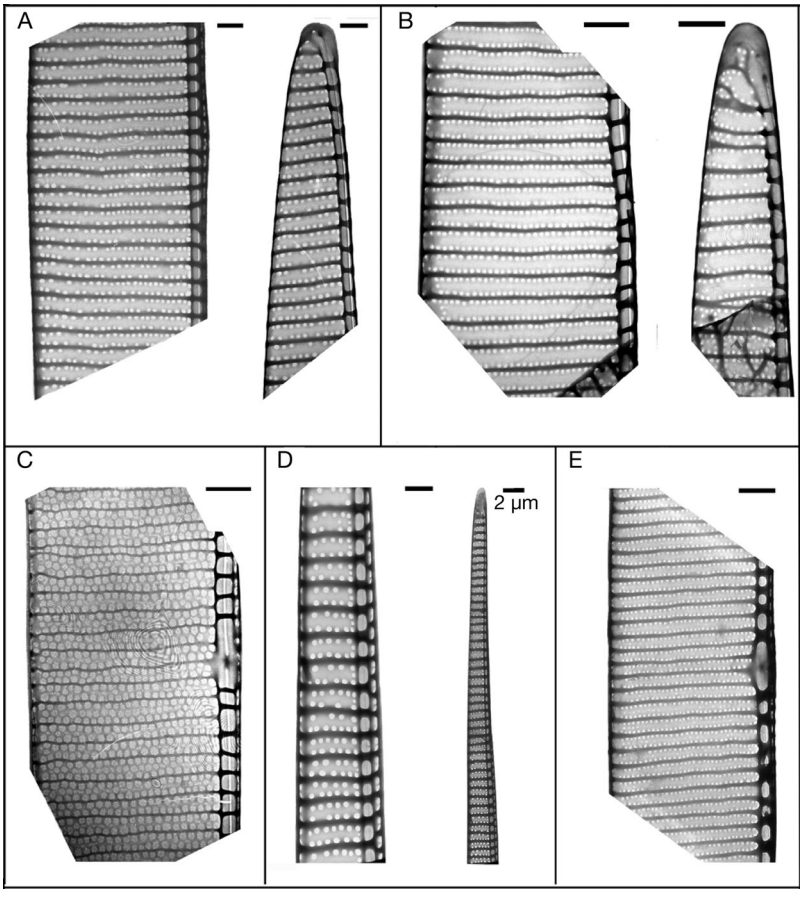

Fig. 7. *Pseudo-nitzschia* spp. TEM micrographs of frustules of Scottish strains (all from cultures) belonging to seriata-group. (A) *P. australis*, (B) *P. seriata*, (C) *P. fraudulenta*, (D) *P. pungens*, (E) *P.* cf. *subpacifica*. Scale bars represent 1 µm where not labelled otherwise

Biomass

The delicatissima-group reached higher cell densities than the seriata-group. However, the larger size of seriata-group cells meant that the converse was true for the C biomass content of the 2 groups, this quantity being much greater for the seriata-group (Fig. 5). The delicatissima-group reached a maximum biomass of 3 µg C l^{-1}, while the biomass of the seriata-group peaked at approximately 18 µg C l^{-1}. When expressed as a percentage of the total diatom biomass (Fig. 5), the delicatissima-group represented up to 14% of all diatoms (August 2002), while at times up to 77% of the total diatom biomass was composed of the seriata-group (July 2003).

