

Seasonal occurrence at a Scottish PSP monitoring site of purportedly toxic bacteria originally isolated from the toxic dinoflagellate genus *Alexandrium*

K. TÖBE¹, C. FERGUSON², M. KELLY², S. GALLACHER² AND L. K. MEDLIN¹

¹ Department of Biological Oceanography, Alfred Wegener Institute, Bremerhaven, Germany

² Fisheries Research Services Marine Laboratory, Aberdeen, Scotland, UK

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There is increasing evidence that bacterial–algal interactions play a role in Harmful Algal Bloom (HAB) ecology. Bacteria that are associated with bloom-forming algal species, specifically toxic dinoflagellate algae, have been implicated in the production and biotransformation of paralytic shellfish toxins (PSTs). To clarify the role that these bacteria may play in the production of PSTs, it is desirable to identify and localize the bacteria associated with the dinoflagellates and enumerate them during the course of the algal blooms that the toxic dinoflagellates produce. Because 16S rRNA-targeted probes offer the possibility of both, we previously made and tested probes for some putatively toxigenic bacteria isolated from cultures of the PSP-related dinoflagellates *Alexandrium tamarense*, *A. affine* and *A. lusitanicum*. The bacteria isolated from the dinoflagellates belong primarily to the alpha-proteobacterial group of *Roseobacter* and the gamma-proteobacterial group of *Alteromonas*. Here, we report the successful application of these probes to Lugol's-fixed seawater samples. We detected these bacteria in high numbers in the water column when *Alexandrium* spp. were both present and absent, and during periods when mussels contained PSTs.

Key words: dinoflagellates, paralytic shellfish toxins, PSP, purportedly toxic bacteria, 16S rRNA probes

Introduction

Paralytic shellfish poisoning (PSP) is a life-threatening illness in humans who consume seafood contaminated with paralytic shellfish toxins (PSTs) (Kao, 1993). These are potent neurotoxins composed of the primary toxin, saxitoxin (STX), and at least 20 known derivatives that vary in toxicity (Oshima, 1995). Bivalve molluscs become contaminated with PSTs primarily through filter-feeding some species of PST-producing dinoflagellates (viz., four species of the dinoflagellate genus *Alexandrium*, *Pyrodinium bahamense* var. *compressum* and *Gymnodinium catenatum*; Cembella, 1998). PSTs are also known to be produced by three species of cyanobacteria: *Aphanizomenon flos-aquae* (Mahmood & Carmichael, 1986), *Anabaena circinalis* (Negri *et al.*, 1997) and *Lyngbya wollei* (Onodera *et al.*, 1997), and by the calcareous rhodophyte, *Jania* sp. (Cembella, 1998).

A bacterial origin of PSTs was suggested by Silva (1982) based on the presence of bacteria-like particles within dinoflagellate cells. A PST-producing intracellular bacterium named 'Moraxella' sp. was

subsequently isolated from *Alexandrium tamarense* (Kodama *et al.*, 1988; Kodama, 1990) and bacteria were observed within the dinoflagellate nucleus (Kodama *et al.*, 1990a). This bacterium was later identified as belonging to a new genus of alpha-Proteobacteria rather than to *Moraxella*, a gamma-Proteobacterium (Kopp *et al.*, 1997). However, claims of intracellular bacteria have not been substantiated by other researchers (Doucette *et al.*, 1998 and references therein), although research on *Alexandrium* cultures has shown the presence of bacteria-like structures underneath the theca (*A. lusitanicum*; Franca *et al.*, 1995, 1996) or attached to the theca (*Prorocentrum micans*; Rausch de Traubenberg & Soyer-Gobillard, 1990). Most recently, Lewis *et al.* (2000) and Córdova *et al.* (2001) have demonstrated unequivocally intracellular bacteria in various species of *Alexandrium* using several different detection methods. Since the early reports, heterotrophic bacteria isolated both from cultures of toxic dinoflagellates and from field samples have been reported to synthesize PSTs, with toxins detected by a number of methods both biological and chemical, including Confocal Laser Scanning Microscopy. However, despite a large body of data, definitive spectral evidence for PST

production by bacteria remains lacking and the bacteria are best described as purportedly toxic bacteria (Kodama *et al.*, 1990; Ogata *et al.*, 1990; Doucette & Trick, 1995; Gallacher & Birkbeck, 1995; Levasseur *et al.*, 1996; Gallacher *et al.*, 1997; Gallacher & Smith, 1999 and references therein; Córdova *et al.*, 2001).

It has also been suggested that bacteria may contribute to dinoflagellate toxicity indirectly via mechanisms that are currently unknown, although bacterial adhesion to the dinoflagellate cell wall has been suggested (Gallacher & Smith, 1999). It is well established that the phenotypic properties of bacteria may be dramatically altered upon attaching to a surface (Costerton *et al.*, 1995). In the case of PSTs, Doucette & Powell (1998) demonstrated that bacterial enhancement of dinoflagellate toxicity levels was contingent upon presumed physical contact between the bacterium *Pseudomonas stutzeri* and the dinoflagellate *A. lusitanicum*. How the bacterium might affect dinoflagellate toxicity levels if they occur intracellularly remains conjectural. However, *Alexandrium* species are not known to phagocytose bacteria directly (Legrand & Carlsson, 1998). Therefore, perhaps not surprisingly, claims of intracellular bacteria have not been widely substantiated by other researchers (Doucette *et al.*, 1998 and references therein). However, Córdova *et al.* (2001), using a vitality test, have demonstrated that intracellular bacteria in *A. catenella* are alive and can divide. It is also of interest to note that dinoflagellate bacteria have been shown to bio-transform PSTs (E. A. Smith, personal communication), although the effect of adhesion or intracellularity on this process has not been investigated. Nevertheless, describing the spatial relationship between bacteria and host algae is essential to understand any mechanism by which bacteria might modulate algal toxin production, either extracellularly or intracellularly.

One of the first steps in investigating this relationship is to identify the bacterial population associated with dinoflagellates. Recent studies have shown that the dinoflagellate microflora in laboratory culture tends to be restricted to the alpha-Proteobacteria (primarily *Roseobacter* spp.), gamma-Proteobacteria (mainly *Alteromonas* spp.) and to the cyto-subclasses of the *Cytophaga* among the Eubacteria (Hold *et al.*, in press). It is also important for understanding of bacteria/dinoflagellate interactions and PST production to conduct studies in the environment. However, progress in this area has been slow and limited to a few publications relating to the detection of PSTs in bacterial-sized fractions in seawater (Kodama *et al.*, 1990b; Sakomoto *et al.*, 1992; Levasseur *et al.*, 1996). Visualizing individual bacterial cells through *in situ* hybridization of fluorescently labelled, taxon-

specific ribosomal RNA (rRNA) probes is one way of furthering these investigations. Ribosomal RNA probes are so designed that they can be used to detect strains of certain bacterial species (Amann *et al.*, 1996). Here we describe the use of rRNA probes (Brinkmeyer *et al.*, 2000) for *in situ* identification in field material of bacteria previously isolated from laboratory cultures of *Alexandrium tamarense* (Hold *et al.*, in press), including several putatively toxigenic bacterial strains. The abundances of the bacteria were traced through integrated water column samples taken at designated monitoring sites at the Orkney Islands, Scotland, by applying rRNA probes designed to be specific for the bacterial clade and species to which these dinoflagellate bacteria belong (Brinkmeyer *et al.*, 2000). The abundance of the dinoflagellate bacteria, including purportedly toxic strains, was compared with the total counts for *Alexandrium* spp. and with mussel toxicity.

