Investigations into the role of bacteria/dinoflagellate interactions in Paralytic Shellfish Poisoning

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1. General Introduction

1.1 Harmful Algal Blooms

Microscopic algae of the oceans are the critical food for marine species, such as filter feeding bivalve shellfish, the larvae of commercially important crustaceans and finfish. In most cases the proliferation of plankton algae up to millions of cells per litre, the so-called 'algal blooms', therefore have a positive effect for aquaculture and wild fisheries operations. Such algal blooms can also have serious negative consequences, such as major environmental and human health impacts, and can cause huge economic losses to aquaculture, fisheries and tourism (Hallegraeff 1995; Li et al. 2001). More than ten thousand known species of planktonic algae are found in the upper layers of the oceans and continental waters. Excluding those species that respond to eutrophication and associated processes and that can give rise to biomass problems, less than a hundred of these algae have so far been recorded as harmful. The severity of their harm can range from nuisance algae, when foam accumulates on tourist beaches, to economically damaging algae, when for example, fish farms lose stock or extraction of resources is prevented, and to possible death, when drinking water or seafood become contaminated with potent toxins (Wyatt 1998).

It is believed that the first written reference to a Harmful Algal Bloom (HAB) appears in the bible, 1000 years B.C.: ''…all the waters that were in the river were turned to blood. And the fish that was in the river died; and the river stank, and the Egyptians could not drink of the water of the river'' (Exodus 7:20-21). This colouration was presumably caused by a non-toxic bloom-forming alga, which became so densely concentrated that it generated anoxic conditions resulting in indiscriminate kills of both fish and invertebrates (Hallegraeff 1995).

In a strict sense, HABs are completely natural phenomena, which have occurred throughout recorded history. However, in recent decades the public health and economic impacts of such events appear to have increased in frequency (Hallegraeff 1993). Until 1970, HABs of Paralytic Shellfish Poisoning (PSP) producing dinoflagellate blooms were only known from temperate waters of Europe, North America and Japan (Dale and Yentsch 1978). By 1990, toxin-producing dinoflagellate blooms were also documented from throughout the Southern Hemisphere in South Africa, Australia, India, Thailand, Brunei, Sabah, the Philippines and Papua New Guinea (Hallegraeff 1993). Possible reasons for this apparent increase of HABs could be an increased scientific awareness of toxic species, increased utilization of coastal waters for aquaculture, stimulation of plankton blooms by cultural

eutrophication and/or unusual climatological conditions and transport of dinoflagellate resting cysts either in ship ballast water or associated with movement of shellfish stocks from one area to another (Hallegraeff 1993 and 1995).

HABs are generated when environmental conditions, such as changes in salinity, water temperature, and increased nutrients and sunlight trigger cyst germination to a vegetative stage that enables rapid reproduction of microalgae (Anderson 1990). Once the dinoflagellate bloom begins, an exponential growth phase causes a tremendous increase in their population. Depletion of nutrients and carbon dioxide in the water and degraded environmental conditions caused by the bloom decrease population growth. A stationary phase ensures levelling off the population. Then the water may become a fluorescent reddish colour, termed a red tide (Fig. 1). Continued environmental degradation increases cell death and leads ultimately to a population crash. At this phase of the bloom, many dinoflagellate species, undergo sexual reproduction and form resting cysts that settle to the bottom, ready for the next bloom (Anderson 1990). It has been suggested that dinoflagellate red tides may represent a set of conditions optimising the process of sexual reproduction (Seliger 1993), which is required for genetic recombination and to provide cysts for inoculation of subsequent blooms (Doucette 1995).

Cysts can be toxic too, as reported for the dinoflagellates *Alexandrium tamarense* and *Gymnodinium catenatum* and can be filtered from the sediments by benthic marine organisms. However, cysts have very robust cell walls and the efficiency of the transfer of the toxins from cysts to benthic organisms is still not known. Therefore, it is possible that some benthic organisms will simply pass cysts through their digestive systems and expel them in their faeces (Granéli et al. 1999). Within this bloom cycle, the most toxic cells occur generally during the middle of the exponential growth phase, whereas older cells tend to undergo more toxin transformation (Anderson 1990).

Different Types of Harmful Algal Blooms

According to Hallegraeff (1995) the following types of HABs can be differentiated:

1. Species that produce basically harmless water discolourations, which under exceptional conditions in sheltered bays can grow so dense that they cause indiscriminate kills of fish and invertebrates due to oxygen depletion. Such species are, for example, the dinoflagellates *Gonyaulax polygramma*, *Noctiluca scintillans*, *Scrippsiella trochoidea* and the cyanobacterium *Trichodesmium erythraeum*.

2. Species that produce potent toxins that can find their way through the food chain to humans, causing a variety of gastrointestinal and neurological illnesses:

-Diarrhetic shellfish poisoning (DSP) is caused by dinoflagellates. Examples: *Dinophysis acuta*, *D. acuminata*, *D. fortii*, *D. norvegica*, *D. mitra*, *D. rotundata* and *Prorocentrum lima*.

-Amnesic shellfish poisoning (ASP) is caused by diatoms. Examples: *Pseudo-nitzschia multiseries*, *P. pseudodelicatissima* and *P. australis*.

-Ciguatera Fish Poisoning is caused by dinoflagellates. Examples: *Gambierdiscus toxicus* and probably also *Ostreopsis* spp. and *Prorocentrum* spp.

-Neurotoxic Shellfish Poisoning (NSP) is caused by dinoflagellates. Examples: *Gymnodinium breve* and *G.* cf. *breve* (New Zealand).

-Cyanobacterial Toxin Poisoning is caused by cyanobacteria. Examples: *Anabaena circinalis*, *Microcastis aeruginosa* and *Nodularia spumigena*.

-Paralytic Shellfish Poisoning (PSP) is caused, mainly by dinoflagellates. Examples: dinoflagellates *Alexandrium acatenella, A. catenella*, *A. cohorticula*, *A. fundyense*, *A. fraterculus*, *A. minutum*, *A. tamarense*, *Gymnodinium catenatum*, and *Pyrodinium bahamense* var. *compressum*.

3. Species that are non-toxic to humans, but harmful to fish and invertebrates by damaging or clogging their gills or by producing haemolytic toxins. Examples: diatom *Chaetocerus convolutus*, dinoflagellate *Gymnodinium mikimotoi*, prymnesiophytes *Chrysochromulina polylepsis*, *Prymnesium parvum*, and *P. patelliferum*, raphidophytes *Heterosigma carterae*, and *Chattonella antiqua* (Hallegraeff 1995).

Fig.1: California red tide generated by a *Noctiluca* bloom (picture made by Peter J.S. Franks)

1.2 Paralytic Shellfish Poisoning and Paralytic Shellfish Toxins

One of the first recorded fatal cases of human poisoning after eating shellfish contaminated with dinoflagellate toxins goes back to the year 1793, when Captain George Vancouver and his crew landed in British Columbia, now known as Poison Cove. He wrote that for local Indian tribes it was forbidden to eat shellfish when the seawater became phosphorescent (Dale and Yentsch 1978). This water colouration was induced by a toxic dinoflagellate bloom. Today the illness is known as Paralytic Shellfish Poisoning (PSP) and the causative alkaloid toxins are now called Paralytic Shellfish Toxins (PSTs). On a global scale, almost 2000 cases of human poisoning with a mortality of 15% through the consumption of fish or shellfish are reported each year. Also whales and porpoises can be poisoned, when they receive toxins through the food chain via contaminated zooplankton or fish (Geraci et al. 1989; Durbin et al. 2002; Cembella et al. 2002).

So far, PSTs have been detected in laboratory cultures of the dinoflagellates *Alexandrium* spp., *Pyrodinium bahamense* var. *compressum* and *Gymnodinium catenatum* (Cembella 1998) and also in freshwater cyanobacteria, *Aphanizomenon flos-aquae* (Jakim and Gentile 1968; Mahmood and Carmichael 1986), *Anabena circinalis* (Humpage et al. 1994; Negri et al.

1997) and *Lyngbya wollei* (Carmichael et al. 1990; Carmichael and Falconer 1993; Carmichael 1997; Onodera et al. 1997).

Toxic dinoflagellate produce higher amounts of PSTs when nitrogen is abundant. Where phosphorus is deficient, individual algal cells become more toxic, probably because the cells continue PST production but reduced cell division prevents distribution of toxins to newly produced cells. These non-dividing cells continue to accumulate toxin (Anderson et al. 1990), and therefore the cell quota for the toxin is higher. PST occurrences tend to be seasonal, occurring most often during late spring and summer. Off-season episodes of PSTs are most likely caused by retention of toxins from the summer (Anderson 1990).

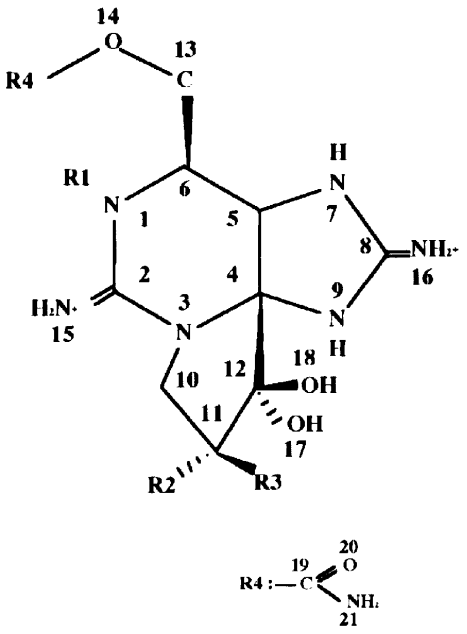
PSTs are termed collectively saxitoxins (Fig. 2), deriving the name from the butter clam, *Saxidomus giganteus*, where saxitoxins were originally extracted and identified (Kao 1993). Approximately two dozen naturally-occurring derivates of saxitoxins exist (Shimizu 1996). All the saxitoxins are neurotoxins that act to block movement of sodium through nerve cell membranes, stopping the flow of nerve impulses causing the symptoms of PSP, which includes numbness, paralysis, and disorientation (Mosher et al. 1964). PSTs have been classified in three groups: 1. The highly potent carbamates, such as STX, neo-saxitoxin, and gonyautoxins (GTX1-4); 2. The weakly toxic N-sulfocarbamoyl toxins (B1, B2, C1-4); and 3 the decarbamoyl analogues of intermediate toxicity (Suárez-Isla and Vélez 2000). PSP toxins have a common tricyclic skeleton differing in toxicity that results from N-1 (=R1) hydroxylation, C-11 O-sulfation (=R2, R3), C-13 carbamolyation (=R4), and N-21 sulfation (Oshima et al. 1989; Shimizu 1993; van Dolah 2000).

The toxicity of PSP toxins is estimated to be 1000 times greater than cyanide and symptoms appear soon after consuming toxic shellfish (Kao 1993). These toxins are so potent that the small amount of about 500 µg, which can be easily accumulated in just one 100 gram serving of shellfish, can be fatal to humans. There is no antidote for PSP, and all cases require immediate medical attention that may include application of life support equipment to save a victims life. If the dosage is low and proper medical treatment is administered, symptoms should diminish in approximately nine hours (Kao 1993).

Saxitoxin molecules undergo chemical transformations that change one molecular form to another. Transformations are performed by the dinoflagellate cell and by many animals that acquire saxitoxins. One common transformation, named epimerisation, occurs when a part of the original saxitoxin molecule rearranges (Oshima 1995). Scallops and mussels, for example, can perform the epimerisation of saxitoxin, when hydrogene and hydroxsulfate switch locations on the number 11 position of the saxitoxin molecule (Oshima et al. 1990). This

transformation can decrease the toxicity of the original saxitoxin by 11 times. Numerous other types of transformations occur as well as eventual detoxification that can render the shellfish safe for consumption (RaLonde 1996).

Only few studies have been published that have investigated the genes involved in PST production and it is not yet clear whether chromosomal, chloroplast or mitochondrial DNA from dinoflagellates, or nucleic acids from bacteria, plasmids or viruses are involved (Ishida et al. 1998; Sako et al. 1995). Sako et al. (1995) demonstrated that F1 progeny of mating dinoflagellates, with different toxin profiles, inherited their toxin profile in a Mendelian manner. The authors suggested that genes for toxin synthesis were coded by chromosomal genes in these algae and are a hereditary characteristic (Sako et al. 1995). Mendelian inheritance did not occur in all cases and recombination between genes was proposed (Ishida et al. 1998). An alternative explanation could be the involvement of genes other than dinoflagellate chromosomal DNA (Cembella 1998). Taroncher-Oldenburg and Anderson (1997 and 2000) suggested that up or down regulated genes during a narrow time frame in early G1-phase are related to toxin biosynthesis in dinoflagellates and found that STX is accumulated in *A. fundyense* in this time period of the cell cycle. Because of these findings, the authors assumed that STX biosynthesis could be regulated at the transcriptional level, and the genes responsible for toxin production are activated in a cyclic pattern (Taroncher-Oldenburg and Anderson 2000).