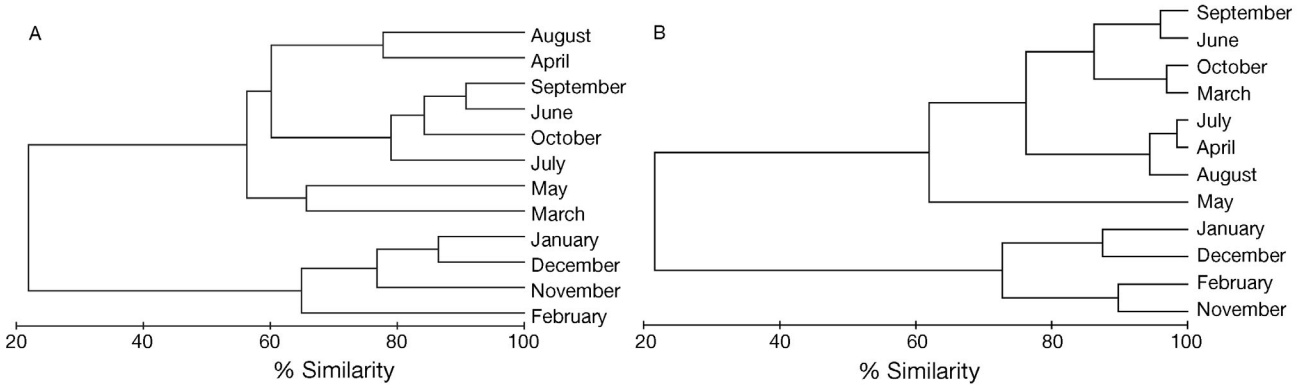

Fig. 8. *Pseudo-nitzschia* spp. Dendrogram generated by cluster analysis of abundance data grouped by month over the study period. (A) Analysis with seriata-group and delicatissima-group separately, (B) analysis with the 2 groups combined

Cluster and redundancy analysis

Fig. 8 is a dendrogram of the sum of *Pseudo-nitzschia* spp. abundance each month based on both total and group (delicatissima and seriata) counts. Data were square root transformed prior to analysis. In both cases, there is a marked difference between winter and summer populations, with November to February exhibiting only 20% similarity with the rest of the year, indicative of the low phytoplankton densities observed in winter. When analysed on a group specific basis, a summer (June, July, September, October) population was evident. August showed only 64% similarity with the 2 mo preceding and following it, and clustered most closely with the spring month of April, a result of the peak in the delicatissima-group in these 2 mo. March and May clustered together, but with only 68% similarity and were representative of the increa-se and decline of the spring *P. delicatissima* bloom.

Analysis based on total counts failed to recognise these patterns in the data. In particular, the dendrogram based on total *Pseudo-nitzschia* spp. found high similarity between April, July and August, and hence did not discriminate the difference in species composition at these times. Applying taxonomic discrimination to group level therefore indicated that the 2 *Pseudo-nitzschia* groups had different temporal distributions, with the former occurring in both spring and summer, and the latter only in summer and early autumn.

Fig. 9 is an RDA ordination plot of samples and environmental variables. The RDA axes separate the 2 *Pseudo-nitzschia* groups. The seriata-group was most closely related to temperature, consistent with its appearance in summer. In contrast, the delicatissima-group (that appeared in both spring and summer) was closely related to both salinity and ammonium concentration. A negative relationship between both groups and the 3 major inorganic nutrients (nitrate, phosphate and silicate) was evident, indicating the requirement for these for growth and nutrient depletion prior to the peak of the bloom of either group. Photoperiod (the duration of the light phase during a 24 h period) showed a strong positive relationship with both *Pseudo-nitzschia* groups. The 7 environmental variables included in the analysis accounted for 31% of the variance in the species composition. Of this, 90% was explained by the 3 environmental variables that were significant in the Monte Carlo permutation test. Photoperiod explained 16% of the variance in species composition ($p = 0.002$), temperature 8% ($p = 0.002$) and salinity 4% ($p = 0.028$).

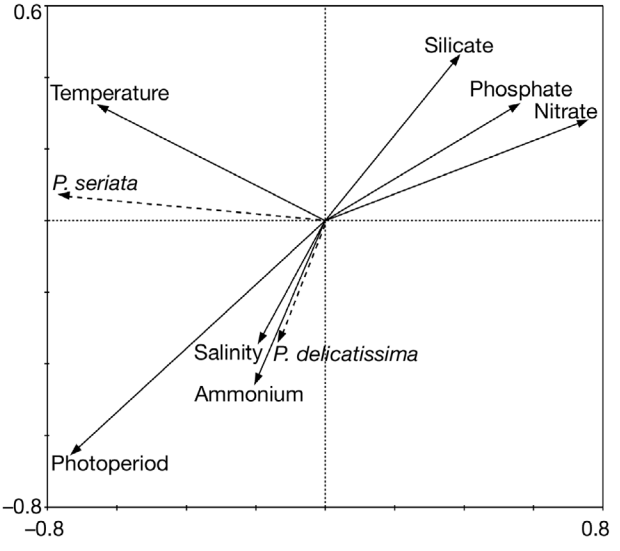

Fig. 9. *Pseudo-nitzschia* spp. Redundancy analysis of relationship between *Pseudo-nitzschia* group abundance and 7 measured environmental variables