Materials and methods

Sample sites

Integrated water samples were collected using a 10 m hose at several different sites at the Orkney Islands (see Fig. 2), immediately fixed in acid Lugol's preservative (Thronsen, 1978) and then transferred to brown bottles. Between samplings, the hose was flushed before the next sample was taken. Most samples were taken at Scapa and String, whereas four other sites were randomly sampled throughout the 1999 monitoring season (see Fig. 2, Table 2). The phytoplankton-monitoring programme of the FRS Marine Laboratory in Aberdeen, UK, analysed these samples in 1999. A modification of normal monitoring procedure was implemented in attempts to maximize the number of bacteria recovered in the settled sample. This involved allowing the samples to settle for 1 week, after which 900 ml of supernatant was removed to give the 'supernatant sample'. The bottom 100 ml was allowed to settle for a further 4 h. These were examined by inverted light microscopy and the number of *Alexandrium* spp. cells recorded.

For the purpose of bacterial analysis, fractions from both the settled and supernatant (approximately 40 ml of total seawater) were examined because a preliminary study showed that not all the bacteria present in the water column would settle after 1 week in a settling chamber. We examined 41 samples collected approximately weekly between February and November 1999 and stored in brown bottles at 4 °C until analysed.

Mouse bioassay

The mouse bioassay procedure was performed in accordance with the official mouse bioassay methodology (AOAC, 1990) for the detection of PSP in shellfish by FRS Marine Laboratory as part of the Scottish monitoring programme.

Table 1. Sequences of the oligonucleotide probes used in this study, with protocol details for detection

| Probe | Sequence | Compensating % formamide | NaCl (M) in wash buffer |
|---|----------------------------------|-----------------------------|----------------------------|
| Eubacterial probe 338R (Amann <i>et al.</i> , 1990) | 5'-ACT CCT ACG GGA GGC AGC-3' | 20 | 0.9 |
| A. <i>Roseobacter</i> -clade/536R | 5'-CAA CGC TAA CCC CCT CCG-3' | 18% | 0.27 |
| Competitor for <i>Roseobacter</i> -clade/536R | 5'-CAA CGC TAG CCC CCT CCG-3' | 18% | 0.27 |
| B. 407-20/1446R | 5'-GTC CGC TGC CTC AAA AGT T-3' | 10% | 0.45 |
| C. 667-19/1241R | 5'-TAA CCC ACT GTA GAT GCC-3' | 8% | 0.54 |
| D. 667-12/191R | 5'-GG GCT AAT CCT TCC TTC CCC-3' | 20% | 0.23 |
| G. AMAC/137R | 5' TGT TAT CCC CCT CGC AAA 3' | 10% | 0.45 |
| H. 4 α vs3/210R | 5'-TCT CTT TGC GCC AGA GCT-3' | 10% | 0.45 |
| I. 407-2/209R | 5'-CT TTG CGT GGG AGC CGG-3' | 20% | 0.23 |
| J. 253-19/175R | 5'-CAA GTG CAC ATT ATG CGG-3' | 0 | 0.9 |

Use the letters beside each probe in Fig. 1 to compare the probes listed above with the taxa recognized by each probe. See Brinkmeyer *et al.* (2000) for details of probes shown in Fig. 1 and not listed here.

Fluorescent in situ hybridization (FISH)

A 4 ml aliquot of the settled water sample plus 40 ml of the corresponding supernatant sample were well mixed to make a total seawater sample of 40 ml and filtered at 15 kPa onto a 0.2 μ m pore-size white polycarbonate membrane (Millipore, Eschborn, Germany), diameter 2.5 cm, with a glass microfibre supporting filter, diameter 2.5 cm (Whatman, Maidstone, UK) in a glass vacuum filter holder (Sartorius, Göttingen, Germany). Each sample was filtered in duplicate for the *in situ* hybridizations. One filter was for the hybridization of the *Alteromonas*-clade probe and its taxon-specific probes. The replicate was for the hybridization of the *Roseobacter*-clade probe and its taxon-specific probes. The filters were fixed in 4% paraformaldehyde (PFA) (pH 7.2, freshly made, i.e. not older than 2 weeks, in 1 \times phosphate-buffered saline (PBS)) and filter sterilized (0.2 μ m), then incubated overnight at 4 °C directly in the filter chamber. The samples were rinsed with 10 ml 1 \times PBS and with 10 ml sterile distilled water. Then the filter was dried at room temperature for 5 min. The upper side of the filter was marked with a pencil. Each filter was dehydrated through a graded ethanol series: 50%/80%/100% for 5 min each, then dried at room temperature and cut into four pieces. Each filter quarter was hybridized with two different oligonucleotide probes (Table 1).

The probe combinations on the first filter from each sample were as follows:

Quarter 1: *Alteromonas*-clade probe/137R, labelled with Cyanin 3 (CY3), a red fluorescent dye and the eubacterial probe 338R, labelled with Fluoresceinisothiocyanate (FITC), a green fluorescent dye (Amann *et al.*, 1995).

Quarter 2: 4 α vs3/210R CY 3-labelled and the *Alteromonas*-clade/137R probe, FITC-labelled.

Quarter 3: 407-2/209R CY 3-labelled and the *Alteromonas*-clade/137R probe, FITC-labelled.

Quarter 4: 253-19/175 R CY 3-labelled and the *Alteromonas*-clade/137R probe, FITC-labelled.

The probe combinations on the second filter from each sample were as follows:

Quarter 1: *Roseobacter*-clade/536R probe CY 3-labelled and the eubacterial probe 338R, FITC-labelled.

Quarter 2: 667-12/191R CY 3-labelled and the *Roseobacter*-clade 536R probe, FITC-labelled.

Quarter 3: 667-19/1241R CY 3-labelled and the *Roseobacter*-clade 536R probe, FITC-labelled. 667-19/1241R is no longer taxon-specific as determined by our most recent probe match searches done during the preparation of this paper.

Quarter 4: 407-20/1446R CY 3-labelled and the *Roseobacter*-clade 536R probe FITC-labelled.

The 5'-end labelled probes were obtained from MWG (Ebersberg, Germany).

In this manner, each filter quarter was hybridized with a taxon-specific and a higher taxonomic probe. The hybridizations were performed in a 35 mm Petri dish. Each filter quarter was saturated with 20 μ l hybridization buffer (20 mM Tris-HCl pH 8.0; 0.9 M NaCl; 0.01% SDS, x % formamide)+ 50 ng ml⁻¹ of each oligonucleotide probe + 50 ng ml⁻¹ of competitor probe, if necessary. The formamide concentration varied with each probe and is based on the annealing temperature of the probe to its target (Table 1). Only the *Roseobacter*-clade probe presents a single mismatch with its closest neighbour and must be used with a competitor probe to block the sites of the nearest neighbours to prevent false positives from occurring (Amann *et al.*, 1990 and references therein). The lid of the Petri dish also contained a filter paper, saturated with hybridization buffer to create a moisture chamber for the hybridization. The dishes themselves were put into another box, which contained more wet tissues to ensure enough humidity for the hybridization. The hybridizations were performed at 46 °C for 2–3 h in the dark, to prevent fading of the probes in the light. The hybridization was stopped by adding 5 ml of wash buffer (20 mM Tris-HCl; x M NaCl (where X is the content of NaCl, replacing the formamide in the hybridization buffer, as given in Table 1); 5 mM EDTA, 0.01% SDS). The filter pieces were washed two times for 20 min at 48 °C with gentle shaking. After the washes, the filter pieces were dipped in sterile distilled water and dried at 48 °C for 15 min. The filter quarters from one filter were assembled together onto a slide. Counter-staining was performed using 25 μ l 4',6-diamidino-2-phenylindole (DAPI) solution: 1 ml Citifluor (Citifluor Products, Canterbury, UK)+0.5 ml sterile

distilled water + 1.5 μl DAPI (stock solution 1 $\mu\text{g ml}^{-1}$). The quarters were fixed with a coverslip and sealed with nail polish. The slides were viewed with a Zeiss Microscope equipped for epifluorescence microscopy under oil immersion ($\times 1000$ enlargement) with the appropriate filter set (Zeiss filter sets 02, 09 and 14). The entire filter quarter was scanned under $\times 1000$ enlargement and all bacteria exhibiting a positive signal were counted. Because of the limited material available for analysis, we did not replicate the counts but instead scanned and counted the entire filter rather than counting replicate fields.