	R1	R2	R3	R4
STX	H	H	H	$CONH_2$
neoSTX	OH	H	H	$CONH_2$
GTX1	OH	H	OSO_3^-	$CONH_2$
GTX2	H	H	OSO_3^-	$CONH_2$
GTX3	H	OSO_3^-	H	$CONH_2$
GTX4	OH	OSO_3^-	H	$CONH_2$
GTX5	H	H	H	$CONHSO_3^-$
GTX6	OH	H	H	$CONHSO_3^-$
epiGTX8 (C1)	H	H	OSO_3^-	$CONHSO_3^-$
GTX8 (C2)	H	OSO_3^-	H	$CONHSO_3^-$
C3	OH	H	OSO_3^-	$CONHSO_3^-$
C4	OH	OSO_3^-	H	$CONHSO_3^-$
dcSTX	H	H	H	H
dcneoSTX	OH	H	H	H
dcGTX1	OH	H	OSO_3^-	H
dcGTX2	H	H	OSO_3^-	H
dcGTX3	H	OSO_3	H	H
dcGTX4	OH	OSO_3^-	H	H
11-α-hydroxySTX	H	H	OH	$CONH_2$
11-β-hydroxySTX	H	OH	H	$CONH_2$

Fig. 2: Chemical structure of Paralytic Shellfish Toxins. STX, saxitoxin, GTX, gonyautoxin, neo-STX, neo-saxitoxin, dc, decarbamoyl (Sako et al. 2000)

1.3 Measurement of Paralytic Shellfish Toxins

The detection of saxitoxins causes problems because of the large differences concerning the specific toxicity of STX derivates, the small amount of STX in natural samples and the tendency for the toxins to undergo biotransformation in the shellfish digestive glands and during sample extraction and preparation. The analytical methods provide an instrumental response proportional to the concentration of each derivative in a complex sample. Pure standards of known concentrations enable this proportionality, whereas assays provide a single response proportional to the toxicity of a toxic mixture. The assay response could be, e.g., a colorimetric or fluorescence change or a loss of a physiological response (Suárez-Isla and Vélez 2000).

1.3.1 Mouse Bioassay

The preferred method for analysing shellfish for PSP is the mouse bioassay (MBA), which is used in shellfish monitoring programs world-wide (AOAC 1990). This test measures simultaneously the total of all the saxitoxin toxicities from a sample of shellfish tissue. The saxitoxin level is measured by timing the death of an 18-20 gram mouse following injection of fluid extracted from shellfish tissue (AOAC 1990). Toxicity is expressed as microgram

STX equivalents (equiv.) /100 g shellfish tissue standardised against a reference STX solution. Harvesting or consumption is not permitted in many countries when toxicity reaches or exceeds 80 µg STX equiv. /100 g shellfish tissue (Suárez-Isla and Vélez 2000). The mouse bioassay has many disadvantages, such as the lack of specifity and precision. This test does not specify the toxins, it only reveals the total PSP toxicity of a sample (Luckas 1992). Additionally, the test is very expensive and time consuming, especially when large numbers of samples have to be analysed. The main analytical limitations are the high variability of ± 20% and the low sensitivity very near to the detection limit of about 35 µg STX equiv. /100 g (Suárez-Isla and Vélez 2000). Increasingly, the use of this technique is becoming unacceptable for ethical reasons in a number of countries. Therefore, alternative techniques were evaluated for use in PSP monitoring programs (Gallacher et al. 1998).

1.3.2 Alternatives to the Mouse Bioassay

In the mouse neuroblastoma (MNB) assay a mouse neuroblastoma cell line is used, which originally arose from a spontaneous tumor in mice. This cell line allows detection of sodium channel blocking toxins (SCBs), such as PSP toxins (Gallacher et al. 1993).

The expose of MNB cells to two chemicals, oubain and veratridine, results in an influx of sodium ions and subsequently to cell death. In the presence of toxins that exhibit sodium channel blocking activity, such as saxitoxin, the cells survived. By incorporating the use of chemicals like neutral red, which gave a colorimetric reaction, quantification can be carried out with a microtiter plate reader. The technique determines total toxicity, regardless of which PSTs are present. Therefore, several workers suggested that this method could be used to detect PSP in shellfish monitoring programs (Jellet et al. 1992; Gallacher et al. 1993). However, although the MNB is highly specific for the detection of SCBs and more sensitive than the MBA, it does not differentiate which PSTs are present nor does it distinguish PST from the other SCB group, the tetrodotoxins (TTX) (Gallacher et al. 1997).

Several immunoassays have been developed for the detection of PSTs. These assays have the advantage of being relatively sensitive and no special apparatus is needed. Saxitoxin and neo-saxitoxin enzyme linked immunosorbent assay (ELISA) kits were found to be as sensitive as the MBA (Usleber et al. 1997).

Bioassays using animals other than mice, like houseflies, chick embryos, brine shrimp, and bacteria have been suggested (Ross et al. 1985). A bioassay that uses the desert locus,

Schistocerca gregaria, for the detection of saxitoxin and related compounds in cyanobacteria and shellfish may be useful for routine screening of PSP toxins (McElhiney et al. 1998).

The housefly assay is more sensitive than the mouse bioassay but requires considerable technical experience to perform microinjections, whereas the other bioassays do not have the required sensitivity for regulatory work (Isla and Vélez 2000). So far, none of the alternative techniques to determine PSP in seafood are validated for monitoring purposes, but the chemical methods for the determination of PSP toxins already reduce the animal experiments (Whittle and Gallacher 2000).

1.3.3 Spectrophotometric Assay

Bates and Rapaport (1975) developed a fluorimetric method for the measurement of saxitoxins in shellfish samples. The nonfluorescent PSTs are oxidised in an alkaline solution to a fluorescent purine derivative by hydrogen peroxide treatment. The fluorescence of the oxidised products is measured after acidification the solution (Bates and Rapaport 1975). Individual PSTs differ in toxicity and fluorescence intensity after oxidation (Franco and Fernández-Vila 1993).

1.3.4 Chromatography

The methods based on the oxidation/fluorescence assay do not specify the individual toxins. Therefore, alternative methods have been developed by many researchers. High performance liquid chromatography (HPLC) is the most widely used technique for PSP toxin detection. HPLC is very sensitive and enables the separation of each PSP toxin. All HPLC methods are based on the fluorescence detection of the oxidised PSP toxins (Luckas 2000). HPLC developed for the detection of PSP in shellfish and dinoflagellates requires treating the samples with either a pre–or post-column alkaline oxidation procedure followed by fluorescence detection (Franco and Fernández-Vila 1993; Oshima 1995; Flynn and Flynn 1996) to exclude insecurities during the PSP measurement with HPLC (Luckas 2000).

A *pre-column* HPLC oxidation method proposed by Lawrence and Ménard (1991) and Lawrence et al. (1996), does not separate all toxins, but can produce fast and sensitive results. A *post column* derivatization HPLC method for PSTs has the ability to quantitate each toxin in a crude sample of small size and simple clean-up procedures can avoid toxin transformations, which can occur during purification and concentration procedures (Oshima

1992). The HPLC method of Oshima (Oshima 1995) can separate all PSP toxins, but has the disadvantage that it is a very time consuming measurement method, due to the need of three separate runs in order to determine all toxins (Vale 2001).

1.3.5 Capillary electrophoresis

The HPLC method offer good sensitivity for the separation and detection of different PSTs, but the sensitivity is dependent on parameters, such as reagent concentrations, reaction time, pH, and temperature of the oxidation reaction (Luckas 2000). Therefore, the Capillary Electrophoresis-Mass Spectrometry (CE-MS) with UV detection was developed for the separation and determination of the underivatized PSP toxins (Thibault et al. 1991). The separation of the toxins is conducted by differential migration of solutes in an electric field. The advantage of this technique is the short analysis time and high efficiency and resolution. However, for the capillary electrophoresis highly purified extracts are necessary, to obtain reproducible separations. Additionally, the detection limit is one magnitude higher than the HPLC technique with fluorescence detection, because of the very low volumes for injection (Luckas 2000).

1.4 Interactions between bacteria and microalgae

1.4.1 Symbiotic relationship

Bacteria can be found in loose and tight associations in the physical environment of phytoplankton (Caldwell 1977; Rothaupt and Güde 1992; Gallacher and Smith 1999; Alavi et al. 2001) and also inside microalgae cells (Cole 1982; Franca et al. 1995; Alverca et al. 2002). The latter symbiotic bacteria live inside their host cells in special compartments that are rich in metabolites, such as the cytoplasm (Jeon 1991), nuclei (Görtz 1983), or perinuclear space (Fokin and Karpov 1995). Interactions of bacteria and algae range from symbiotic, via commensal, to parasitic interactions (Schäfer et al. 2002) and are highly variable in space and time (Grossart 1999). In the symbiotic relationship, bacteria benefit from phytoplankton products, such as exudates (Bell et al. 1974; Cole 1982), whereas phytoplankton profits of bacterial products, such as remineralized nutrients (Golterman 1972), vitamins (Haines and Guillard 1974), and other growth factors (Paerl and Pickney 1996). Commensalic bacteria in or around algae benefit from the algae without having any negative effect on it (Barbeyron

and Berger 1989). Bacteria can be also parasites of phytoplankton, and penetrate into the algae to lead to cell lysis and death (Cole 1982).

Also, a gene transfer from symbiont to host nucleus is postulated, which could result in an advantage in contrast to cells without a symbiotic relationship (Henze et al. 1995). However, little is known about metabolic interactions of intracellular bacteria and their host protozoa (Görtz and Brigge 1998).

Intracellular bacteria or cyanobacteria have been found so far in the dinoflagellate *Glenodinium foliaceum* and *Gonyaulax diacantha*, (Silva 1962; Silva 1978), *Amphidinium herdmanii* and *Katodinium glandulum* (Dodge 1973), *Gymnodinium lebourae* (Lee 1977), *Gymnodiniumm splendens* (Silva 1978), *Noctiluca scintillans* (Lucas 1982; Kirchner et al. 1999), *Peridinium balticum* (Chesnick and Cox 1986) in the genera *Ornithocercus*, *Histioneis* and *Citharistes* (Lucas 1991; Gordon et al. 1994), *Gyrodinium instriatum* (Silva and Franca 1985; Alverca et al. 2002), in *Alexandrium minutum* (Lu et al. 2000), *A. tamarense* and *A. fundyense* (Lewis et al. 2001).

Lewis et al. (2001) investigated the association of bacteria with various vegetative growth phases and sexual life-cycle stages of *Alexandrium* spp. The authors found bacteria to be associated with the surfaces of vegetative cells, planozygotes, hypnozygotes and planomeiocytes by using transmission electron microscopy (TEM) and the presence of intracellular bacteria in vegetative cells were also shown (Lewis et al. 2001). Bacteria were generally 0.2-1 µm across with a thin outer wall and membrane, and a dense peripheral cytoplasmatic layer. Often no bacteria were found in the cut sections, but when present, there were usually less than 10 bacteria per section. The highest bacterial numbers were found endocytoplasmatic in dinoflagellates isolated as individuals or found in loose clusters, particularly in log and stationary phase cultures of *A. tamarense* and *A. fundyense*. Bacteria were frequently located adjacent to chloroplasts and were detected also outside the theca but under the outer cell membrane, in the cingular region of the dinoflagellate (Lewis et al. 2001).

Hold et al. (2001b) showed with isolation and culture studies that a number of different bacterial species are extracellularly associated with dinoflagellates, some of which are common to each of the dinoflagellate cultures examined, whereas others appear to be unique to a particular dinoflagellate. The phylogenetic diversity of the observed bacteria was limited to two bacterial phyla, the Proteobacteria and the Cytophaga-Flavobacter-Bacteroides, with the former restricted to the α-and γ-Proteobacteria subclasses (Hold et al. 2001b). This is similar to the findings of Babinchak et al. (1998) who found similar classes of bacteria in a range of dinoflagellates. The α-Proteobacteria, particularly those of the *Roseobacter*-clade,

dominated the microflora of all dinoflagellate cultures in the study of Hold and colleagues (Hold et al. 2001b). Prokic et al. (1998) investigated the diversity of bacteria associated with *Prorocentrum lima*, a known diarrhoetic shellfish poison producing dinoflagellate. The authors also found that bacteria of the genus *Roseobacter* dominated the microflora of *P. lima*. Córdova et al. (2002) showed that Chilean clones of *A. catenella* are simultaneously infected by several bacterial species and that these bacteria were alive inside the cell. Furthermore, the authors hypothesised that bacterial infection may be clone specific and that some dinoflagellates are more susceptible to infection than others (Córdova et al. 2002).

1.4.2 Nutrient exchange

Algae represent the primary source of organic nutrients for heterotrophic microbes in the marine environment and the abundance of bacteria is frequently positively correlated with algal concentrations (Kjelleberg et al. 1993). The major flow of organic matter produced by phytoplankton is transferred via dissolved organic compounds, e.g., exudates and leakage from broken cells, to bacteria and the microbial loop (Azam 1998), whereas remineralisation by bacteria supplies nutrients to the phytoplankton. Heterotrophic bacteria utilise carbon ultimately by photosynthetically (algal-) derived carbon sources (Bell 1984) and bacteria are important in processing of phytoplankton derived particulate organic matter. Bacteria rapidly colonise different sized aggregates and their ectoenzymatic activities have important implications, e.g., for reducing the export of organic carbon and the release of dissolved organic carbon to the deeper ocean layers, or into the surrounding medium (Azam and Long 2001). The stimulation of bacterial growth by extracellular release of organic carbon by photosynthetic algae has led to the formulation of the phycosphere as an important region of interactions between bacteria and algae (Bell and Mitchell 1972). In this phycosphere, bacteria and algae interact with each other and produce both stimulatory and inhibitory substances towards each other. The bacteria can be free in this zone (Blackburn et al. 1998), attached to the surface of algal cells (Kogure et al. 1982; Vaqué et al. 1990; Worm and Sondergaard 1998), or occur intracellularly (Silva and Franca 1985; Lewis et al. 2001; Alverca et al. 2002). Motile bacteria in the phycosphere of phytoplankton cells utilise nutrients and exudates released from the algae, which then ultimately cannot be utilised by bacteria outside this zone (Bell and Mitchell 1972). Bacteria and phytoplankton cells can also compete for nutrients under certain environmental conditions where nutrients are limited for both organisms (Cole 1982; Manage et al. 2000). Changes in bacteria and phytoplankton

interactions because of environmental conditions, such as eutrophication and pollution may have huge impacts on global carbon cycling (Azam et al. 1998; Grossart 1999).