DISCUSSION

Hydrographic conditions and nutrients

The Firth of Lorne (Fig. 1) is a large, multi-basin fjord from which smaller fjords branch. Water exchanges between its inner parts, especially between the 2 basins of Loch Linnhe, have been described in several papers (Allen & Simpson 1998, Watts et al. 1998). Physical processes in the main basin in the Firth of Lorne are less well known, but logic, the presence of the Firth of radioisotopes from the Irish Sea (McKay et al. 1986), and freshwater budgets (Simpson & Hill 1986) suggest that its water exchanges with that of the Scottish Coastal Current. Site LY1, with a water depth of about 50 m, lies on the sill that separates the main basin of the Firth of Lorne from the Outer Loch Linnhe basin.

The density structure of the water column (Fig. 10) is typical of distributions observed in Scottish fjords (e.g. Wood et al. 1973), and while there is no obvious step in the profile suggesting flow separation, it is suggestive of a 2-layer structure in the upper part of the water column. Other data (Grantham 1983, Laurent & Tett unpubl.) show that the lower layer extends to the seabed, with typically little further increase in density below 15 m. We suppose that the superficial layer is on average seawards-tending, and that its lower salinity is the result of outflows from Loch Linnhe, Loch Creran and Loch Etive.

Typical salinities (Fig. 2) at the sampling depth of 10 m at LY1 were between 32 and 34, with the highest values (around 33.5) tending to occur during spring, the driest season in this region. These values, and the maximum nutrient concentrations (in late winter) of 6 to 8 µM nitrate and 0.5 to 0.7 µM phosphate support the hypothesis that the 10 m LY1 water originated in the main basin of the Firth of Lorne. Ultimately this water might have come from the Scottish Coastal current, being modified during transit.

Phytoplankton counts along the 7 stations of the Spelve-Creran transect that included LY1 (Fig. 1) were used to generate Fig. 11, whose data indicates that *Pseudo-nitzschia* spp. densities were relatively similar at all stations during each of the 3 transects. For the delicatissima-group the density at LY1 was always close to the mean for all stations, and for the seriata-group always within 1 standard deviation of the mean. The count data from LY1 can therefore be seen as representative of intermediate-depth waters in the inner Firth of Lorne, and thus, arguably, of the coastal inflow to the Firth.

In the last 20 yr, the region has experienced a marked increase in nutrient input as a result of the development of a large fish farming industry (Black 2001). The substantial mixing of coastal water with the adjacent open sea would seem to suggest that such nutrient inputs are unlikely to perturb the ambient nutrient concentrations sufficiently to cause shifts in the phytoplankton community composition that would promote *Pseudo-nitzschia spp.* dominance over other phytoplankton or an elevation in their toxicity (Tett & Edwards 2002). While our laboratory studies (Fehling et al. 2004a) have demonstrated the influence of variability in dissolved nutrient stoichiometry on toxin production by a *Pseudo-nitzschia* species (*P. seriata*) from the region, as yet there is insufficient evidence to confirm or deny that local nutrient enrichment actually influences the occurrence of *Pseudo-nitzschia* species at a particular location.

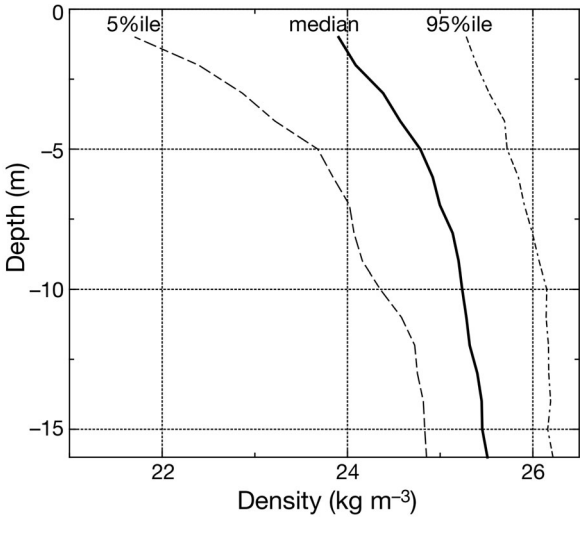

Fig. 10. *Pseudo-nitzschia* spp. Variation in density with depth. Almost 100 CTD profiles were available. For each 1 m depth increment, density values were sorted into lower 5%ile, median, and upper 95%ile. Lower 5%ile was ≥5% of values, and upper 95%ile was ≥95% of the values