Total bacterial counts

To count the total bacterial population in the environment sample a similar proportion of settled and supernatant sample was filtered onto a 0.2 μm pore size black polycarbonate membrane (Millipore, Bedford, MA) as described above. The cells on the filter were fixed in 4% PFA buffered with 1 \times PBS for at least 1 h at 4 °C. The filters were air-dried and incubated in 80% ethanol for 5 min. The ethanol bleached the black filters and the bacteria were counted on these filters. The bacteria were counter-stained with DAPI (as above), viewed with a Zeiss microscope equipped with epifluorescence microscopy under oil immersion ($\times 1000$ enlargement) and counted using the DAPI filter set (Zeiss 02). DAPI counts were used for total bacterial counts instead of using counts obtained with the EUB 338R probe because of the small taxonomic limitations of this probe and because of the potential difficulty in detecting cells with low rRNA content in field material.

Total bacterial counts and counts for *Alteromonas*- and *Roseobacter*-clade bacteria were very high. Therefore, we counted at least 20 microscope fields in three different areas of the filter pieces rather than scanning the entire filter as we did for the species-specific probe counts, which were rarer events. The total bacterial cell numbers per millilitre were calculated with following formula:

$$\text{Bacterial numbers ml}^{-1} = \frac{\text{Bacteria per microscope field (average)} \times (\text{effective filter surface/microscope field surface})}{\text{ml of sample filtered}}$$

Statistical analysis

To determine the correlation between the increase/decrease in the bacteria and the increase/decrease in cells of *Alexandrium* spp. and mussel toxicity, we determined the correlation coefficient according to Pearson product moment correlations using the statistical software MINITAB. We tested both untransformed and transformed (square root transformation) correlations of either the total counts for the *Roseobacter*-clade or *Alteromonas*-clade bacteria with *Alexandrium tamarense* counts or with mussel toxicity. To test the correlation of the taxon-specific bacteria with *Alexandrium* or with mussel toxicity, we determined the correlation of all of

the specific bacteria pooled together and with 4zvs3 alone, because this bacterium was the most abundant of the various bacterial species tested. Significance was tested at the 0.05 level.

Results

Probes and sampling sites

Probes to bacteria obtained from PSP-producing dinoflagellates (Brinkmeyer *et al.*, 2000; Hold *et al.*, in press; Table 1, Fig. 1) were successfully applied to Lugol's-fixed water samples. These samples were collected from six sites in the Orkney Isles during 1999 as part of the Scottish phytoplankton monitoring programme (Tables 2, 3; Fig. 2). The phylogenetic affiliations of the bacteria for which clade-, genus- and taxon-specific probes were available are shown in Fig. 1. The probes were originally tested for specificity with laboratory cultures employing dot blot and *in situ* hybridization formats (Brinkmeyer *et al.*, 2000); however some further method development was required before the probes could be applied to Lugol's-fixed water samples. This involved fixing the filters containing the trapped bacteria from the water samples overnight in 4% PFA in 1 \times PBS prior to hybridization and maintaining a good moisture chamber to prevent the filters from drying. This procedure resulted in stronger hybridization signals.

Additionally, as several weeks could elapse between collection of the water samples and analysis, the stability of the bacterial numbers in the water samples was examined. Repeated counts of total bacteria counter-stained with DAPI from water samples at different intervals in a time period over 10 months showed no significant loss in the number of fixed bacteria (see below).

FISH

The total abundance of bacteria belonging to both *Alteromonas*- and *Roseobacter*-clades was determined by counting the number of cells exhibiting a fluorescent signal from the 16S rRNA probes. *Alteromonas*-clade and *Roseobacter*-clade bacteria and several of the taxon-specific bacteria in these clades were present in the preserved water samples (Figs 3–16).

With water samples from the two main sites (Scapa and String), the total bacterial counts as defined using DAPI staining remained relatively

Fig. 1. Phylogenetic reconstructions using the 16S rRNA gene placing the bacteria isolated from various toxic dinoflagellates near their closest neighbours. (A) *Roseobacter*; (B) *Alteromonas*. The regions/species recognized by the rRNA probes are highlighted in bold on the trees (redrawn from Brinkmeyer *et al.*, 2000). A, the *Roseobacter*-clade from the alpha-Proteobacteria; G, the *Alteromonas*-clade from the gamma-Proteobacteria. See Table 1 for the identification probes associated with the other taxa.

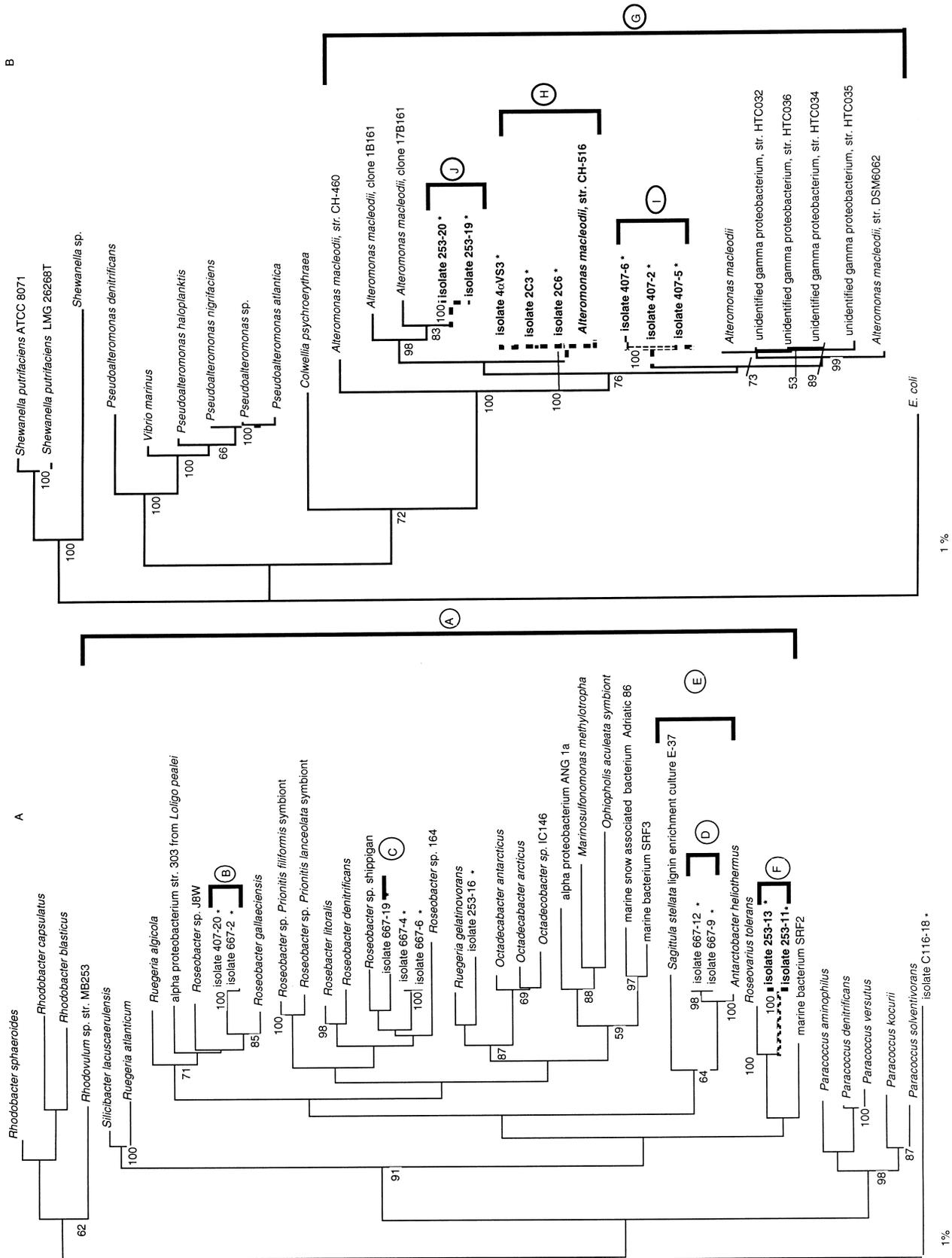


Fig. 1. For legend see facing page.