1.4.3 Bacteria and Harmful Algal Blooms (HABs)

Interactions between algae and bacteria are commonly observed in both freshwater and marine ecosystems, and bacteria are increasingly postulated as important regulators in processes of algal bloom initiation, maintenance and decline (Doucette 1995). So far, the interactions of bacteria and HABs have not been investigated to a great extent (Doucette et al. 1999), despite the known coupling of both organisms through the microbial loop (Azam et al. 1983). The bacterial community change quantitatively during an algal bloom and may either play a beneficial or a detrimental role in controlling algal growth (Doucette 1995; Plumley 1997; Doucette et al. 1999). The bacterial effects may be either direct, e.g., through intracellular symbionts (Franca et al. 1995; Plumley 1997) or indirect through, e.g., the release of soluble compounds into the surrounding water that could impact algal growth responses (Yoshinaga et al. 1995). The free-living bacterioplankton in a phytoplankton bloom has been shown to be dominated by □-Proteobacteria, Cytophaga-Flavobacter Bacteroides, □-Proteobacteria, and the *Planctomycetes* groups (De Long et al. 1993; González and Moran 1997).

Fukami et al. (1991) showed that in *Gymnodinium nagasakiense* red tides bacteria could either stimulate or inhibit dinoflagellate growth depending on the stage of the bloom. Bacteria of undetermined composition at times up to and including peak algal concentrations increase the growth of *G. nagasakiense* cultures, whereas bacteria obtained during bloom decay produced a strong negative effect on algal growth. A number of dinoflagellates undergo sexual reproduction, which comprises a number of life stages and can lead to the production of cysts, the resting state of dinoflagellates (Dale 1983). Marine bacteria associated with surfaces were shown to inhibit germination of algal spores (Egan et al. 2001) and the inhibition of mating was suggested to have an adverse effect of the dynamics of HABs and also on the genetic fitness of the population (Doucette 1995).

A potentially more positive bacterial influence on HABs is the production of cytokinins (plant hormones) by numerous species of marine bacteria (Maruyama and Simidu 1986). Bacteria which are able to synthesize these compounds may promote the development of algal blooms (Doucette 1995), because cytokinins can stimulate the growth of some red tide species (Iwasaki 1979). Moreover, bacteria may substantially alter their phenotypic

expression by attaching to algae cells and synthesize different lipopolysaccharide and proteins and exhibit markedly enhanced antibiotic resistance (Costerton 1987).

It has been reported that several bacteria are capable of killing various HAB species and consequently are involved in the termination of HABs and the regulation of population dynamics of marine phytoplankton (Doucette et al. 1998). These algicidal bacteria have different requirements for physical contact with the algal cell, some kill by direct attack (Imai et al. 1998), whereas others release the active compound into the surrounding water (Lovejoy et al. 1998). Several algicidal marine bacteria that kill bloom-forming phytoplankton have been isolated from different geographic locations (Sakata 1990; Imai et al. 1991; Fukami et al. 1992; Yoshinaga et al. 1995; Lovejoy et al. 1998; Doucette et al. 1998 and 1999). So far, only one bacterially produced algicidal agent, a 10 kD heat labile compound, has been characterised chemically to any extent (Yoshinaga et al. 1995).

Viruses are also proposed to be important regulators of phytoplankton population dynamics and HABs (Doucette 1995; Imai et al. 1998). They can also kill marine phytoplankton (Shuttle et al. 1990) and have been detected in the cells of bloom-forming phytoplankton at the end of phytoplankton blooms. Their importance in bloom dynamics has been suggested in the rapid termination of the blooms (Sieburth et al. 1988; Milligan and Cosper 1994; Brussaard et al. 1996). Algicidal bacteria and viruses are suggested to be useful tools in reducing the effects of HABs (Imai et al. 1998). However, the use of these organisms as natural regulators of HAB dynamics needs further investigations about how these organisms interact with algae at the molecular, cellular, and population levels (Imai et al. 1998; Doucette et al. 1999).

1.4.4 Bacterial Saxitoxin Synthesis

It has been proposed that prokaryotic cells are able to synthesize saxitoxins. This has changed the way HABs have been viewed and has lead to a closer look at prokaryotes involved. The identification of a bacterial strain within non-toxic dinoflagellate cultures (Silva and Sousa 1981; Silva 1990), the modification of the toxin profile after a bactericidal treatment of a toxic dinoflagellate culture (Hold et al. 2001a) and the autonomous bacterial production of phycotoxins (Gallacher et al. 1997; Doucette et al. 1998) have caused doubts, that these toxins are indeed produced by the algae alone. Several authors have reported on the production of PST by ‘axenic’ dinoflagellate cultures with levels of toxicity similar to those observed for non-axenic strains (Dantzer and Levin 1997; John and Flynn 1999), but the

absence of bacteria was not clearly demonstrated. Silva and Sousa (1981) made an important discovery, when they transformed a non-toxic dinoflagellate strain to a toxin producer by simply inoculating the non-toxic strain with the bacterium *Pseudomonas* sp., isolated from a toxin-producing dinoflagellate. However, this observation did not show which organism, the bacterium or the dinoflagellate, produced the toxins. This observation, linked to the fact that dinoflagellates routinely harbour intracellular bacteria (Bold and Wynn 1979), prompted the question 'Are bacteria the real source of saxitoxins?' Kodama and co-workers attempted to prove the hypothesis that bacteria can produce saxitoxins by isolating bacteria from cultured dinoflagellates and even removing bacteria individually from inside the dinoflagellate cells. They found that under certain precise growing conditions bacteria could synthesize saxitoxins (Kodama et al. 1988; 1990a). A PST-producing intracellular bacterium named '*Moraxella*' sp. was subsequently isolated from *Alexandrium tamarense* (Kodama et al. 1988; Kodama 1990a) and bacteria were observed within the dinoflagellate nucleus (Kodama et al. 1990a). Doucette and Trick (1995) demonstrated PST synthesis by this bacterium, named PTB-1, and another bacterial strain, *Pseudomonas stutzeri* by HPLC (Doucette and Trick 1995). *Pseudomonas stutzeri* was originally isolated as an intracellular symbiont of a saxitoxin producing *Alexandrium lusitanicum* strain (Franca et al. 1995 and 1996). In contrast, Plumley (2001) showed that *Pseudomonas stutzeri* does not synthesize saxitoxins. Available evidence indicates that this bacterium does synthesize compounds that compete for saxitoxin at sodium channel binding sites, and hence could contribute to PSP events in coastal areas (Plumley 2001). Córdova and colleagues (2002) isolated intracellular bacteria from a Chilean *A. catenella* clone and they showed by HPLC analysis that two of the isolated intracellular bacteria were capable to produce small amounts of saxitoxin.

Several independent research groups have isolated overall 18 eubacteria (Table 1), which have been reported to produce PSTs. In most cases, taxonomic information are lacking and so it is difficult to conclude if these are different species. The exception are studies incorporating 16S rRNA sequence analysis which demonstrated that five of the strains consisted of three species of the genus *Alteromonas* and one from the *Roseobacter*-clade (Gallacher and Smith 1999). These bacteria are distinctly different from the putatively toxic PTB strains, isolated from *A. tamarense* by Kodama et al. 1988, where sequence analysis has suggested similarity to the non-validated species *Agrobacterium stellulatum*, belonging to the α-subclass of Proteobacteria (Kopp et al. 1997). In 1997, the first spectral evidence of PST production by bacteria was published, which demonstrated the presence of saxitoxin, neo-

saxitoxin and several gonyautoxins (GTX's) using selective ion monitoring with capillary electrophoresis-mass spectrometry (CE-MS) (Gallacher et al. 1997).

It has been suggested that bacteria associated with dinoflagellates in nature and also under laboratory conditions, may influence the production of PST by dinoflagellates (Doucette and Powell 1998; Gallacher and Smith 1999). Tosteson et al. (1989) and Doucette et al. (1998) suggested that bacteria could modify algal toxicity via attachment. However, mechanisms by which physical interactions bacteria can influence toxin production of the dinoflagellate remain unknown (Simon et al. 2002). Some authors report higher toxicity in axenic cultures (Singh et al. 1982; Dantzer and Levin 1997), whereas others show the reverse (Doucette and Powell 1998). Hold et al. (2001a) investigated the PST production of *A. tamarense* and *A. lusitanicum* cultures in the presence and absence of bacteria. The authors showed that removal of bacteria either did not inhibit toxin production and/or change the toxin profile in *A. lusitanicum*. Also, the growth rate of *A. lusitanicum* was unaffected by removal of bacteria. However, the concentration of individual toxins and total toxicity generally was greater in axenic cultures. In contrast, the same *A. lusitanicum* strain was shown to be 50% less toxic in the absence of bacteria compared to control cultures in a study of Doucette and Powell (1998). Hold et al. (2001) showed that the removal of bacteria had a different effect on the toxicity of *A. tamarense*. In general, the toxin profile was similar in axenic and non-axenic cultures. However, the removal of bacteria from this dinoflagellate resulted in a decrease in overall toxicity of the culture. The non-axenic culture produced more of some toxin compounds, depending on the growth rate of the dinoflagellate and the toxin derivate. Therefore, the authors concluded, that bacteria directly influence toxin production in some dinoflagellate cultures whereas in others they may have an indirect effect through influencing the growth rate, although the mechanisms remain unknown (Hold et al. 2001a).

To date, evidence for bacterial production of PST has mainly been based on chromatographic behaviour and sodium channel blocking activity and capillary electrophoresis-mass spectrometry (Gallacher et al. 1997). The topic that several bacteria isolated from *Alexandrium tamarense, A. affine*, *A. lusitanicum*, *Gymnodinium catenatum*, shellfish and seawater are capable of producing a range of PSTs remains controversial, because there is a lack of unequivocal spectral evidence and the quantities of bacterial PST production seem low in comparison to dinoflagellates (Gallacher and Smith 1999), and therefore these bacteria remain only putatively toxic (Groben et al. 2000).

Table 1. Paralytic shellfish toxins produced by marine bacteria (redrawn from Gallacher and Smith 1999, modified).

Bacterial strain	Detection method	Toxin (x 10^{-4} pg STX equiv. cell $^{-1}$)	Toxin Profile	Origin	Reference
Ten unidentified strains* *Moraxella* sp.	1, 2, 3 2, 4	0.06-0.004	STX neo-STX, GTX1-4	*A. tamarense* OF 84423D-3	Kodama et al. 1988 and 1990a
PTB-1**	2, 4 5	0.08-3.26	GTX5, C1 B2/neo-STX, C1, GTX2	*A. tamarense* PT-1	Ogata et al. 1990; Doucette and Trick 1995; Levasseur et al. 1996
PTB-6 GCB-2***	2, 4	Not available	GTX1-4 STX, GTX1-4 unidentified peaks	*A. tamarense* PT-5 *G. catenatum*	Ogata et al. 1990
Vibrio sp. *Pseudomonas* sp.	2, 6,7	1.47 3.71	STX, neo-STX GTX1-4	*Perna perna* (Mussels)	Freitas et al. 1992
667-2 407-2 UW4-1 UW2c-6 253-19	2, 8, 4 9,10	0.87-2.94	STX, neo-STX, GTX1-4, B2, C toxins	*A. affine* NEPCC 667 *A. tamarense* NEPCC 407 *A. tamarense* UW4 *A. tamarense* UW2c *A. lusitanicum* NEPCC 253	Gallacher et al. 1996 and 1997
Pseudomonas stuzeri *Pseudomonas diminuta*	2	Not available	GTX4, C4 GTX1,3,4,C2-4	*A. lusitanicum* *G. catenatum*	Franca et al. 1996
Sp. 1 and 2	2, 11,4	1.00	STX, neo-STX	*A. tamarense* Ipswitch strain	Shimizu et al. 1996
Pseudomonas sp. DCM10	5	0.005-0.18	C1/GTX2, C2/GTX3 B2/neo-STX	Gulf of St. Lawrence, Canada	Levasseur et al. 1996
Alteromonas sp. 6SM1		0.05-0.59	C4/GTX4		
Acinetobacter sp. 6SN9		0.02-0.98	C3/GTX1, C4/GTX4		
Acinetobacter sp. 5Ms5		0.01-0.78	C3/GTX1, C4/GTX4		

* Later classified as *Moraxella* sp. (Kodama et al. 1988). ** It is not noted if this is the original PST producing *Moraxella* sp. strain isolated by Kodama et al. (1988)
*** Gram positive 1: Mouse bioassay; 2: HPLC (Oshima 1995); 3: Thin layer chromatography; 4: Mouse Neuroblastoma Assay; 5: HPLC (Sullivan 1993); 6: Crab leg sensory nerve bioassay; 7: Toad sciatic nerve bioassay; 8: ELISA; 9: Capillary Electrophoresis Mass spectrometry; 10: HPLC Flyn and Flynn (1996); 11: HPLC Shimizu et al. (1996).

1.4.5 Bacterial Biotransformation of Paralytic Shellfish Toxins

It has been reported that toxin profiles of contaminated shellfish vary from that of the causative dinoflagellate. Studies performed in the eighties demonstrated that two bacterial isolates from shellfish were capable of biotransforming PSTs (Kotaki 1989) and it has been shown that *Vibrio* and *Pseudomonas* spp. isolated from the viscera of marine crabs, snails, and a marine red alga were capable of transforming hydroxysulfate carbamate derivatives to saxitoxins through reductive elimination (Kotaki et al. 1985; Kotaki 1989). Smith et al. (2001) have also demonstrated that bacteria isolated from both shellfish viscera and dinoflagellate cultures are capable of metabolising PSTs through reductive elimination. This was demonstrated by *de novo* appearance of GTX2 and 3 following incubation with GTX1-4 by bacterial isolates M12 and R65. A bacterial transformation of GTX1-4 to neo-STX or STX was not detected in this study and the reaction rates and the reaction products varied significantly between the investigated bacterial isolates (Smith et al. 2001). The authors suggested that the bacterial transformation of PSTs occurs during accumulation and depuration processes in shellfish (Smith et al. 2001). However, the biological role of this transformation could not be clarified.