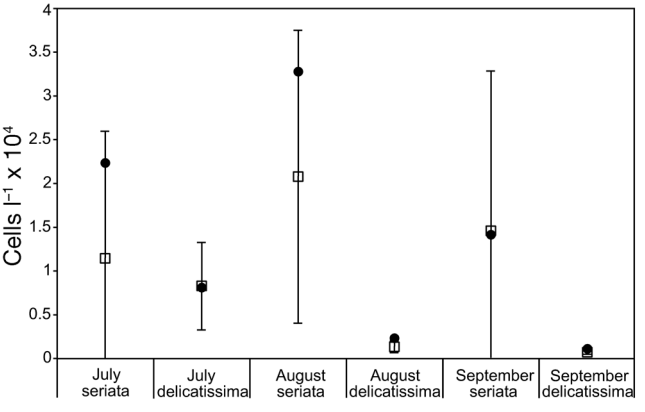

Fig. 11. *Pseudo-nitzschia* spp. Abundance at Site LY1 (●) compared to mean (± SE) of seriata- and delicatissima-groups at 6 stations sampled during each of the 3 Spelve–Creran transects (□)

Pseudo-nitzschia identification and categorisation

The study demonstrated that a diverse *Pseudo-nitzschia* community exists in Scottish waters, the *Pseudo-nitzschia* population at Site LY1 being composed of a number of different species but with a repeatable annual succession. The almost complete lack of *P. multiseries* (the dominant toxic species in Canadian waters of similar latitude) highlights the necessity for location dependent studies of *Pseudo-nitzschia* dynamics. Both potentially toxic and non-toxic species were present, their abundance fluctuating markedly throughout the year. The dominant potentially toxic species present were *P. seriata* and *P. australis*, both identified frequently from TEM analysis. Both species were abundant in the summer months, when potentially toxic *P. pseudodelicatissima* (Pan et al. 2001, Trainer et al. 2002) and toxic *P. calliantha* (Lundholm et al. 1997, Martin et al. 1990) were also present.

Identification of *Pseudo-nitzschia* to species level under the light microscope is only partially possible. However, as indicated in Tables 1 & 2, even TEM identification of the species composition proved problematic, as strains of the same species exhibited differences in morphological fine structure. Hence, molecular identification methods are necessary to provide unambiguous genetic identification (Fehling et al. 2004b). However, such analysis indicated that, in general, cells exhibiting small differences in fine structure did belong to the same species.

Pseudo-nitzschia group dynamics and relation to environmental variables

The requirement to isolate living cells into culture prior to conducting molecular analysis places a limitation on the routine use of such techniques for *Pseudo-nitzschia* identification. Hence, to gain a degree of taxonomic resolution that could be routinely applied with light microscopy, we used cell width and aspect ratio as the criteria to separate cells into the delicatissima and seriata-groups, as suggested by Hasle (1965). The distribution of the *Pseudo-nitzschia* population over the annual cycle and the benefit of separating *Pseudo-nitzschia* into 2 groups was then shown by cluster analysis, which clearly favoured our taxonomic discrimination between the 2 groups. It indicated that the 2 groups had different temporal distributions, with the delicatissima-group occurring in both spring and summer, and the seriata-group occurring only in summer and early autumn. As no toxic cells were found within the delicatissima-group, this categorisation may be of particular benefit to monitoring programs in Scottish waters.

The results of the RDA revealed 3 significant variables, the most significant being photoperiod. The importance of photoperiod is consistent with our previous laboratory study of *Pseudo-nitzschia seriata* and *P. delicatissima* (Fehling et al. 2005), in which we found growth rate and cell yield of both species to be related to the duration of the light phase experienced by the cells.