Table 2. Summary of sites and dates in 1999 for which bacterial or algal counts or mussel toxicity were available for this study

| Date | Day no. | Scapa | Gairsay | String | Cava | Clestrain | Yinstay |
|----------|---------|-------|---------|--------|-------|-----------|---------|
| 24 Feb. | 55 | | X | | | | |
| 8 Mar. | 67 | X A M | | X A | | | |
| 19 Mar. | 78 | | | M | | | |
| 20 Mar. | 79 | M | | | | | |
| 22 Mar. | 81 | X A | | X A | X A | X A | |
| 6 Apr. | 96 | X A M | | X A M | | | |
| 16 Apr. | 106 | X A | | X A | | | |
| 19 Apr. | 109 | M | | M | | | |
| 3 May | 123 | X A | | X A | | | |
| 14 May | 134 | X A | | X A | | | |
| 16 May | 136 | M | | M | | | |
| 27 May | 147 | A | | X A | | | |
| 1 June | 152 | M | | M | | | |
| 2 June | 153 | X A | | X A | | | |
| 8 June | 159 | x M A | | X A M | | | |
| 15 June | 166 | X A M | | X A M | | | |
| 22 June | 173 | X A M | M | X A M | | | |
| 23 June | 174 | | | A | X A M | X A | |
| 29 June | 180 | X A M | | X A M | | | |
| 5 July | 186 | M | | M | | | |
| 7 July | 188 | X A | | X A M | | | |
| 20 July | 201 | A | A | A | | | |
| 27 July | 208 | X A | | X A | | | |
| 30 July | 211 | X A | X | X | | | x A |
| 17 Aug. | 229 | X A | | | | | |
| 24 Aug. | 236 | A | | | | | |
| 7 Sept. | 250 | A | | A | | | |
| 24 Sept. | 267 | A | | A | | | |
| 27 Sept. | 270 | | | A | | | |
| 26 Oct. | 294 | X A | | X A | | | |
| 10 Nov. | 314 | X A | | A | | | x |

Sites marked with an X represent those where bacterial counts were obtained on that date; those marked with an A represent those where *Alexandrium* counts were obtained; those marked with an M are those where mussels were collected for toxicity measurements. Lone *Alexandrium* counts do not appear in Figs 3–8, whereas lone mussel values on 19 and 20 March were plotted on 22 March, that on 1 June on 2 June, that on 5 July on the 7 July as a 5–15% reduction in mussel toxicity per day is reasonable if no reinfection occurs (Bricelj & Shumway, 1998). Other lone mussel values were not plotted.

Table 3. Total counts of bacteria labelled with clade- and species-specific probes at four infrequently sampled sites in 1999

| Site and date | Total counts (cells ml ⁻¹) | Species-specific probes (cells ml ⁻¹) | | |
|------------------------------------|---|---|------------------|--------|
| | | 4αvs3 | 407-2 | 253-19 |
| <i>Alteromonas</i> -clade bacteria | | | | |
| Cava | 22 Mar. | 2200 | 0 | 0 |
| | 23 June | 13300 | 3 | 2 |
| Clestrain | 23 June | 2600 | 1 | 0 |
| Gairsay | 30 July | 5700 | 0 | 0 |
| Yinstay | 30 July | 1200 | 0 | 0 |
| <i>Roseobacter</i> -clade bacteria | | | 667-9 and 667-12 | 667-19 |
| Cava | 22 Mar. | 2300 | 0 | 0 |
| | 22 Mar. | 13900 | 39 | 6 |
| Clestrain | 22 Mar. | 9200 | 0 | 0 |
| Gairsay | 30 July | 11100 | 0 | 0 |
| Yinstay | 30 July | 1600 | 0 | 0 |

stable (average cell count \pm SD over the entire sampling period): String, 2.20×10^5 cells ml⁻¹ \pm 237.10; Scapa, 1.43×10^5 cells ml⁻¹ \pm 231.5. One example of the stability of cell counts at one site over

time is from Scapa, where the average count \pm SD over two separate counts taken several months apart was $6.95 \times 10^4 \pm 500$. In contrast the cell counts for the *Alteromonas*-clade using clade-

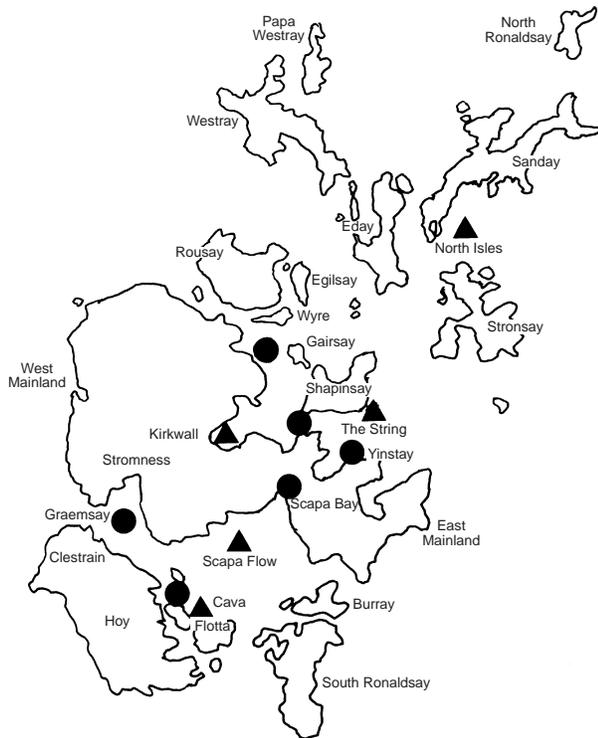


Fig. 2. Detail of the Orkney Islands showing the sites where water samples and mussels were taken and processed as described in the text.

specific probes ranged over the entire sampling period from 0 to 66×10^3 cells ml^{-1} at String and from 0 to 63×10^3 cells ml^{-1} at Scapa, representing 30–44% of the total bacteria at String and Scapa. Similarly, for *Roseobacter*-clade bacteria as determined by clade-specific probes, counts ranged from 0 to 48×10^3 cells ml^{-1} at String and from 0 to 54×10^3 cells ml^{-1} at Scapa or about 22–37% of the total bacterial cells present at the two sites, respectively.

Bacteria belonging to the *Alteromonas*- and *Roseobacter*-clades occurred at low levels at both sites in April (days 91–120), accumulated from May (days 121–151) onwards, peaked in June (days 152–181) and dropped markedly in July (days 182–212), with only low numbers detectable in August (days 213–243) through to November (Figs 3, 4). For total counts of *Alteromonas*-clade bacteria, peak population densities at String occurred about 2 weeks after peak densities at Scapa (Fig. 4). Abundance of individual species basically followed this same pattern, again with maximum bacterial numbers occurring in June with the exception of *Alteromonas* at String where two peaks in *Alteromonas*-clade bacteria occurred at Scapa: one in April and another in July (Figs 5–8), although these were an order of magnitude lower than the maximum counts for these bacteria at String.