1.4.6 Bacterial Tetrodotoxin Synthesis

Tetrodotoxin (TTX) is a potent marine neurotoxin originally found in the ovary and liver of puffer fish. It has now become clear that TTX is not synthesized in puffer fish and that this toxin is produced by certain bacteria and reaches the fish via the food chain (Kodama et al. 1995; Gallacher et al. 1996; Narahashi 2001; Carroll et al. 2002). TTX has also been identified in amphibians, fish, echinoderms, arthropods, mollusks, nemerteans and platyhelmenthes (Miyazawa and Noguchi 2001). Animals bearing TTX are much more resistant to the lethal effect in contrast to animals having no tetrodotoxin (Koyama et al. 1983; Nagashima et al. 2002). Many species of TTX producing bacteria have been identified, including *Pseudomonas*, *Vibrio*, *Alteromonas*, *Shewanella*, *Pasteurella*, *Aeromonas*, *Plesiomonas*, and *Pseudoalteromonas* (Cheng et al. 1995; Gallacher et al. 1996; Ritchie et al. 2000).

Several parallels exist between saxitoxins and tetrodotoxins. Both marine neurotoxins have different chemical structures but act in the same manner by blocking the sodium channels of excitable membranes (Strichartz and Castle 1990; Narahashi 2001). The toxins

have similar effects on human health, similar molecular weights and coexist in the same animals (Kodama et al. 1983; Fusetani et al. 1983; Yasumura et al. 1986). Both toxins consist of a range of closely related isomeric forms (Gallacher et al. 1996). Additionally, TTX is also synthesized under laboratory conditions only in minor quantities, although in nature high quantities of the neurotoxin were found in affected animals. Therefore, Gallacher et al. (1996) suggested an inability to reproduce *in vivo* conditions in the laboratory for TTX and PSTs producing bacteria, particularly in relation to the changes in phenotypic expression of the bacteria which may occur upon surface attachment and upon exposure to exudates (Doucette 1995).

1.5 Toxification of Shellfish

Bivalves are ideal conveyers of PSP toxin (Li et al. 2001) because they are relatively indiscriminate filter feeders, consume massive amounts of algae, are not generally killed by saxitoxins, and pass the accumulated saxitoxins on to any animal that eats them (RaLonde 1996). Shellfish nerve cells are not entirely immune to the effects of saxitoxins and the degree of tolerance influences the shellfish's ability to feed and accumulate toxins. The concentration of saxitoxin in shellfish is very variable. It depends on the amount of toxic algae in the water as determined by the bloom size and patchiness, the toxin content of the individual dinoflagellate cell, the feeding rate of the shellfish, the avoidance of toxic algae by the shellfish and the transformation of the consumed saxitoxins by the shellfish (RaLonde 1996).

Changes in toxin profiles of shellfish tissues may arise from selective retention or elimination of individual toxins, by epimerisation or by a variety of enzymatic conversions through the shellfish (Bricelj and Shumway 1998) or by bacterial transformation of PSTs (Smith et al. 2001).

The extreme toxicity of blue mussels is primarily a result of their relatively insensitivity to high toxin accumulation that allows them to continue feeding on toxic algae, which can lead from being initially toxin-free to exceeding the action level of 80 µg saxitoxin level in less than a hour (Bricelj et al. 1990). *Saxidomus giganteus*, the butter clam, can be highly toxic partially because their nerve cells appear to have a special resistance to STX saxitoxin, one of the two most potent forms of the saxitoxins (Twarog et al. 1972; Beitler and Liston 1990). In addition, the butter clam has a distinctive ability to bind chemically the highly toxic STX saxitoxin in their siphon tissue (Beitler and Liston 1990), and can retain PSP toxins for up to two years after initial ingestion (Hall 1982).

The feeding behaviour of the bivalves influences also the saxitoxin content. *Crassostrea gigas*, the pacific oyster, an important species for aquatic farming, tends to consume toxic algae readily during initial contact but decreases and eventually stops feeding when tissue toxin levels become high (Bardouill et al. 1993). Saxitoxin concentrations also differ among various shellfish tissue. In the Pacific giant scallop, *Patinopectin caurinus*, the adductor muscle seldom accumulates saxitoxins above 80 µg STX equiv./100 g mussel tissue, but other tissues regularly have higher levels (RaLonde 1996). The maximum level of PSP detected in shellfish from Scottish waters fluctuates on a yearly basis, with the highest level recorded being 6000 µg STX equiv./100 g flesh from mussels obtained in 1995 (Gallacher et al. 1998).

1.6 Monitoring of Phytoplankton in affected areas in Scotland

A monitoring program for the detection of PSTs has operated in Scotland since 1968 and was increased in scale in 1990, when record levels of toxicity were detected on the Scottish west coast. Currently 43 inshore sites are examined; the testing frequency varies according to the perceived risk of toxicity, based on historical data and the importance of the area to aquaculture. High-risk sites are monitored weekly and low-risk sites at 2-week intervals from April to September. All sites are monitored monthly, from October to March. If toxicity is detected, sampling frequency at affected and adjacent areas is increased, and the number of examined shellfish species is also increased (Gallacher et al. 1998). If the action level of 80 µg STX equiv./100 g mussel tissue is exceeded, restrictions on fishing and harvesting are imposed. A phytoplankton monitoring program was incorporated in 1996 and has demonstrated that potentially toxic dinoflagellate species are present in Scottish coastal waters (Kelly and MacDonald 1996).

1.7 Aim of the thesis

Marine bacteria are suggested to play either a direct or indirect role in the occurrence of Paralytic Shellfish Toxins in dinoflagellates and shellfish. Progress to evaluate bacterial interactions with Paralytic Shellfish Toxin-associated dinoflagellates in the environment or their potential involvement in the toxicity of shellfish is lacking and therefore further investigations are needed. Molecular methods were applied to gain informations about the bacterial communities associated with dinoflagellates, the physical interactions between bacteria and dinoflagellates, the appearance of putatively toxic bacteria in HAB affected areas and their possible role in toxification of bivalves.

1.8 Outline of the thesis

1.8.1 Physical associations between bacteria and dinoflagellates

One of the first steps in investigating the relationship between bacteria and dinoflagellates is to identify the bacterial community associated with algae and their physical association with dinoflagellates. It has been shown that several dinoflagellates possess, both an extracellular and/or intracellular bacterial flora (Silva 1978; Rausch de Trauenberg et al. 1995; Biegala et al. 2002; Simon et al. 2002; Alverca et al. 2002). Intracellular bacteria are suspected to synthesize nutrients for the alga, whereas the dinoflagellate supplies an optimal habitat for the bacteria (Fenchel et al. 1993; Gordon et al. 1994; Alverca et al. 2002).

It has not been clarified in these studies, if the bacteria that were within the dinoflagellates were alive or dead. Only living bacteria can be expected to interact in a symbiotic relationship with their host cell and, e.g., influence their growth rate and/or toxin profile and the quantity of toxins produced. Therefore, it is of great importance to distinguish between bacteria that live inside the dinoflagellate cells from those that are phagocytised and will be digested by the host dinoflagellate cell. Most dinoflagellates are at least mixotrophic and a bacterial uptake could be another nutrient source for the alga (Hansen 1991; Schnepf and Ellbrächter 1992; Jacobson and Anderson 1996; Skovgaard 2000).

In **Publication I** the bacterial population and their physical association with dinoflagellates of the genus *Alexandrium* was identified and localised using two approaches. First, fluorescently-labelled 23S rRNA probes for the major subclasses of Proteobacteria (Manz et al. 1992) and 16S rRNA clade specific probes designed for dinoflagellate associated

bacteria (Brinkmeyer et al. 2000) were used to determine, if *Alexandrium* spp. harbour an attached or intracellular bacterial flora by fluorescence *in situ* hybridization (FISH) in conjunction with confocal laser scanning microscopy (CLSM). The hybridization with fluorescently labelled oligonucleotide probes does not differentiate between dead or alive cells and with it does not deliver any information about intracellular living bacteria or possible phagocytised bacteria. Therefore, in the second approach the redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) was applied succeeding the fluorescence *in situ* hybridization, to identify metabolically active bacteria inside the dinoflagellate cells.

1.8.2 Field studies to investigate the presence of dinoflagellate bacteria in seawater during PSP seasons

There is a large gap of knowledge concerning the investigations on bacteria and dinoflagellate interactions connected to Paralytic Shellfish Toxin (PST) production by conducting studies in the environment. To date progress in this area has been slow and limited to a few publications relating to the detection of PST in bacterial-sized fractions in seawater (Kodama et al. 1990a; Sakamoto et al. 1992; Levasseur et al. 1996). The complications arose because of technical complications in determination of the bacterial groups in the marine environment. Many marine bacteria are not culturable with known culture conditions and hence cannot specified by conventional methods (Schut et al. 1993).

Visualising individual bacterial cells by using *in situ* hybridization with fluorescently-labelled, species-specific ribosomal RNA (rRNA) probes is a suitable tool to perform such investigations. The rRNA has regions with different degrees of conservation, which makes it possible to develop probes for higher taxonomic groups, probes for groups of related species, named ‘clades’, genus specific probes, probes down to species or even strain level (FAIR Project Report 2001). In general, progress of 16S rRNA gene sequence data for identifying bacteria associated with toxic and non-toxic dinoflagellates in the environment has been slow.

In **Publication II** 16S rRNA probes were used to determine the presence of bacteria originally isolated from bloom forming species of the genus *Alexandrium* spp. in environmental samples. The aim of this study was to obtain information about the seasonal distribution of the dinoflagellate associated bacteria and if they are present during *Alexandrium* spp. blooms and periods of shellfish toxicity in a HAB affected area. Specifically fluorescently labelled 16S rRNA oligonucleotide probes recognising these bacteria (Brinkmeyer et al. 2000) were applied to Lugol’s preserved environmental samples

taken at designated monitoring sites at the Orkney Islands, Scotland from March to November in 1999. At this location a yearly bloom of toxic dinoflagellates occurs and mussel beds are closed for harvesting when toxicity levels in the mussels exceed those allowed by the EU. The total abundance of the putatively toxic bacteria was determined by counting the number of cells exhibiting a fluorescence signal from the 16S rRNA probes. The seasonal abundance of the dinoflagellate associated bacteria was compared with cell counts of *Alexandrium* spp. and with mussel toxicity. The monitoring of the purportedly toxic bacteria at the Orkney Islands was repeated from March to August in 2000. In this second monitoring year (**Appendix to Publication II**) samples were collected from two main sampling sites by the Scottish sampling monitoring program, to evaluate the physical association and the simultaneous presence of the putatively toxic bacteria in relation to *Alexandrium* spp. counts and mussel toxicity.

1.8.3 Studies on the effect of a bacterial contribution to shellfish toxicity

Today, virtually nothing is known about the effect of bacteria on shellfish toxicity. Evidence for the involvement of bacteria on shellfish toxicity has been implicated by Kodama and colleagues (1990) who reported that PST were present in particles of a similar size fraction (0.45-5 µm) to bacteria in seawater from Ofunto Bay, Japan, during times when bivalve toxicity increased in the absence of toxic dinoflagellates. The same group (Sakomoto et al. 1992) later reported that during a bloom of *A. catenella*, in Tanabe Bay, Japan, particles smaller than dinoflagellates (5-20 µm) contained a considerable amount of PST, whereas no significant toxicity was detected in dinoflagellate cells. It was concluded that these particles could be small ciliates or flagellates, but such organisms were not observed in high numbers at the time of sampling although many detritus or silt particles were seen in water samples. Hence, it was speculated that these toxic particles could be detritus/silt with attached toxin producing bacteria (Sakamoto et al. 1992). This particles can be readily accumulated by animals, such as bivalves and possible toxify the animal. However, a definitive link between purportedly toxic bacteria and mussel toxicity has not been established.

In **(Publication III)** the ability of putatively toxic bacteria to invoke toxicity in the blue mussel, *Mytilus edulis* was examined. The aim of this study was to determine, if bacteria were capable of toxifying shellfish and if so, how prevalent were such bacteria in shellfish organs. Feeding experiments with putatively toxic bacteria were performed with different combinations and concentrations of bacteria and together with and without silt to enable an

attachment of the bacteria to particulate matter. In this way, differences should be determined in the filtration rate of bacteria attached or not attached to particulate matter by the mussels. The digestive gland, the hepatopancreas, where toxins become concentrated in mussels, was dissected and paraffin and cryosections were made. Using oligonucleotide probes, the presence of the fed bacteria in the tissue sections of the hepatopancreas were examined. The toxin content of the shellfish tissue was monitored over the course of the experiment with HPLC.

1.8.4 Reassociation experiments with putatively toxic bacteria into a toxic Alexandrium tamarense clone

Bacteria have often been found in the physical environment of toxic dinoflagellates (Doucette and Trick 1995; Gallacher et al. 1997; Doucette et al. 1998; Prokic et al. 1998). Several putatively toxic bacteria have been isolated from toxic and non-toxic cultures of *Alexandrium tamarense* (Ogata et al. 1990; Doucette and Trick 1995; Gallacher et al. 1997). Hold et al. (2001b) have shown that a number of different bacterial species are associated with dinoflagellates, some of which are common to each of the dinoflagellate cultures examined, whereas others appear to be unique to a particular dinoflagellate. So far, only little is known about the attachment behaviour of bacteria to algal cells (Kogure et al. 1982; Vaqué et al. 1990; Worm and Sondergaard 1998). The authors used in their studies phase contrast and epifluorescence microscopy for detection of bacteria attached to algae cells. Also, indirect methods have been proposed, in which the number of attached bacteria was estimated by subtracting the total number of bacteria collected before and after chemical treatment and sonication of the bacterial culture (Albright et al. 1986). However, these treatments dispersed attached bacteria in the medium. A more suitable way to monitor the attachment behaviour of bacteria to algae is the use of fluorescence *in situ* hybridization together with tyramide signal amplification (TSA-FISH) in conjunction with CLSM (Schönhuber et al. 1997 and 1999). TSA-FISH enables a strong amplification of the hybridization signal *in situ,* e.g., to overcome the autofluorescence of algae cells. Thus, it is possible to monitor the specific attachment behaviour of bacteria to algae cells and to locate bacteria within the cells. With CLSM it is possible to enumerate attached bacteria surrounding the entire dinoflagellate cell and also within the dinoflagellate cells.