While no significant relationship between macronutrient concentration and *Pseudo-nitzschia* spp. was found, the availability of these nutrients is an undeniable requirement for phytoplankton growth. In late spring, and prior to the establishment of the summer phytoplankton population, the concentration of all 3 macro-nutrients decreased through their uptake by phytoplankton. Subsequently, a general increase in silicate concentration was evident over the summer months, with only 2001 exhibiting silicate concentrations consistently less than the 2 µM threshold of Egge & Asknes (1992). As a summer diatom bloom was evident in all years, including 2001, there was little evidence of silicate limitation of the summer population. Rather, *Pseudo-nitzschia* spp. growth seemed to be related to the availability of nitrate. The decline of the early summer seriata-group bloom coincided with the point that nitrate concentrations declined to a near zero threshold value. Subsequently, the late summer seriata-group bloom began to increase only when nitrate concentration had also begun to increase.

The dynamics of the delicatissima-group in summer were also most readily related to nitrate availability but in a contrasting manner to the seriata-group. The beginning and end of the delicatissima-group summer bloom was closely related to the start and end of the period of nitrate depletion respectively. This suggests that the species making up the delicatissima-group summer bloom are effective scavengers of low nitrate concentrations, but are out-competed at higher concentrations of this nutrient. Alternatively, they are capable of efficiently using the ammonium that would be regenerated from zooplankton grazers. However, this latter hypothesis does not explain the decrease in the bloom when nitrate concentrations increase again.

Spring bloom

The initial bloom of the delicatissima-group, in which presumably only *Pseudo-nitzschia delicatissima* occurred, corresponded to the period of the spring phytoplankton increase. This increase comprised relatively few species and was dominated in numbers and biomass by *Skeletonema* sp. (Fehling 2004). High concentrations of *P. delicatissima* in spring were also reported by Hasle et al. (1996), who studied the Skagerrak and Norwegian

coasts between 1980 and 1993, and by Miralto et al. (2003) in the Adriatic (Mediterranean Sea), who found the spring bloom mainly composed of *Skeletonema costatum* and *P. delicatissima*. Both *P. delicatissima* and *S. costatum* are small and lightly silicified diatoms, which may help ensure their success under permanently stratified conditions. Their success in spring suggests that both are able to divide at the low illuminations and water temperatures of late winter.

RDA indicated a significant influence of salinity and a close relationship with the delicatissima-group in particular. Such results are consistent with those of Thessen et al. (2005), who found an influence of salinity on growth and distribution of *Pseudo-nitzschia* spp. in the waters of Louisiana, USA.

Nutrient concentrations prior to the spring bloom were typical of temperate coastal waters. The maximal concentrations in spring were observed in late February/early March and are indicative of the quantity of nutrient available to fuel the spring bloom. As noted above (last subsection), RDA did not yield a significant relationship between inorganic nutrients and *Pseudo-nitzschia* spp. occurrence. In addition no significant correlation was found between magnitude of the spring *P. delicatissima* bloom in terms of numbers or biomass and the availability of any single inorganic nutrient. This lack of correlation is most probably a result of the dominance of the diatom *Skeletonema* sp. in spring, making the contribution of *P. delicatissima* to diatom biomass relatively low (<5%); hence any modest changes in nutrient availability due to natural or anthropogenic forcing are unlikely to significantly modify the species composition of the diatom assemblage, with *Skeletonema* sp. acting as a buffer against this. Furthermore, the mono-specific and benign nature of the spring *Pseudo-nitzschia* bloom suggests that any increase of nutrients at this time would not results in an ASP event.

The decline of a phytoplankton bloom is commonly related to nutrient limitation, grazing or both. The decline of the spring *Pseudo-nitzschia delicatissima* bloom was rapid in each year. Finite concentrations of nitrate, phosphate and silicate were evident at this time, suggesting an important role for grazing and parasitism/disease. However, Egge & Asknes (1992) suggested that diatoms dominate only when inorganic silicate concentrations exceed 2 µM. As the silicate concentration had been reduced to approximately this value, silicate limitation may have been a factor in the decrease of the population.