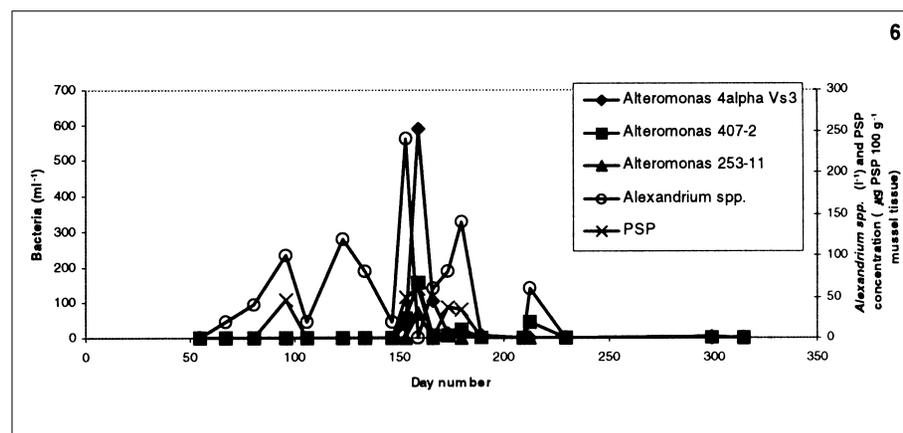
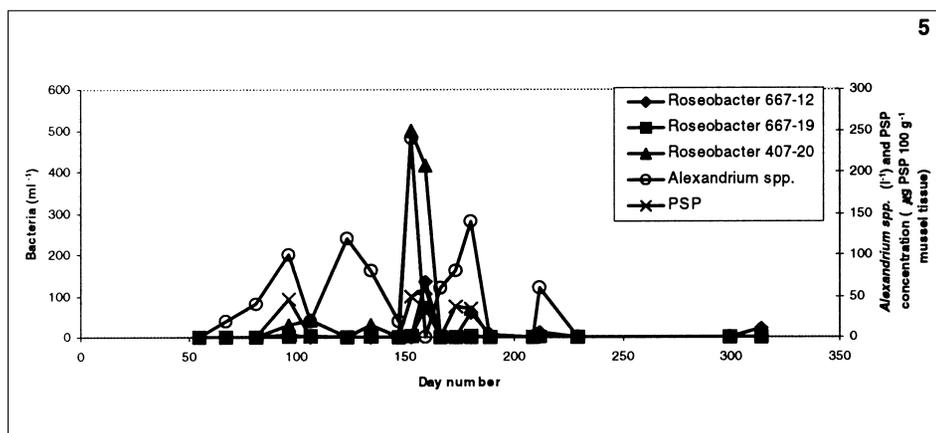
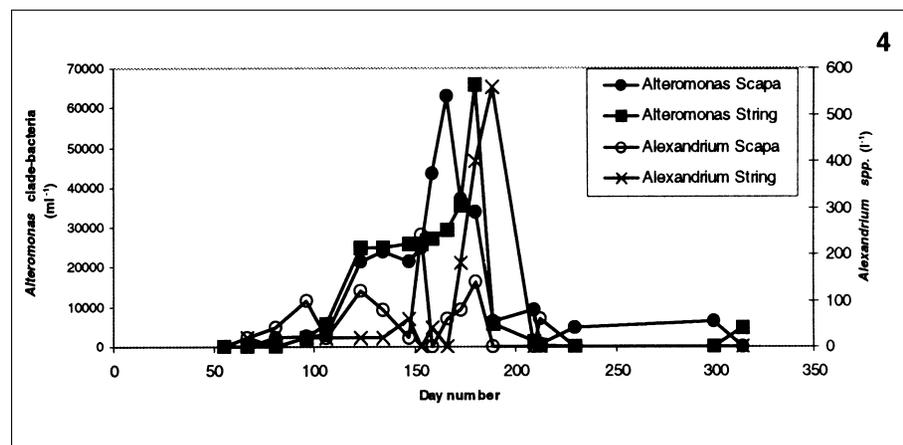
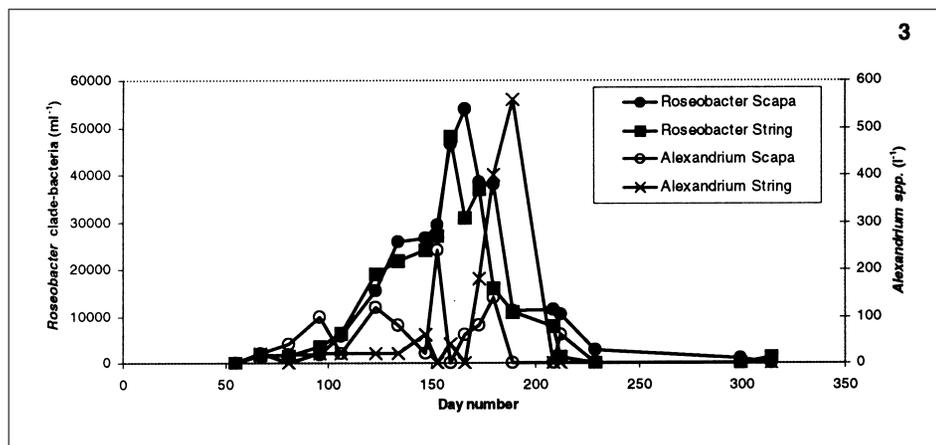
Although the taxon-specific probes identified populations of both clades, the numbers identified

by the species probes were only about 1% of those detected by the clade probes. Sites other than Scapa and String were randomly sampled during the phytoplankton and shellfish monitoring programmes (Table 2). In the few samples tested, *Alteromonas* species-specific bacteria were detected at two of the four sites in low numbers whereas particular *Roseobacter* species were detected at one site, again in low numbers (Table 3).