In **Publication IV** the attachment behaviour of two phylogenetically related bacterial species PTB-1 and PTB-6 to a highly toxic *A. tamarense* clone was investigated, from which

the PTB-1 bacteria were originally isolated. It should be determined if bacteria specifically attach or become intracellular within a specific dinoflagellate strain, or if the attachment occurs randomly. Group, genus, and species-specific eubacterial probes targeting the 16S rRNA of these bacteria were tested on this dinoflagellate in culture to simultaneously identify, localise and quantify the associated bacteria, either attached or intracellular.

2. Publications

2.1 List of publications

This doctoral thesis is based on the following publications:

I. TÖBE, K.; CÓRDOVA, J. AND MEDLIN, L.K.
DETERMINATION OF INTRACELLULAR AND EXTRACELLULAR ASSOCIATED BACTERIA WITH DINOFLAGELLATES OF THE GENUS *ALEXANDRIUM*
J. of Plankton Research, to be submitted

II. TÖBE, K.; FERGUSON, C.; KELLY, M.; GALLACHER, S. AND MEDLIN, L.K. 2001.
SEASONAL OCCURRENCE AT A SCOTTISH PSP MONITORING SITE OF PURPORTEDLY TOXIC BACTERIA ORIGINALLY ISOLATED FROM THE TOXIC DINOFLAGELLATE GENUS *ALEXANDRIUM*
European Journal of Phycology. 36: 243-256

IIa TÖBE, K.; FERGUSON, C.; KELLY, M.; GALLACHER, S. AND MEDLIN, L.K.
APPENDIX TO PUBLICATION II: MONITORING OF PURPORTEDLY TOXIC BACTERIA AT TWO PSP MONITORING SITES OF THE ORKNEY ISLANDS IN 2000

III. TÖBE, K.; SMITH, E. A.; GALLACHER, S. AND MEDLIN, L.K.
DETECTION OF BACTERIA ORIGINALLY ISOLATED FROM *ALEXANDRIUM* SPP. IN THE MIDGUT DIVERTICULA OF *MYTILUS EDULIS* AFTER WATER-BORNE EXPOSURE
Harmful algae, in press

IV. TÖBE, K.; MEDLIN, L.K.; DOUCETTE, G. AND MIKULSKI, C.
REASSOCIATION EXPERIMENTS WITH BACTERIA INTO A HIGHLY TOXIC *ALEXANDRIUM TAMARENSE* CLONE
Microbial Ecology, to be submitted

2.2 Statement of my part of the publications

Publication I

The experiments were planned together with all authors. The experiments were carried out and analysed by myself. The manuscript was written by me.

Publication II and Appendix to Publication II

The experiment concept was developed together with L.K. Medlin, C. Ferguson and S. Gallacher, performed and analysed by myself. The manuscript was written in collaboration with L.K. Medlin. M. Kelly was involved in the collection of environment samples and presenting *Alexandrium* counts from the Orkney Islands. Sampling of field samples and detection of PSP in shellfish was conducted by the Scottish Phytoplankton Monitoring Program of the FRS Maine Laboratory in Aberdeen.

Publication III

The experiments were planned together with L. K. Medlin, S. Gallacher and E. Smith. The experiments have been carried out by E. Smith and me. The manuscript was written together with E. Smith.

Publication IV

The experiments were planned and carried out in cooperation with L. K. Medlin, G. J. Doucette and C. Mikulski. I have analysed the data and wrote the manuscript.

2.3 Publication I

DETERMINATION OF INTRACELLULAR AND EXTRACELLULAR ASSOCIATED BACTERIA WITH DINOFLAGELLATES OF THE GENUS *ALEXANDRIUM*

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J. of Plankton Research, to be submitted

ABSTRACT

Fluorescently-labelled 16S and 23S rRNA oligodeoxynucleotide probes, group or clade specific, respectively, were used to identify bacteria associated with *Alexandrium* spp. by confocal laser scanning microscopy (CLSM). Bacteria were found to be associated with *Alexandrium tamarense*, *A. lusitanicum and A. andersonii,* intra- and/ or extracellulary, respectively. The presence of metabolically active intracellular bacteria in dinoflagellates of the genus *Alexandrium* was documented with the use of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). This compound is reduced by living, respiring bacteria into a water-insoluble fluorescent formazan (CTF), which fluorescences bright red. Actively respiring bacteria were detected intracellularly in log and stationary phase-fixed *A. tamarense* and *A. lusitanicum* by epifluorescence microscopy (FM) and CLSM. These results support the given theory that specific bacteria are an integral part of the physical environment of *Alexandrium* spp.

Key words*: Alexandrium*, bacteria, confocal laser scanning microscopy, cyanoditolyltetrazolium chloride (CTC), dinoflagellates, epifluorescence microscopy, 16S rRNA probes, 23S rRNA probes, Paralytic Shellfish Poisoning

INTRODUCTION

Harmful algal blooms (HABs) are increasing in frequency along the coastal regions of many oceans around the world (Alavi et al. 2001). The production of toxins in HABs phenomena are often documented, but not always (Plumley 1997). Many of the HAB-toxin producers are single-cell dinoflagellates that produce Paralytic Shellfish Toxins (PSTs). PSTs are termed collectively saxitoxins (Kao 1993). Saxitoxins are neurotoxins that block movement of sodium through nerve cell membranes, thus stopping the flow of nerve impulses and causing the symptoms of an intoxification called Paralytic Shellfish Poisoning, leading to paralysis and subsequent death in humans (Mosher et al. 1964; Lassus et al. 1992). Prokaryotic cells associated with toxic microalgae are postulated to be able to synthesize autonomously saxitoxins and thus enhance algal toxicity (Kodama et al. 1988; 1990a; Ogata et al. 1990; Gallacher et al. 1997; Gallacher and Smith 1999; Córdova et al. 2002). Paralytic Shellfish Toxins (PSTs) have been detected in several bacteria isolated from *Alexandrium* spp. (Kodama et al. 1990; Doucette and Trick 1995; Gallacher et al. 1997; Córdova et al. 2002). However, unequivocal spectral evidence of autonomous bacterial saxitoxin production is still lacking (Gallacher and Smith 1999).

The ecological role of the naturally occurring bacterial-algal associations has mostly not been clarified and most of the involved bacterial have not been identified (Hold et al. 2001). One important algal-bacterial interaction is the role they play in the rise and demise of HABs (Doucette 1995). The presence of bacteria within dinoflagellates has been demonstrated in a number of vegetative stages of microalgae (Silva 1978; Lucas 1982; Gordon et al. 1994; Rausch de Trauenberg et al. 1995; Doucette et al. 1998; Lewis et al. 2001). These endocytic bacteria likely synthesize nutrients for the host, for example, by nitrogen fixation or by an increase in enzyme activity, whereas hosts supply optimal habitats (Fenchel et al. 1993; Gordon et al. 1994).

It has not been clarified, if the bacteria, which have been found within the dinoflagellates were alive or dead, but it is of great importance to differentiate between those, that live inside the dinoflagellate cells and those that are phagocytised. Most dinoflagellates are at least mixotrophic and bacterial uptake could be another nutrient source for the alga (Schnepf and Ellbrächter 1992; Skovgaard 2000). Phagocytising organisms bear a high risk of infection by the phagocytised micro-organisms, which resist the cell's mechanisms of killing and digestion (Görtz and Brigge 1998). However, *Alexandrium* species are not known to phagocytose bacteria directly (Legrand and Carlsson 1998) and are generally considered to be

mainly autotrophic. However, ciliates or phytoplankton cells have been found in food vacuoles of *A. ostenfeldii* (Jacobson and Anderson 1996).

In this study, the presence of bacteria within and around the dinoflagellates by application of 16S- and 23S ribosomal RNA (rRNA) oligodeoxynucleotide probes was investigated. It has been shown that the marine bacteria are dominated by □- and □□Proteobacteria and also by Cytophaga-Flavobacterium-Bacteroides bacteria (Glöckner et al. 1999) and have been found in association with different species of dinoflagellate (Lafay et al. 1995). 23S rRNA probes recognising the major subclasses of Proteobacteria (Manz et al. 1992) and 16S rRNA clade specific probes detecting dinoflagellate associated bacteria were used to identify the bacterial community of *Alexandrium* spp. by fluorescence *in situ* hybridization (FISH) and CLSM. *Alteromonas*-clade bacteria belonging to the □-subclass of Proteobacteria, and *Roseobacter*-clade bacteria, belonging to the □-subclass of Proteobacteria, are common in marine environments and are often found associated with microalgae (Lafay et al. 1995; Doucette and Trick 1995; Gallacher et al. 1997; Hold et al. 2001; Alavi et al. 2001). Bacteria of the genus *Alteromonas* are often found with toxic dinoflagellates in culture and many strains have been reported as purportedly toxic (Buck and Pierce 1989; Tosteson et al. 1989; Doucette and Trick 1995; Onji et al. 1995; Gallacher et al. 1997; Gallacher and Smith 1999). Probes recognising bacteria of the *Alteromonas*-clade, and probes recognising bacteria of the *Roseobacter*-clade, were designed from bacteria originally isolated from *Alexandrium* spp. (Brinkmeyer et al. 2000).

The presence of metabolically active bacteria inside dinoflagellates of toxic and non-toxic species of the genus *Alexandrium* was investigated by the use of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), a monotetrazolium redox dye that produces a fluorescent formazan (CTF) when it is chemically or biologically reduced. Rodriguez and co-workers (1992) described the first application of CTC for microscopic visualisation of actively respiring bacteria in native and nutrient amended environmental samples and in bacterial biofilms formed on microscope slides. The oxidised form of CTC is nearly colourless and is non-fluorescent, but this compound is readily reduced via electron transport activity to fluorescent, insoluble CTF, which accumulates intracellularly (Rodriguez et al. 1992). In microalgae, the fluorescent formazan is easily detected intracellularly by FM and CLSM, because of its red fluorescence when illuminated by longwave UV light (>350 nm), after differential killing procedures of the algae (Córdova et al. 2002). Córdova et al. (2002) showed that after killing the infected dinoflagellate, intracellular bacterial still remain alive, and reducing the CTC. Confocal laser scanning microscopy enables the possibility to detect in

real time, the localization of the intracellular bacteria in the infected dinoflagellates, by generating optical thin sections (< 1 µm).

MATERIAL AND METHODS

Species and culture methods. The dinoflagellates *Alexandrium tamarense* BAHME182 (Orkney Islands, UK, toxic), *Alexandrium andersonii* 012b (Golf of Naples, Italy, toxic), *Alexandrium lusitanicum* BAHME91 (Laguna de Obidos, Portugal, toxic), *Alexandrium ostenfeldii* KO324 (Limfjord, Denmark, toxic) and *Alexandrium taylorii* AY1T (Lagoon of Marano, Italy, non-toxic) were used in this study. The dinoflagellates were maintained in IMR/2 medium (Eppley et al. 1967) at a photon flux density of 100 µmol m^{-2} s^{-1} at 15°C and a 14:10 h light/dark photon cycle.

Fluorescence in situ *hybridization (FISH).* Seven mlof each dinoflagellate culture were filtered down in duplicate from each fixation time with a Millipore filter manifold (Millipore, Bedford, USA) onto black polycarbonate filters, (0.2 µm pore size, 25 mm diameter [Osmonics, Minnetonka, USA]) and fixed in freshly made 4% paraformaldehyde (PFA, pH 7.2, set up in 1 X PBS, 0.2 µm filter sterilised) for 1 h at 4°C. The filter was rinsed in 1 X PBS, dried and dehydrated by a graded ethanol series (50, 80, 100%) for 5 minutes each. One ml lysozyme solution (5 mg ml^{-1} in sterile Milli-Q) was applied to each filter and incubated for 30 minutes at 37°C in a humid chamber to improve probe penetration through the dinoflagellate cell walls and into the bacterial cells. The enzymatic reaction was stopped by rinsing the filters three times for 1 minute in 5 ml sterile Milli-Q-water. The filters were dehydrated again by a graded ethanol series (50, 80, 100%) for 5 minutes each, air dried (Amann et al. 1995; Schönhuber et al. 1997 and 1999) and cut in four equal pieces with a sterile razor blade. One quarter of each duplicate was used as a negative control. Each filter quarter was hybridized together with two different fluorescently labelled oligonucleotide probes (hybridization pattern, see Fig. 1). The fluorescence label of the oligonucleotide probe (purchased from Interactiva, Ulm, Germany) was fluoresceinisothiocyanate (FITC), a green fluorescence dye, cyanin 3 (CY3) a red fluorescence dye or horseradish labelled probes (HRP) for TSA-FISH. Twenty µl hybridization buffer (20mM Tris/HCL, [pH 8.0], 0.9 M NaCl, X% deionized formamide (concentration see Table 1), 0.01% SDS) + 2 µl labelled oligonucleotide probe (50 ng μl^{-1}) were added to each filter quarter. The negative control quarter was hybridized with the hybridization solution without a probe. Labelled probe BET

42a was hybridized with a 10-fold excess of unlabelled GAM 42a and labelled GAM 42a hybridized with a 10-fold excess of unlabelled BET 42a to block unspecific binding sites (Manz et al. 1992). Labelled probe ROSEO 536R was hybridized 1:1 with unlabelled ROSEO C536R. This competitor probe matches non-target bacterial strains with 1 mismatch to the ROSEO 536R probe (Brinkmeyer et al. 2000). Hybridizations were performed at 46°C for 2-3 h in the dark in 25 mm petri dishes, in which the lids were lined with Whatman paper and soaked in hybridsation buffer. The hybridizations were stopped by rinsing each filter piece in 10 ml wash buffer (20 mM Tris /HCl [pH 8.0], X M NaCl, 5 mM EDTA, 0.01% SDS). The formamide is replaced in the wash buffer by the NaCl solution with equivalent stringency (molarity of NaCl, see Table 1). Then the filter pieces were incubated in 25 ml wash buffer two times for 10 minutes at 48°C while shaking (Amann et al. 1990). The filters were rinsed in sterile Milli-Q and air dried. Mounting and counterstaining of the cells was performed with a mixture of 4',6-diamidino-2-phenylindole, DAPI, (Sigma, USA) and the antifade Citifluor (Citifluor Products, London, UK). Thirty µl of this mixture (1 ml Citifluor, 0.5 ml sterile water, 1,5 µl DAPI [stock 1 µg μl^{-1}]) were given directly onto the filter quarters. Coverslips were placed over the filters and sealed with nail varnish. The slides were kept at –20°C in the dark until analysed by FM and CLSM.