Summer bloom

The second, and most striking, feature of the *Pseudo-nitzschia* spp. seasonal cycle is the summer bloom. The bloom of the delicatissima-group was less marked than that during spring in numerical terms, but comprised a number of species and reached a greater proportion of total diatom biomass (albeit at a time when phytoplankters other than diatoms were important). In contrast to spring, the delicatissima-group bloom was composed of potentially toxic species including *P. calliantha*, *P. pseudo delicatissima* and *P.* cf. *pseudo delicatissima*, together with non-toxic *P. delicatissima. P. pseudodelicatissima* cells resembled the *P. pseudo delicatissima* description of Lundholm et al. (2003), while *P.* cf. *pseudodelicatissima* differed slightly in the number of sectors of the poroid hymen from any described species within the '*pseudodelicatissima/cuspidata*' complex (Lundholm et al. 2003). Hasle et al. (1996) also found greatest *P. pseudodelicatissima* densities (later identified as *P. calliantha* by Lundholm et al. 2003) mainly in summer. However, as we did not have any species of the delicatissima-group other than *P. delicatissima* in culture, we could not explore their molecular species identity, nor test their toxicity.

The summer bloom of the seriata-group lasted for about 4 mo, comprised several species, and was the only event of the year for the group, which did not take part in the spring bloom. During this time, the seriata-group was amongst the most characteristic organisms. Typical other organisms were a variety of dinoflagellates (mostly <50 µm in size), the photosynthetic ciliate *Myrionecta rubra*, the centric diatom *Leptocylindrus* spp., another lightly silicified raphid pennate *Cylindrotheca closterium*, and the araphid *Asterionellopsis glacialis*.

It was at this time of year that Tett (1973) observed (in 1970 and 1971) at a similar location to LY1, the greatest species diversity and evenness in phytoplankton. However, in our study, *Pseudo-nitzschia* spp. belonging to the seriata-group contributed a majority of diatom biomass in summer (Fig. 5). Moreover, comparing these results with estimates of total autotrophic biomass based on chlorophyll *a* data collected concurrently with our samples for microscopy and carbon: chlorophyll conversion, values derived for Scottish west coast waters by Edwards et al. (2003) suggest that *Pseudo-nitzschia* spp. can contribute up to 70% of total autotrophic biomass at this time. However, as the toxin produced by *Pseudo-nitzschia* spp. is bioaccumulated by filter-feeding shellfish, its relevance to health may well be independent of its relative abundance, biomass or ecological significance. Within our samples, the seriata-group contained at least 2 toxic species, *P. australis* and *P. seriata* (Fehling et al. 2004b). These frequently occurred together with non-toxic *P. fraudulenta*, *P. pungens* and *P.* cf. *subpacifica.* Should we wish to achieve real 'early warning' monitoring of potential future toxic events in these waters, a major challenge

will be to develop methodologies capable of discriminating between toxic and non-toxic species on a routine basis.

As N concentrations are very low at times, these results suggest that the seriata-group is particularly capable of utilising regenerated ammonium (preventing any buildup of this quantity), and hence might be expected to also respond to anthropogenic additions of this nutrient. The RDA ordination plot (Fig. 9) shows little relationship between the seriata-group and ammonium; however, this may be due to the rapid utilisation of this nutrient when the seriata-group is present.

Growth of these summer species was favoured by longer daylengths. The duration of light availability at the latitude of the study changes markedly over the year from a minimum of 6.7 h in December to a maximum of 17.8 h in June. While water temperature and nutrient concentration may potentially change over time scales of years through natural and anthropogenic perturbation, photoperiod will remain constant. As photoperiod was the most significant variable in the RDA, this may indicate its potential to act as a stabilising factor for community composition.

Potential for toxicity

During the bloom periods, densities of each group of *Pseudo-nitzschia* were in the order of 10^4 cells l^{-1}, with maxima of 1.5×10^5 cells l^{-1} of the delicatissima-group and 10^5 cells l^{-1} of the seriata-group. Counts of *Nitzschia seriata* from Loch Creran in 1979 and 1980 (Tett & Edwards 2002) were of similar magnitude. While it is impossible to verify if these species are the same as those present today, these results indicate that potentially toxic species have occurred in densities of ~10^5 cells l^{-1} for some time, suggesting that DA contamination of marine life might have been possible 2 decades before its first report in Scottish waters in 1999. However, shellfish were then not tested for DA, and reports of shellfish poisoning in humans or marine wildlife in Scottish waters are not known. These numbers are also similar to the maximal abundances of *Pseudo-nitzschia* spp. at most sites surveyed by the Scottish authorities for shellfish hygiene purposes during 1996 to 1999. A few sites, however, exceeded 10^6 cells l^{-1} (Gallacher et al. 2001 and Scottish Fisheries Research Service internal reports reviewed by Tett & Edwards 2002).