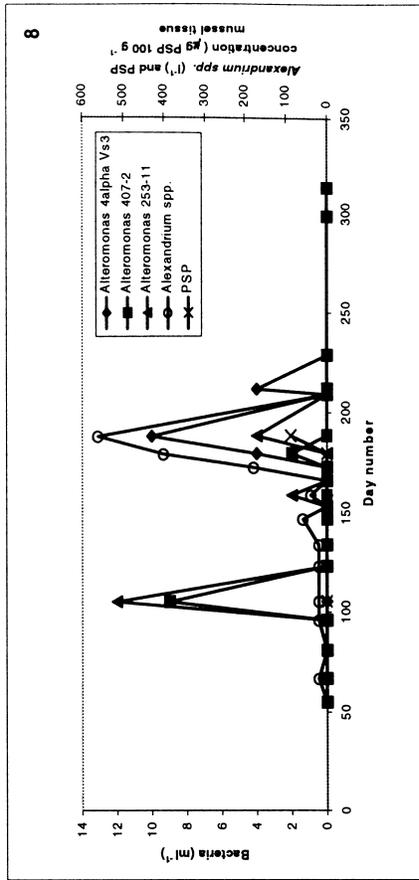
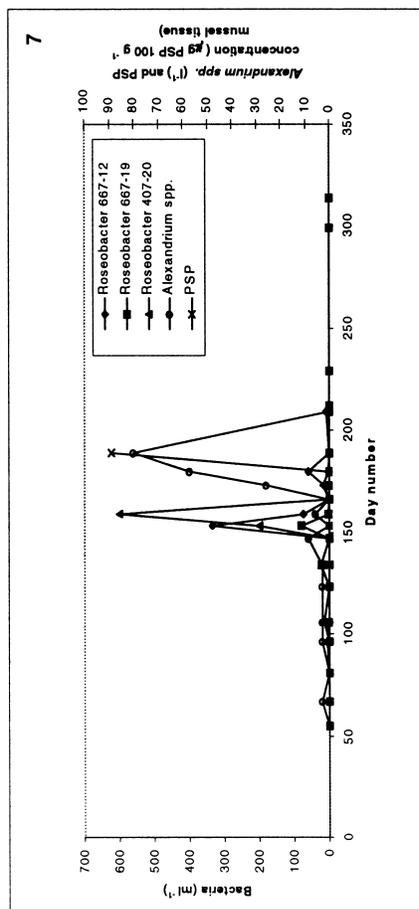
At the same sites at the Orkney Islands in 1999, toxic *Alexandrium* spp. counts were obtained by the Scottish phytoplankton monitoring programme (Figs 5–8). At Scapa, *Alexandrium* exhibited several pulses in abundance during the 1999 sampling season, with highest counts of 240 cells l^{-1} on 2 June 1999 (days 153; Figs 3–6). At String, *Alexandrium* had only one major maximum of 560 cells l^{-1} on 7 July 1999 (day 188; Figs 3, 4, 7, 8; Table 4). At Scapa, the highest *Alexandrium* count occurred as numbers of *Roseobacter*-clade bacteria were increasing (Fig. 3). This resulted in a weak, significant positive correlation between the two data-sets (Table 4). The peak in *Alexandrium* numbers also coincided with a peak in the *Roseobacter* species probe for strain 407-20 (Fig. 5). However, there was no significant correlation between the *Alexandrium* counts and the specific *Roseobacter* species (Table 4, Fig. 3). The numbers of *Alteromonas*-clade counts were positively correlated with increasing *Alexandrium* numbers, which did lead to a weakly significant correlation between the two parameters (Fig. 4, Table 4). For specific *Alteromonas* species, the highest counts occurred just after the *Alexandrium* peaks and were negatively but non-significantly correlated (Fig. 6, Table 4).

At String, *Roseobacter*-clade numbers increased as the *Alexandrium* numbers increased but this correlation was not significant (Fig. 3, Table 4). With the *Alteromonas*-clade bacteria the same pattern emerged as with *Roseobacter*-clade; however, in this instance the two were more highly positively correlated, which led to a weakly significant correlation between the *Alteromonas*-clade and *Alexandrium* numbers (Fig. 4, Table 4).

At String, peaks of *Roseobacter* species identified by the probes preceded the peak in *Alexandrium* numbers by 3 weeks to 1 month (Fig. 7), with a positive but non-significant correlation. Highest *Alteromonas* species counts at String either coincided with the peak in *Alexandrium* counts or preceded it by several weeks (Fig. 8). This led to a significant positive correlation between the *Alteromonas* spp. counts and the *Alexandrium* numbers (Table 4). *Alteromonas* strain 4xvs3 was negatively correlated with *Alexandrium* spp. at Scapa. However, the number of times at String when this strain was detected was too low (only two positive dates) to permit statistical evaluation of the data.



Figs 3–6. Distribution of putatively toxic bacteria in water samples collected at different sites at the Orkney Islands in 1999 as determined by probe hybridization and compared with total *Alexandrium* counts and mussel toxicity. Fig. 3. Clade-specific counts of *Roseobacter*-clade bacteria at both String and Scapa. Fig. 4. Clade-specific counts of *Alteromonas*-clade bacteria at both String and Scapa. Fig. 5. Total counts of bacterial strains 667-2 or 407-20, 667-19 or 667-12 and 667-19, which belong to the *Roseobacter*-clade, at Scapa compared with total counts of *Alexandrium* spp. and with PSP concentrations in mussel at Scapa. Fig. 6. Total counts of bacterial strains 4 α vs3, 407-2 and 253-19, which belong to the *Alteromonas*-clade, at Scapa compared with total counts of *Alexandrium* spp. and with PSP concentrations in mussel tissue at Scapa.



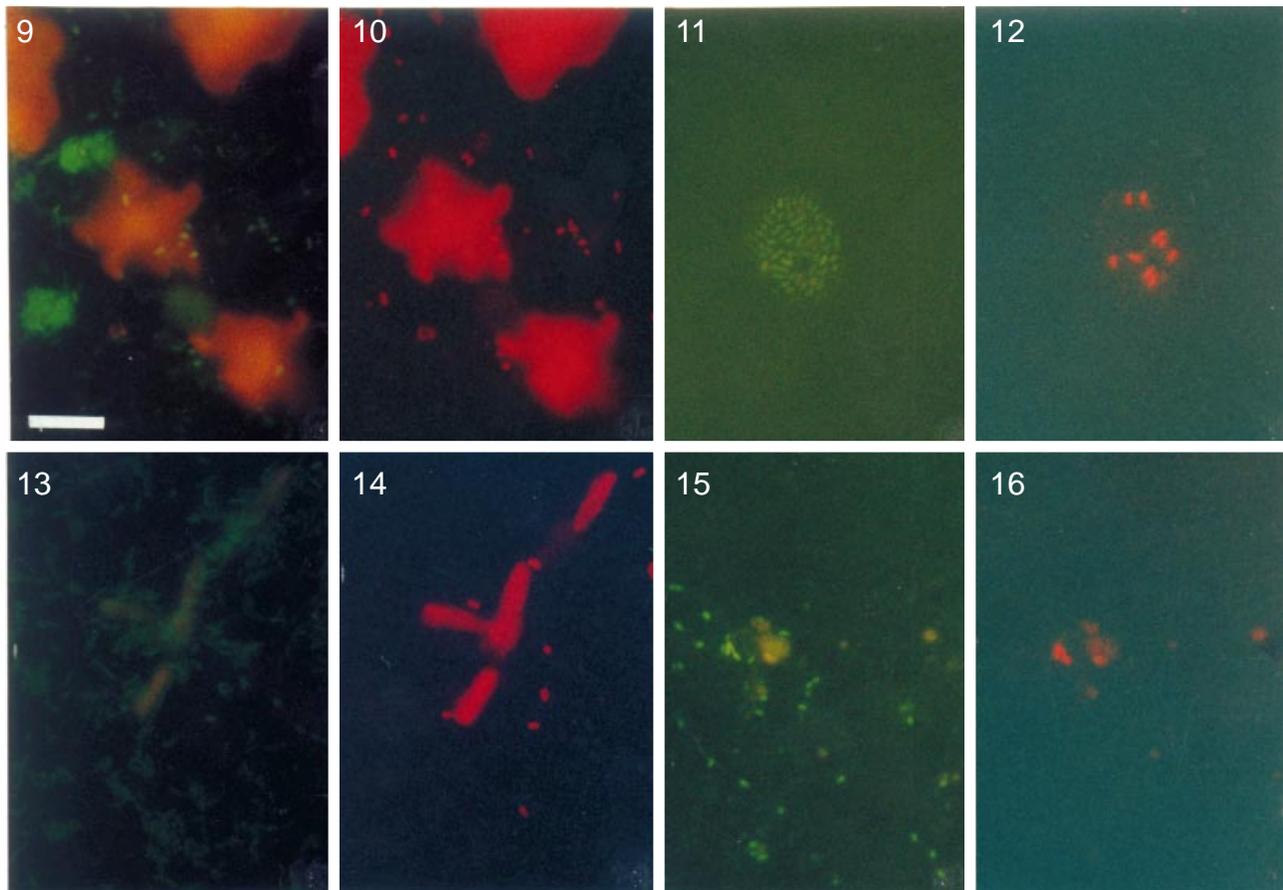
Figs 7–8. Fig. 7. Total counts of bacterial strains 667-2 or 407-20, 667-12 and 667-19 or 667-12 and 667-19 at String and compared with total counts of *Alexandrium* spp. at string and PSP concentrations in mussel tissue at String. Fig. 8. Total counts of bacterial strains 4αvs3, 407-2 and 253-19, which belong to the *Alteromonas*-clade, at String compared with total counts of *Alexandrium* spp. and with PSP concentrations in mussel tissue at String.