TSA-FISH. To determine bacteria within the dinoflagellate it is necessary to overcome the autofluorescence of the target cells. Therefore, additionally a tyramide signal amplification (TSA) method coupled with fluorescence *in situ* hybridization was performed according to Schönhuber et al. (1997 and 1999, slightly modified). The filter pieces hybridized with HRP-labelled probes (see Table 1) were rinsed in sterile Milli-Q and equilibrated for 15 minutes in TNT-Buffer (0.1 M Tris-HCL, [pH 7.5], 0.15M NaCl, 0.05% Tween 20). Forty per cent dextran sulfate (w/v, in sterile Milli-Q-water) used for reducing unspecific staining of non-target cells during long term incubation was mixed 1:1 in 2 X Amplification diluent of the TSA-direct Kit (NEN Life Science Product Inc., Boston, USA). One µl fluorescein tyramide (TSA-Direct Kit) was given to 50 µl of this mixture, to create the FT-working solution. Twenty five µl of this FT-working solution per filter quarter was used and the filter pieces were incubated for 35 minutes at room temperature in the dark. To remove unreacted fluorochrome-tyramide and to stop the enzyme reaction, the filter quarters were washed twice in TNT-Buffer for 15 minutes at 55°C, rinsed in sterile Milli-Q water, air-dried and counterstained (see above).

Epifluorescence microscopy. To show the actively respiring bacteria by FM or CLSM, respectively, 15 µl of the resuspended CTC samples were added on a glass slide and covered with small coverslips. The FISH samples needed no further treatment and were analysed directly after sealing. Epifluorescence was viewed with a Zeiss Microscope (Axioskop 2 plus, Oberkochen, Germany) fitted for FM under oil immersion (Leica immersion oil, Oberkochen, Germany) with the appropriate filter set (Zeiss filter sets, 03: UV G 365; 04: blue BP 450-490 nm; 05: green BP 510-560 nm). The epifluorescence images were aquired with a X 100 objective (Plan-Apochromat, numerical aperture 1,40 Oil DIC) and analysed with the Leica program Axio vision 3.1.

Confocal laser scanning microscopy. Optical sections were acquired with a confocal laser scanning microscope (TSNT, 165081, Leica, Oberkochen, Germany) equipped with an argon-krypton laser. The dinoflagellates were observed using excitation/emission lines of the krypton argon laser: blue (excitation 488 nm, emission 522/32 nm) to visualise the fluorescence of CTF and FITC, and green (excitation 568 nm, emission 605/32 nm) to visualise CY3. The confocal images were acquired with a X 100 objective under oil immersion (NA Oil Planapo, numerical aperture 1,40 x 1,70, Leica) and a X 40 objective (NA Oil Planapo numerical aperture 1,25 x 0,75, Leica). The confocal images were analysed using the Leica-TCSNT program.

CTC treatment of dinoflagellates. Dinoflagellates were harvested at log and stationary phase by spinning down the cells for one minute at 1000 *g*. To determine viable bacteria inside the dinoflagellate cells 1.8 ml of both log and stationary phase algal culture were each fixed with 2% glutaraldehyde (Polysciences*,* Warrington PA, USA), or freshly made 2% PFA in 1 X PBS, for varying times (5, 10, 15, 20 and 30 min). The cells were gently centrifuged twice for 1 min in sterile seawater. The pellet was resuspended carefully in 250 µl 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Polysciences, Inc., Warrington PA, USA), stock solution: 1 mg ml^{-1} in sterile seawater, and incubated for 2 h at 15°C in the dark (Rodriguez et al. 1992; Córdova et al. 2002). After incubation, the samples were gently centrifuged and the pellet were resuspended in 1 ml sterile seawater. The cells were gently centrifuged again for 15 min and the pellets were resuspended in 0.5 ml sterile seawater. After a last gently centrifugation for 5 minutes, the pellets were carefully resuspended in 150 µl seawater and analysed by FM or CLSM. The samples could be analysed directly or stored up to several days after CTC treatment without any loss of signal.

RESULTS

FISH experiments. Hybridization with ALF1B, EUB 338R and the *Roseobacter*-clade probe (CY3), respectively, revealed bacteria located extracellular attached to log- and stationary phase dinoflagellates of the species *Alexandrium tamarense* and *A. lusitanicum* (Figs. 2-6). Moreover, intracellular bacteria of the β-subclass of Proteobacteria were found intracellularly in *A. tamarense* (Fig. 4). Hybridization with EUB338 revealed bacteria attached to the cell surface of *A. andersonii*. Some of these attached bacteria were identified as *Alteromonas*-clade bacteria. These bacteria which often produce mucilage were also found free in the culture of this dinoflagellate (Figs.7-8). FISH with the dinoflagellate *A. taylorii* and *A. ostenfeldii* revealed no bacteria either attached or intracellular.

Treatment of Dinoflagellates with CTC. The different fixation time series of the dinoflagellates showed that the optimal fixing time was 20 minutes fixation with 2% glutaraldehyde or 2% PFA to get the best fluorescence signals of intracellular bacteria. CTC-treated *Alexandrium tamarense* and *A. lusitanicum* cells analysed by FM showed bright red signals inside the log and also stationary dinoflagellate cells at an illumination with UV light (excitation G 365 nm, Zeiss filter channel 3) and a bright orange signal at an illumination with bluelight (BP 450-490 nm, Zeiss filter channel 4). These signals appeared at a high magnification to be bacteria in the cells of *A. tamarense* and *A. lusitanicum*. These endocytoplasmatic bacteria were alive, because they were able to reduce CTC, which remained intracellular, and this resulted in bright clear fluorescence signals, easily visible in FM and CLSM. No metabolically active bacteria were observed in the nucleus of any species and also no intracellular bacteria were found in *A. taylorii*, *A. andersonii* and *A. ostenfeldii* by treatment with CTC. The bacteria found within *A. tamarense* and *A. lusitanicum* were small cocci or rod-shaped (Fig. 9). The dinoflagellates *A. lusitanicum* and *A. andersonii* were visibly damaged by treatment with CTC, as seen by FM and CLSM. No damage of the dinoflagellate *A. tamarense*, *A. taylorii* and *A. ostenfeldii* cells were visible. However, the bacterial cell integrity was still intact in all species noticed by FM and CLSM. The optical thin sections (0,7 µm) generated by CLSM show intracellular bacteria in *A. tamarense* (Fig. 10) and *A. lusitanicum* (Fig. 11) especially in the outer layers of the dinoflagellate cells.

DISCUSSION

Many dinoflagellates have been reported to possess intracellular bacteria (Silva 1978; Lucas; 1982; Gordon et al. 1994; Seibold et al. 2001; Lewis et al. 2001; Alverca et al. 2002). Bacteria could be also found in loose and tight associations in the physical environment of phytoplankton (Caldwell 1977; Rothaupt and Güde 1992; Gallacher and Smith 1999). Members of the genera *Aeromonas*, *Alteromonas*/*Pseudomonas* and *Vibrio* have been frequently found in association with toxic dinoflagellates in culture and some isolated strains have been reported as toxic (Buck and Pierce 1989; Tosteson et al. 1989; Ogata et al. 1990; Doucette and Trick 1995; Gallacher et al. 1997; Babinchak et al. 1998; Gallacher and Smith 1999; Simon et al. 2002). There are very few studies in which rRNA probes have been used to identify the bacteria associated with algae. In our study using rRNA probes, Eubacteria, the α-subclass of Proteobacteria and bacteria of the genus *Roseobacter*, were identified extracellularly attached to the cell surface of stationary phase cells of toxic *Alexandrium tamarense* and *A. lusitanicum* strains. *Roseobacter*-clade bacteria were found in high numbers associated with stationary phase *A. tamarense* and *A. lusitanicum* and free in the culture media, whereas *Alteromonas* bacteria were not found attached to or within the dinoflagellates, but sometimes found in limited numbers free in the culture medium. However, *Alteromonas*-clade bacteria were found attached to the cell surface of *A. andersonii*. In *A. tamarense* intracellular bacteria of the γ-subclass of Proteobacteria were detected. No intracellular bacteria were found in *A. lusitanicum* and *A. andersonii*. Hybridization with the used oligonucleotide probes failed to detect associated bacteria either inside nor attached to the cell surface of *A. taylorii* and *A. ostenfeldii*.

Also no intracellular bacteria were found in other *A. tamarense* strains (PLY173a and NEPCC 407) after FISH with the universal eubacterial probe by Biegala and co-authors (2002). The authors suggested that these strains did not contain any intracellular bacteria at the time of sampling or that intracellular bacteria are able to change from intracellular to extracellular locations, because they are not necessarily endocellular bacteria. (Biegala et al. 2002). However, in the same *Alexandrium*-strains bacteria were detected by transmission electron microscopy (TEM), although in low abundance (Lewis et al. 2001). This shows that great differences in the bacterial population exist between single species of *Alexandrium* and should be investigated further to get more deep insights in the relationship between algae and their associated bacteria.

The *Roseobacter*-clade bacteria detected associated with toxic species of *A. tamarense* and *A. lusitanicum* in this study are widely distributed in a wide range of marine habitats (Rappé et al. 1997; Giuliano et al. 1999) and have been isolated from toxic dinoflagellate (Lafay et al. 1995; Hold et al. 2001). A dominance of *Roseobacter*-clade bacteria, belonging to the α-subclass of Proteobacteria, together with the occurrence of toxic and non-toxic dinoflagellates was also described in studies investigating field material of HAB areas (Gerdts et al. 2000; Töbe et al. 2001). Gerdts et al. (2000) reported a domination by Proteobacteria during toxic *A. tamarense* blooms in the area of the Orkney Islands and the Firth of Forth in 1998. Their results indicated, that α-Proteobacteria were closely associated with the blooms of toxic *A. tamarense*. *Roseobacter*-clade bacteria were also found as the major bacterial group in toxic dinoflagellate of the genus *Prorocentrum lima*, a known diarrhoetic shellfish poison producing dinoflagellate (Prokic et al. 1998).

It is possible that intracellular bacteria are difficult to detect by the use of oligonucleotides, e.g., because of weak hybridization results and/or a decreased probe penetration into the dinoflagellate cell. Only metabolic active bacteria can be expected to live in a symbiotic relationship with their host algae cell and, e.g., influence their growth rate and/or toxin profile and the quantity of toxins produced. Therefore, it is of great importance to distinguish between bacteria that live inside the dinoflagellate cells from those which are phagocytised and digested by the host cell. This was achieved with the use of the CTC compound. CTC serves as an indicator for the detection of metabolically active bacteria within microalgae, because it can only be reduced to the water insoluble fluorescent formazan, when metabolically active cells, such as bacteria, reduce it by capturing electrons derived from the respiratory chain and therefore preventing its release from the cell (Rodriguez et al. 1992; Córdova et al. 2002). However, CTC caused sometimes a background coloration of the dinoflagellates probably because of a residual respiratory activity of the algae (Córdova et al. 2002) and could hide small bacteria.

Metabolically active bacteria were identified in *Alexandrium tamarense* and in *A. lusitanicum*. However, no intracellular bacteria could be detected by FISH in *A. lusitanicum* with the used oligonucleotide probes. Therefore, further characterisation of these metabolically active bacteria by FISH applications is necessary. Nevertheless, our findings are very important suggesting that these bacteria live as symbionts or as commensals in the microalgae. The other investigated dinoflagellates *Alexandrium taylorii*, *A. andersonii* and *A. ostenfeldii* revealed no metabolically active bacteria within the dinoflagellate cells. Our study has made significant progress in the study of the bacteria associated with dinoflagellates.

Using rRNA probes it was possible to identify the genus/species of the bacteria attached to the cell surface of *Alexandrium* spp. and also endocytic in *A. tamarense*. Moreover, metabolically active bacteria within dinoflagellates of the genus *Alexandrium* were shown by the use of the compound CTC. This data provides important information about the association between bacteria and algae. Further characterisation of the bacterial flora of dinoflagellates is necessary to show single bacterial species, which are associated with dinoflagellates and whether the bacteria are located extra- or intracellular by the application of species-specific molecular probes. Additionally, other strains of the used dinoflagellates should be screened for attached or intracellular bacteria to compare the bacterial flora between different strains of one dinoflagellate species. Also, subcultures of the same dinoflagellate strain needs to be screened for bacteria to confirm our findings. With these strategies in place we can begin to unravel the complex association between bacteria and microalgae in relation to toxin synthesis and their role in HABs.