Currently, large-scale shellfishery closures occur annually in Scottish waters. In 1997 a threshold level of 1.5×10^5 cells l^{-1} of *Pseudo-nitzschia* spp. was set, above which samples of shellfish were tested for ASP toxins (Kelly & Fraser 1999). A density of ~10^5 cells l^{-1} is also the trigger level of *Pseudo-nitzschia* spp. suggested by Bates et al. (1998) to cause ASP. A cell density of similar magnitude had caused ASP in southern California in the 1990s, killing pelicans (Buck et al. 1992, Work et al. 1993) and sea lions (Scholin et al. 2000). On a precautionary basis, the *Pseudo-nitzschia* spp. density above with shellfish testing is required was reduced to 5×10^4 cells l^{-1} in 1998 (Kelly & Fraser 1999). However, there remains a poor understanding of what density and/or species composition of *Pseudo-nitzschia* spp. can lead to toxic accumulations in shellfish. Our typical, rather than peak, bloom density of cells of the seriata-group (10^4 cells l^{-1}) was somewhat below the new ASP trigger density, yet, as blooms of this density persisted for some weeks (Fig. 5), the potential for an accumulation of DA in the food chain remains, should the cells exhibit toxicity.

Toxin production by *Pseudo-nitzschia* spp. has been related to nutrient stress. Laboratory studies (Bates 1998, Fehling et al. 2004a) have indicated that limitation by either phosphate or silicate (but not nitrate) is a necessary condition for toxin production by most *Pseudo-nitzschia* species that produce DA, with most toxin being produced in the nutrient limited stationary phase. Changes in diatom abundance/biomass and nutrient concentrations suggest that the *Pseudo-nitzschia* spp. population is nitrate rather than silicate (or phosphate) limited at our study site, and hence unlikely to produce substantial amounts of DA toxin. However, as suggested in the foregoing subsection, nutrient regeneration may act to alleviate N stress and hence produce the conditions necessary for toxin production.

Toxin production in laboratory cultures of Scottish *Pseudo-nitzschia seriata* has been shown to be related to photoperiod (Fehling et al. 2005). The strong relationship demonstrated by the RDA between photoperiod and *Pseudo-nitzschia* spp. abundance suggests that, through its relationship with cell density alone, this variable may have a strong influence on toxin production at different latitudes.

While more isolates and culture studies of toxin production are required, our results suggest that in spring the *Pseudo-nitzschia* spp. population is mono-specific and composed of a non-toxic form of *P. delicatissima*. However, the mixed composition of the summer blooms containing both *Pseudo-nitzschia* groups (delicatissima and seriata) indicates that an assessment of the potential for toxicity of a *Pseudo-nitzschia* spp. bloom based on simple observation of cell density, as is currently applied for regulatory purposes, will be limited in its effectiveness. An improved understanding of the growth and toxicity of different species in response to environmental conditions is required to predict the potential for toxicity in a particular location.

Implications and conclusions

This study identified several *Pseudo-nitzschia* species as frequently occurring in Scottish waters: *P. delicatissima*, *P. calliantha* and *P. pseudodelicatissima* within the delicatissima-group, and *P. australis*, *P. seriata*, *P. fraudulenta* and (occasionally) *P. multiseries*, *P. pungens* and *P.* cf. *subpacifica* within the seriata-group, plus *P. americana*. It has shown that a combination of morphology and molecular methods is crucial for *Pseudo-nitzschia* spp. identification.

In western Scottish coastal waters both non-toxic and potentially toxic species occur annually, with a timing and abundance that are relatively reproducible between years. Our study indicates that toxic species mainly occur during the summer months. Temporal and multivariate analysis indicated the importance of light, temperature and salinity in determining the bloom dynamics of *Pseudo-nitzschia* spp. An important further challenge is to determine how these factors combine to govern toxicity.

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