Mussels (*Mytilus edulis*) from the two main water sampling sites were analysed for PSTs. At Scapa, PSTs were first detected in mussels on 6 April 1999 (day 153) when the *Alexandrium* numbers first increased (Figs 5, 6). Thereafter mussel toxicity fluctuated alongside that of the dinoflagellate cell numbers, with the highest mussel toxicity being $60 \mu\text{g } 100 \text{ g}^{-1}$ mussel tissue on 8 June 1999 (day 159; Figs 5, 6). One exception to this is that following the high *Alexandrium* counts on 3 May and 14 May 1999 (days 123, 134), the next mussel sampled, on 16 May (day 136), showed no toxicity. On 8 June 1999, the second highest numbers of *Alteromonas*- and *Roseobacter*-clade bacteria were recorded ($4.3 \times 10^4 \text{ cells ml}^{-1}$ and $4.6 \times 10^4 \text{ cells ml}^{-1}$, respectively). Three purportedly toxic bacteria, viz., 407-2, 4αvs3 and 253-19, also reached their highest numbers on 8 June 1999 at Scapa (Fig. 6). However, of the 12 mussel samples analysed for PSTs only six were obtained on the same day as the water samples, of which two were negative. Therefore, at String there were insufficient data to determine whether there was a significant relationship between the PST concentration in mussels and the *Alexandrium* or bacterial counts. At Scapa, bacterial numbers detected by both clade and species probes were positively correlated with mussel toxicity but none of these correlations was significant. The toxic bacterium 4αvs3 was negatively correlated with *Alexandrium* counts but positively correlated with mussel toxicity, both non-significantly.

Discussion

One month later, on 5 July 1999 (plotted two days later on day 188 in Fig. 8), the highest amount of PSP, $89 \mu\text{g } 100 \text{ g}^{-1}$ mussel tissue, was detected at String, with peak dinoflagellate numbers and *Alteromonas* species-specific numbers occurring 2 days later (Fig. 8). However, there were insufficiently matched data or positive shellfish samples to determine an association either with *Alexandrium* or bacterial counts at String. On 7 July 1999 (day 188), the *Alteromonas*-clade bacterial counts were $5.6 \times 10^3 \text{ ml}^{-1}$ and the *Roseobacter* bacteria were $1.1 \times 10^4 \text{ ml}^{-1}$ but the purportedly toxic bacteria were low. Where sufficient data were available to allow a correlation to be calculated, there were no significant correlations between mussel toxicity and the occurrence of bacteria.

Hold *et al.* (in press) have shown that bacteria associated with laboratory cultures of PST-producing dinoflagellates belong to the alpha and gamma *Proteobacteria* and the *Cytophaga*. From the *Proteobacteria*, bacteria of the *Roseobacter*- and *Alteromonas*-clades dominated. This study demonstrates that rRNA probes designed for these bac-



Figs 9–16. Whole cell hybridization of bacteria taken from water samples at the Orkney Islands, summer 1999, and filtered onto 0.2 μm white polycarbonate filter. Scale bar represents 10 μm and can be applied to all figures. All hybridization steps were performed at 46 °C. Fig. 9. Water samples from Cava hybridized with the *Alteromonas*-clade/137R probe labelled with FITC. Fig. 10. Same cells hybridized with the probe 407-2/209R labelled with CY3. Fig. 11. Water samples from String hybridized with the universal eubacterial probe EUB 338R labelled with FITC. Fig. 12. Same cells hybridized with the *Alteromonas*-clade probe 137R labelled with CY3. Fig. 13. Water samples from String hybridized with the *Roseobacter*-clade probe 536R labelled with FITC. Fig. 14. Same cells hybridized with the probe 667-12/994R labelled with CY3. Fig. 15. Water samples at Scapa hybridized with the universal eubacterial probe 338R labelled with FITC. Fig. 16. Same cells hybridized with *Roseobacter* clade probe 536R labelled with CY3.

Table 4. Summary of Pearson correlations (R) between bacterial counts (square root-transformed), numbers of *Alexandrium* and mussel toxicity

| Correlations tested | String | | | Scapa | | |
|---|--------|-------------|---------------------|--------|-------------|---------------------|
| | R | Probability | No. of observations | R | Probability | No. of observations |
| Total <i>Roseobacter</i> vs <i>Alexandrium</i> | 0.354 | 0.15 | 18 | 0.479 | 0.783 | 19 |
| Total <i>Alteromonas</i> vs <i>Alexandrium</i> | 0.477 | 0.045* | 18 | 0.458 | 0.049* | 19 |
| Pooled <i>Roseobacter</i> taxon-specific counts vs <i>Alexandrium</i> | 0.017 | 0.949 | 17 | 0.209 | 0.391 | 19 |
| Pooled <i>Alteromonas</i> taxon-specific counts vs <i>Alexandrium</i> | 0.585 | 0.017* | 16 | -0.033 | 0.893 | 19 |
| Total <i>Roseobacter</i> vs mussel toxicity | n.a. | n.a. | 5 | 0.155 | 0.770 | 6 |
| Total <i>Alteromonas</i> vs mussel toxicity | n.a. | n.a. | 5 | 0.174 | 0.742 | 6 |
| Pooled <i>Roseobacter</i> taxon-specific counts vs mussel toxicity | n.a. | n.a. | 5 | 0.641 | 0.170 | 6 |
| Pooled <i>Alteromonas</i> taxon-specific counts vs mussel toxicity | n.a. | n.a. | 5 | 0.358 | 0.486 | 6 |
| 4zvs3 vs <i>Alexandrium</i> | n.a. | n.a. | 5 | -0.068 | 0.783 | 19 |
| 4zvs3 vs mussel toxicity | n.a. | n.a. | 5 | 0.291 | 0.576 | 6 |

n.a., not applicable, too few data.

* $p < 0.05$.

teria (Brinkmeyer *et al.*, 2000) were successfully used in their detection from Lugol's-fixed water samples obtained from the Orkney Isles, particularly String and Scapa, during the Scottish phytoplankton monitoring programme over the period April to November 1999. The successful application of FISH technology to samples containing Lugol's preservative has not been reported previously and is potentially useful for analysing fresh or archived field samples of both bacteria and algae. We have successfully detected bacteria using FISH in samples from as far back as 1997 (data not shown), but the signal is considerably diminished compared with that obtained in fresh samples in 1999 (Figs 9–13). Therefore, caution must be applied when using the technique described in this paper on archived samples, especially if they have been settled, as not all bacterial cells will settle after 1 week. A possible alternative approach is the use of dot blot hybridizations, as these gave stronger signals than FISH in older samples (data not shown). It may also be possible to improve the FISH signal with a Tyramide signal amplification method (Schönhuber *et al.*, 1997).