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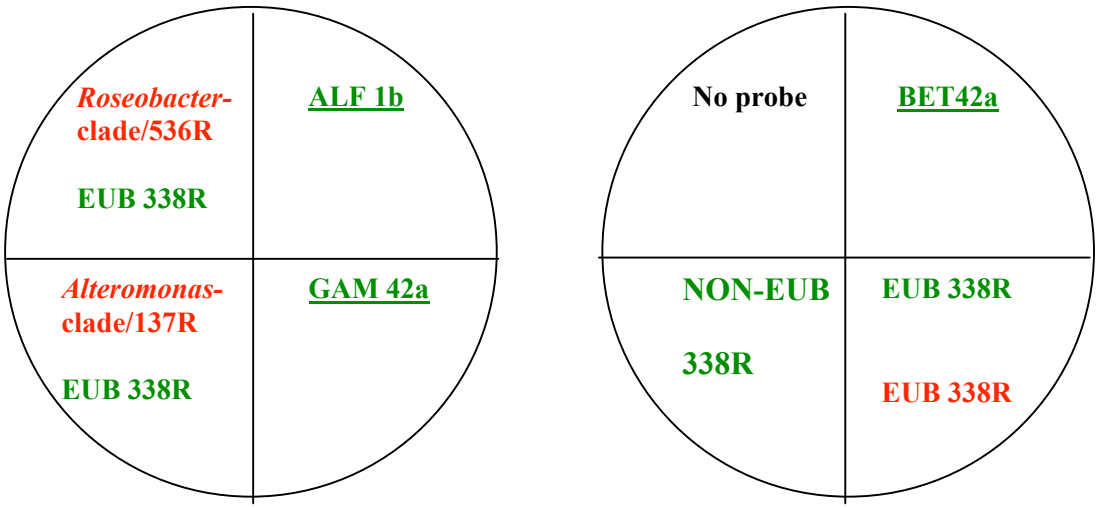

Fig. 1: FISH pattern: FITC-labelled oligonucleotide probes marked in green; CY3-labelled oligonucleotide marked in red; HRP-labelled oligonucleotides marked in green, unerlined.

Table 1: Oligonucleotide probes used in this study

Probe name	**Sequence 5'---------- 3'**	**Target organisms**	***E. coli* target sites and rRNA position**	**Formamide concentration**	**NaCl Molarity wash buffer**
ALF1b	cgt tcg (c/t) tct gag cca g	α-Proteobacteria	19-35 16S	20%	0.225 M
BET42a	gcc ttc cca ctt cgt tt	β-Proteobacteria	1027-1043 23S	35%	80 mM
GAM42a	gcc ttc cca cat cgt tt	γ-Proteobacteria	1027-1043 23S	35%	80 mM
EUB 338R	gct gcc tcc cgt agg agt	Eubacteria	338-355 16S	0-20%	0.225 M
AMAC 137R	tgt tat ccc cct cgc aaa	*Alteromonas*-clade	137-154 16S	0%	0.9 M
ROSEO 536R	caa cgc taa ccc cct ccg	*Roseobacter*-clade	536-553 16S	18%	0.262 M
ROSEO C536R	caa cgc tag ccc cct ccg	*Roseobacter*-competitor probe	536-553 16S	18%	0.262 M

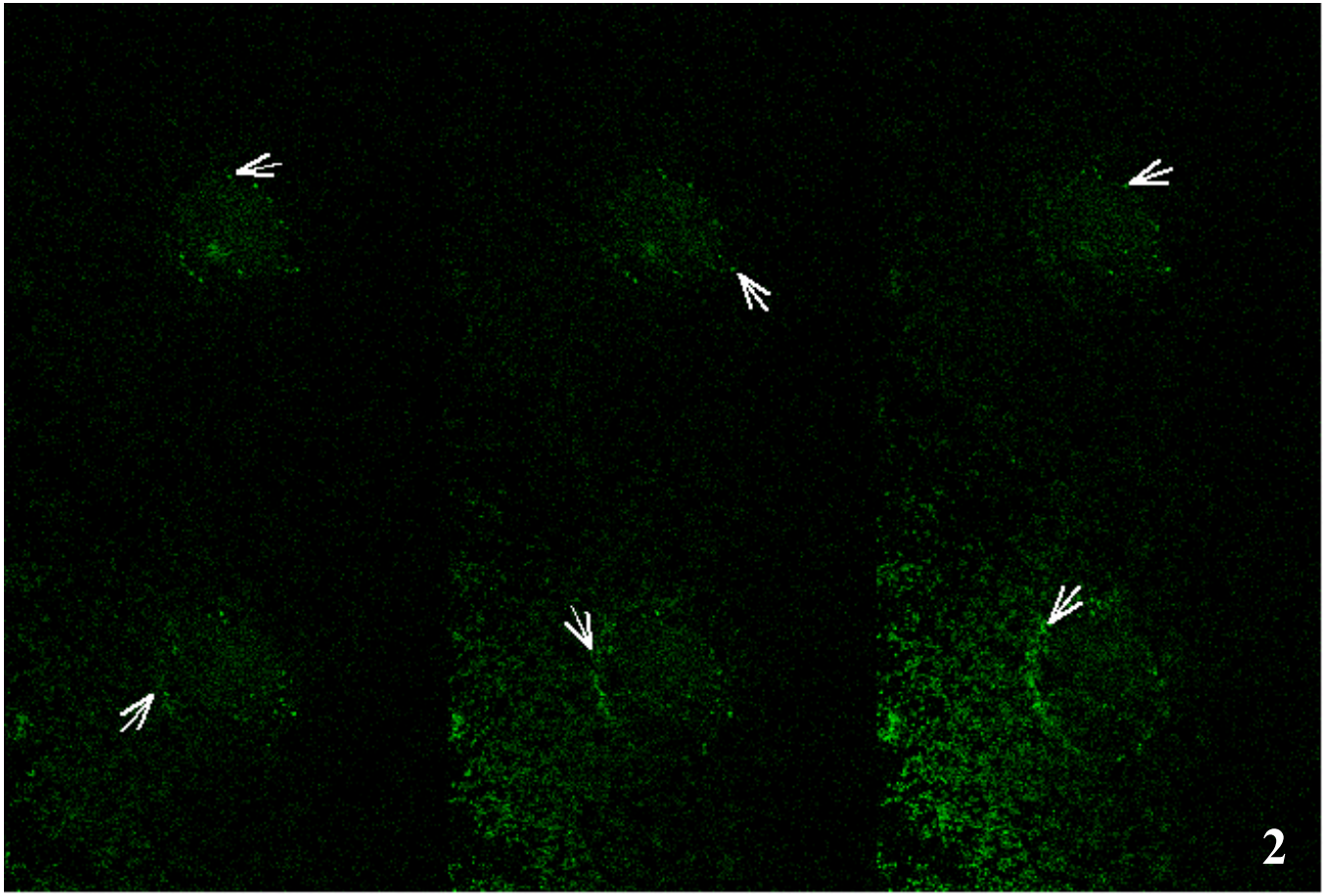

Fig. 2: CLSM optical sections of *Alexandrium tamarense*, 0,7 µm thick; 568 nm excitation FISH with EUB 338R, FITC-labelled. Arrows show hybridized bacteria attached to the cell surface of the dinoflagellate. X40 enlargement.

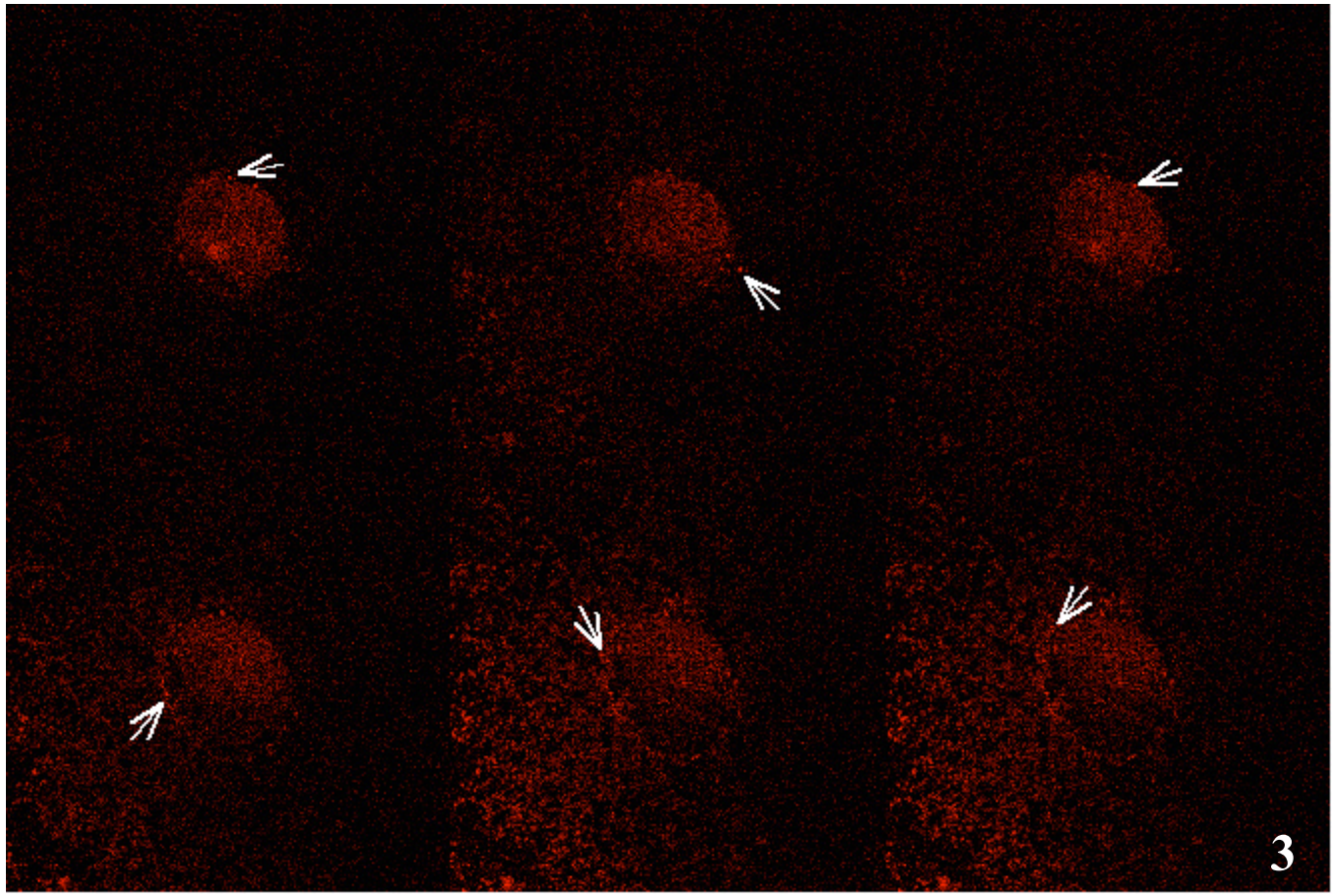

Fig. 3: CLSM optical sections of *Alexandrium tamarense*, 0,7 µm thick; 568 nm excitation FISH with *Roseobacter*-clade/536R, CY3 labelled. Arrows show hybridized bacteria attached to the cell surface of the dinoflagellate. X 40 enlargement.

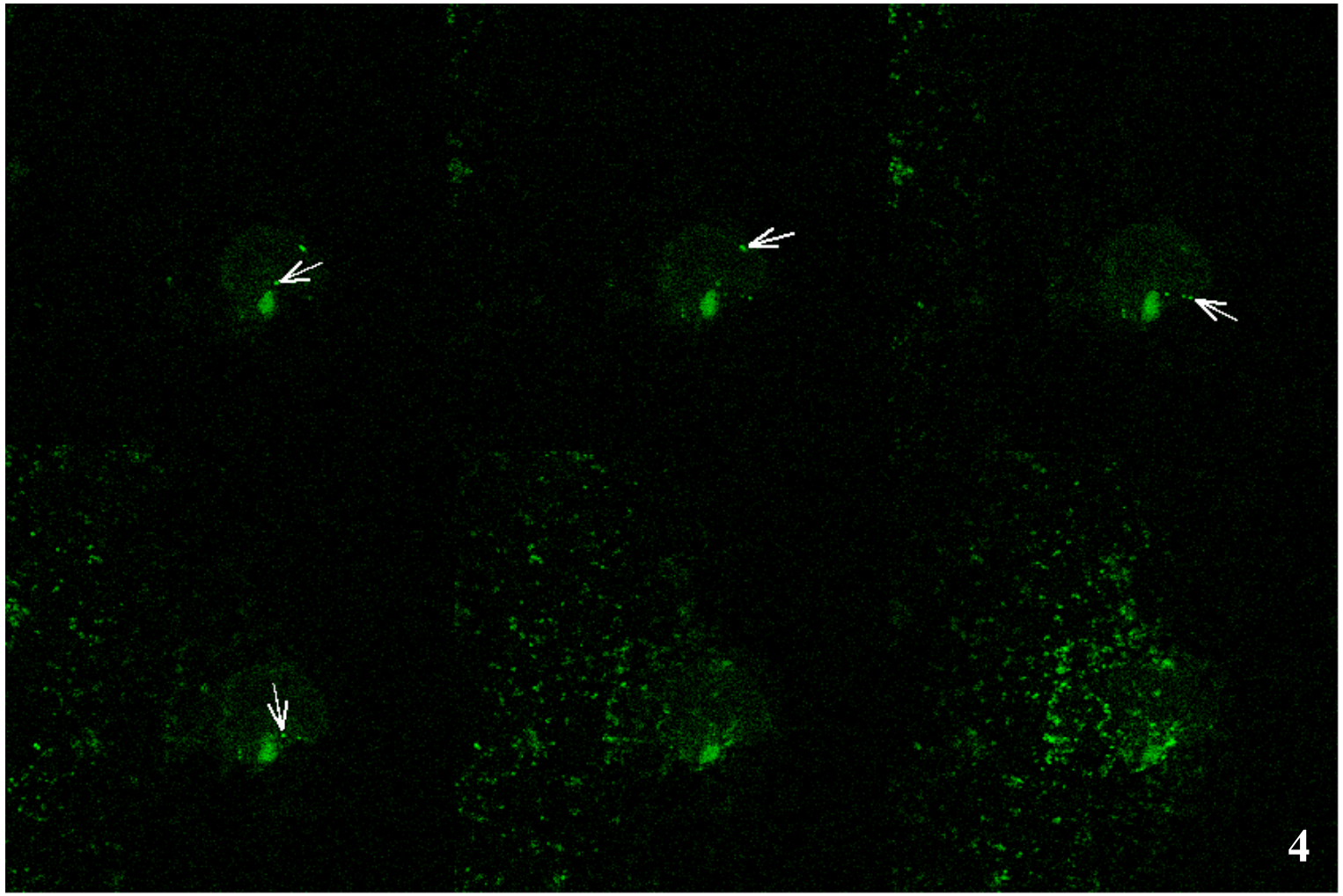

Fig. 4: CLSM Optical sections of *Alexandrium tamarense,* 0,7 µm thick, 450-490 nm excitation. FISH with ALF1b, HRP-labelled. Arrows show intracellular bacteria of the dinoflagellate. X 40 enlargement.