At least three *Alexandrium* species have been reported from waters of the Orkney Isles (*A. tamarense*, *A. ostenfeldii* and *A. minutum*; M. Elbrächter, personal communication) and, although they do co-occur, only one species of the three will dominate at any one time (G. Gerdt, personal communication). The source of these dinoflagellates is unknown; Hummert *et al.* (2001) suggest that *Alexandrium* spp. may be carried into Orkney waters from the open ocean by currents, as determined by drift buoy experiments conducted at single time points in 1999 and 2000 (G. Gerdt, personal communication). Certainly counts 100 times greater than those reported here have been found both north-west and south-east of the Orkney Islands. Conversely, *Alexandrium* cysts have been detected in the area, thereby providing potential seed beds for the vegetative dinoflagellate cells (E. Macdonald, personal communication). Nevertheless, *Alexandrium* spp. and shellfish contaminated with PSTs (G. Howard, personal communication) occur yearly in this area, and are why this site was chosen for this study.

Our work utilized data and samples from a monitoring programme that operates, and is modified throughout the season, to meet regulatory authority requirements. We also used clade-specific probes to the genera *Roseobacter* and *Alteromonas* and species-specific probes for each clade.

The *Roseobacter* species-specific probes were designed to bacteria closely related to *Antarctobacter heliothermus* (667-12), and to a further two bacteria more closely related to *Roseobacter* sp., *Roseobacter* sp. Shippigan strain (667-19) and *Roseobacter*

gallaeciensis (407-20) (Brinkmeyer *et al.*, 2000; Fig. 1). The species-specific probes for the *Alteromonas*-clade consisted of probes to three different unclassified *Alteromonas* species (Fig. 1). All these bacteria were previously isolated from *Alexandrium* cultures taken from different parts of the world (Hold *et al.*, in press).

As bacteria cross-reacting with the probes were easily detected, this would suggest that they were actively growing members of the bacterioplankton. However, a word of caution is warranted. As the vast majority of marine bacterial diversity remains undescribed it is feasible that the probes may have targeted as yet unknown, perhaps closely related bacteria with the same target sequence. Nevertheless, given that the probes were designed to the highest possible specificity given the information currently available in GenBank databases the observations detailed below are considered valid.

Bacteria reacting to probes for the *Roseobacter*- and *Alteromonas*-clades were common, consisting of up to 46% of the total bacterial population. However, the number of bacteria cross-reacting to the species-specific probes were approximately 1% of the number detected by the clade probes. This indicates there is a large percentage of the bacterial community belonging to these two clades that could not be accounted for using the taxon-specific probes.

There was a weak significant positive relationship between *Alteromonas*- and *Roseobacter*-clade counts and those of *Alexandrium* spp. at Scapa Flow. At String there was also a weak correlation between *Alexandrium* and the *Alteromonas* clade counts but not the *Roseobacter*-clade. Interestingly, at String, there was also a significant relationship between the pooled *Alteromonas* species-specific counts (which are purportedly toxic strains), although the bacterial numbers were relatively low. Data were not available on the rest of the phytoplankton community and therefore we were unable to determine whether any relationship existed between the bacteria and other phytoplankton species.

Other researchers have shown that *Roseobacter* and *Alteromonas* bacterial clades are relatively common in the marine environment (Glazebrook *et al.*, 1996; Gonzalez & Moran, 1997; Acinas *et al.*, 1998) although few have investigated their occurrence over time and in relation to dinoflagellate species. Kerkhof *et al.* (1999) have shown that bacteria associated with algal bloom populations are not the same as those found under non-bloom conditions and they inferred from their study that certain groups of bacteria and phytoplankton were tightly coupled in time and space. Chilean isolates of *A. catenella* were infected by a variety of gram-negative bacteria including species from the genera *Pseudomonas*, *Aeromonas*, *Flavobacterium*, *Pasteur-*

ella, *Proteum* and *Moraxella*-like, some of which were shown to produce PSTs (Córdova *et al.*, 2001). Other studies have documented the co-occurrence of similar bacterial genera, such as *Aeromonas*, *Pseudomonas* and *Vibrio*, from within a bloom of dinoflagellates producing red tides (Buck & Pierce, 1989; Romalde *et al.*, 1990). Some bacterial isolates from the bloom and from algal cultures established from the bloom exhibited cytotoxicity (Buck & Pierce, 1989; Romalde *et al.*, 1990). Evans (1973, cited in Buck & Pierce, 1989) postulated a red tide cycle involving (1) initial bacterial growth stimulated by organic and inorganic nutrients, the bacterial populations then providing vitamins to stimulate red tide blooms, (2) other bacteria growth stimulated by dying, decaying target organisms in the red tide bloom, (3) toxins from bacteria working synergistically with the algal toxins to kill fish or toxify shellfish and (4) the cycle perpetuating itself.

Recent investigations of bacterial interactions with HAB species have begun to reveal the complexity of these associations. These describe how bacteria influence algal toxin concentrations and are involved in the decline of algal blooms (see reviews in Doucette, 1995; Plumley, 1997; Doucette *et al.*, 1998; Gallacher & Smith, 1999). However, little information is available with regard to the identity of bacterial populations during the occurrence of toxic *Alexandrium* species in the environment. Babinchak *et al.* (1998) compared bacterial composition at the class level between toxic and non-toxic strains of *A. tamarense*, between toxic species of *Alexandrium* and between toxic strains of the same species taken from different geographic locations. They found different bacterial associations within each level of comparison. Our study infers an association between bacteria of the *Roseobacter*- and *Alteromonas*-clades and some specific *Alteromonas* species to numbers of *Alexandrium* cells. It is noteworthy that there is a co-occurrence in time between *A. tamarense* and bacteria believed to live in some kind of relationship with these algae, but these bacteria may be specific to the hosts from which they were originally isolated, which are from other parts of the world. Córdova *et al.* (2001) also inferred from their western blot data that bacterial infection of *A. catenella* was clone-specific, and their work also suggested that some dinoflagellate clones were more susceptible to bacterial infection than others. Further investigations are required to determine whether these specific bacteria are involved in the increase and decline of *Alexandrium* and its production of PSTs. The latter is particularly pertinent given that bacteria isolated from dinoflagellates can biotransform PSTs (Smith *et al.*, 2001). We therefore plan to conduct further studies over an additional monitoring period using the probes to

investigate this association in more detail. Laboratory investigations are planned to examine the spatial relationship between the bacteria and algae using these probes and confocal microscopy.

Questions also arise with regard to the association of bacteria with shellfish toxicity. At the outset of this study plans also included examining the relationship between the *Alexandrium* numbers, bacterial counts and the concentration of PST in shellfish. However, the adaptive nature of the monitoring programme coupled with the fact that the occurrences of PSTs were lower in 1999 than in previous years (G. Howard, personal communication) meant that there were too few samples taken on the same dates as water samples to allow any inference to be reached. All that can be stated is that the specific bacteria were present during periods when the mussels contained PSTs and the changes in their numbers were positively correlated. Again further work is planned on this aspect at a later date, but it is obvious that a well integrated sampling program must be carried out if any meaningful correlations are to be uncovered. This is an important aspect given that bacteria isolated from shellfish have also been shown to biotransform PSTs (Smith *et al.*, 2001) and hence potentially influence shellfish toxicity. In future environmental studies it would also be of interest to examine shellfish tissue for bacteria using the probes. We have developed *in situ* hybridization using enzymatic, colorimetric detection as well as conventional FISH detection for detecting *Alteromonas* spp. and *Roseobacter* spp. in paraffin-embedded mussel hepatopancreas (K. Töbe *et al.*, unpublished), and this technique will be used in further environmental studies.

In conclusion the data presented in this paper are the most comprehensive to date with regard to examining which bacteria, including purportedly toxic bacteria, are present in the water column during periods of shellfish toxicity and when *Alexandrium* spp. are present. This work is the first step in studying bacterial/dinoflagellate interactions in terms of PSTs in the environment. However, conclusive evidence on the influence of bacteria in relation to the occurrence of PSTs in dinoflagellates and shellfish awaits information on what genes are involved in the production of PSTs and the environmental parameters that trigger them.

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