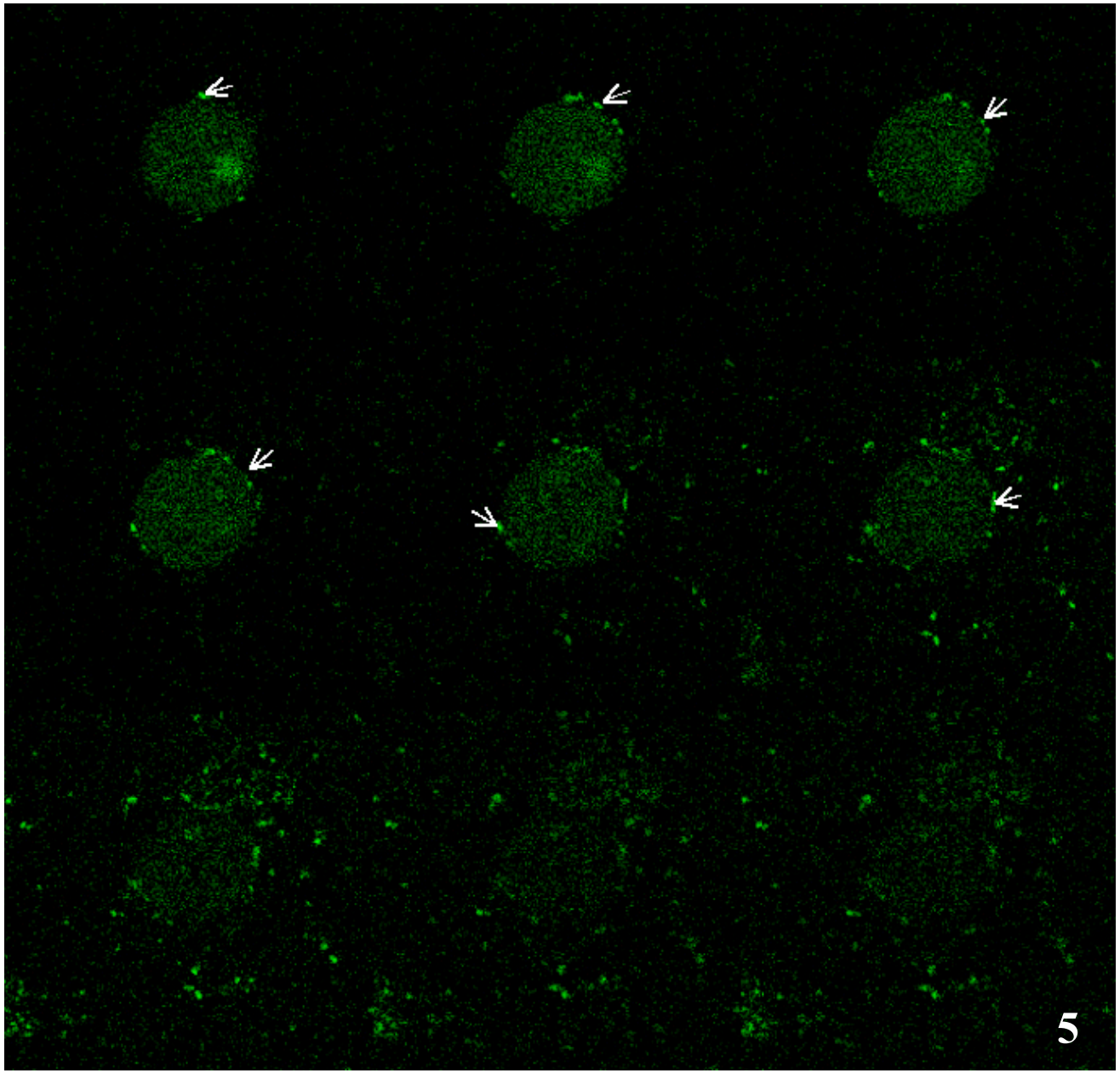

Fig. 5: CLSM optical sections of *Alexandrium lusitanicum,* 0,7 µm thick, 568 nm excitation. FISH with EUB 338R, FITC-labelled. Arrows show hybridized bacteria attached to the cell surface of the dinoflagellate. X 40 enlargement

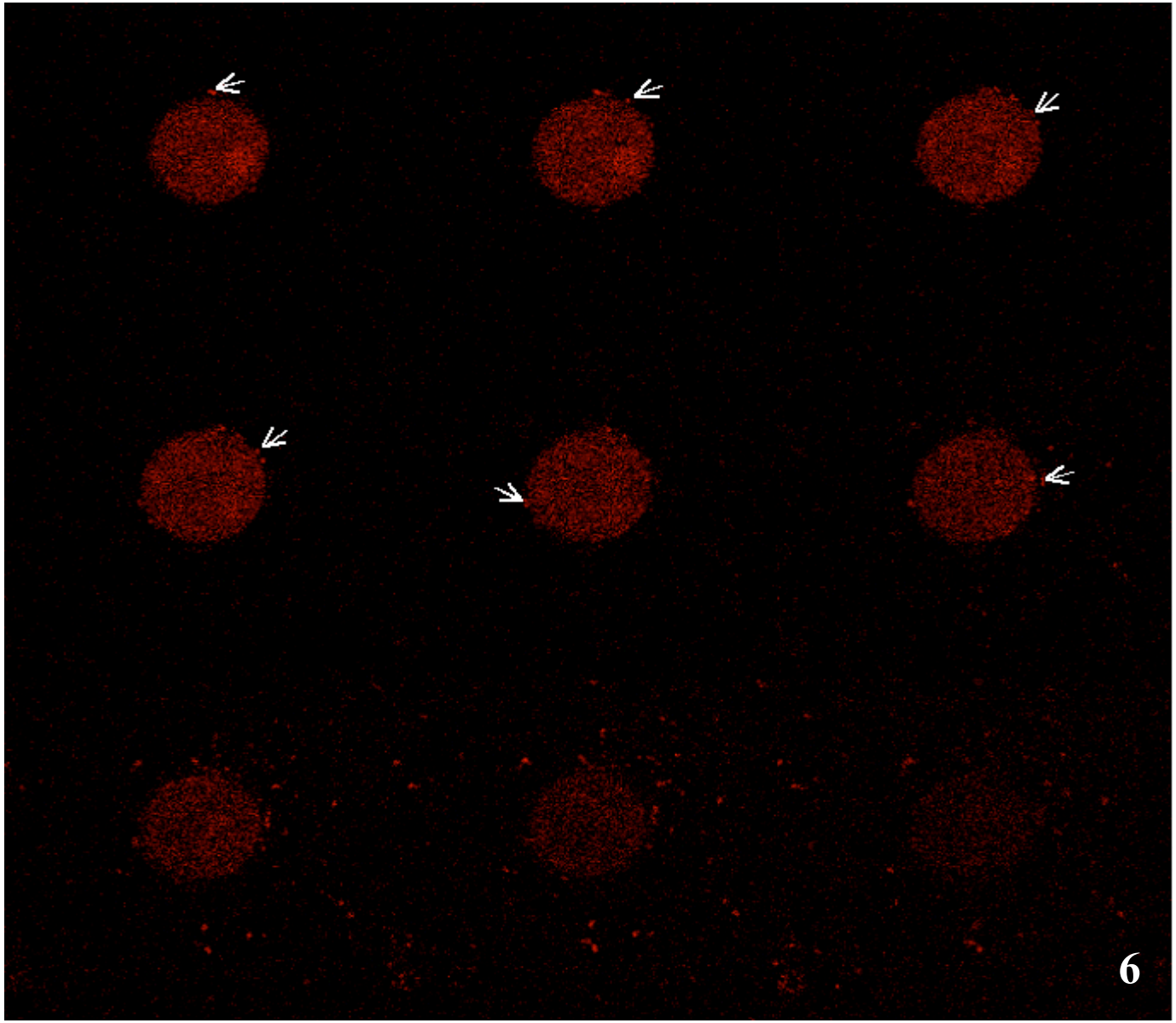

Fig. 6: CLSM optical sections of *Alexandrium lusitanicum,* 0,7 µm thick, 450-490 nm excitation. FISH with Roseobacter-clade/536R, CY3-labelled. Arrows show hybridized bacteria attached to the cell surface of the dinoflagellate. X 40 enlargement.

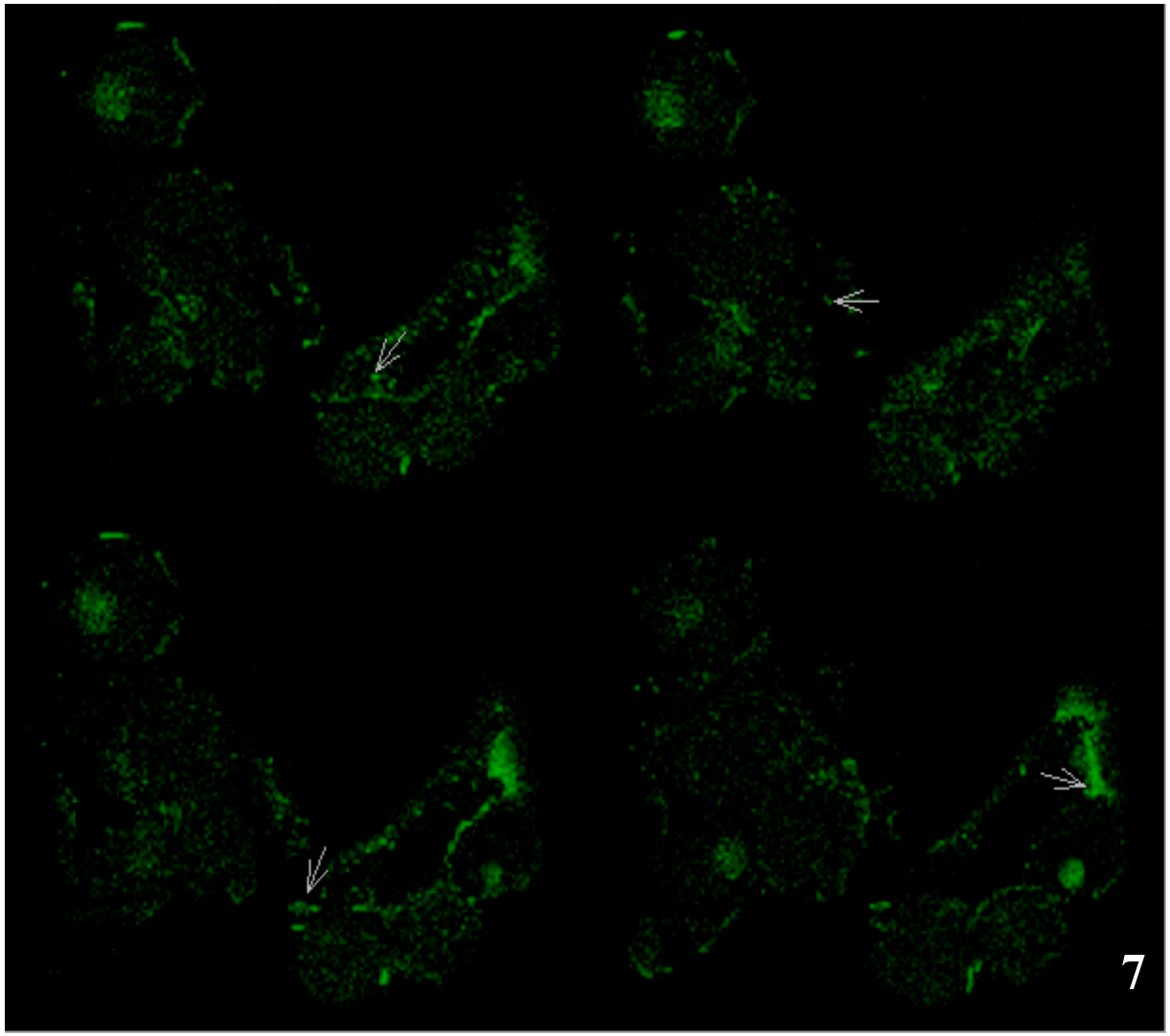

Fig. 7: CLSM optical sections of *Alexandrium andersonii,* 0,7 µm thick, 450-490 nm excitation. FISH with EUB 338R, FITC-labelled. Arrows show hybridized mucilage producing bacteria attached to the cell surface of the dinoflagellate. X 40 enlargement.

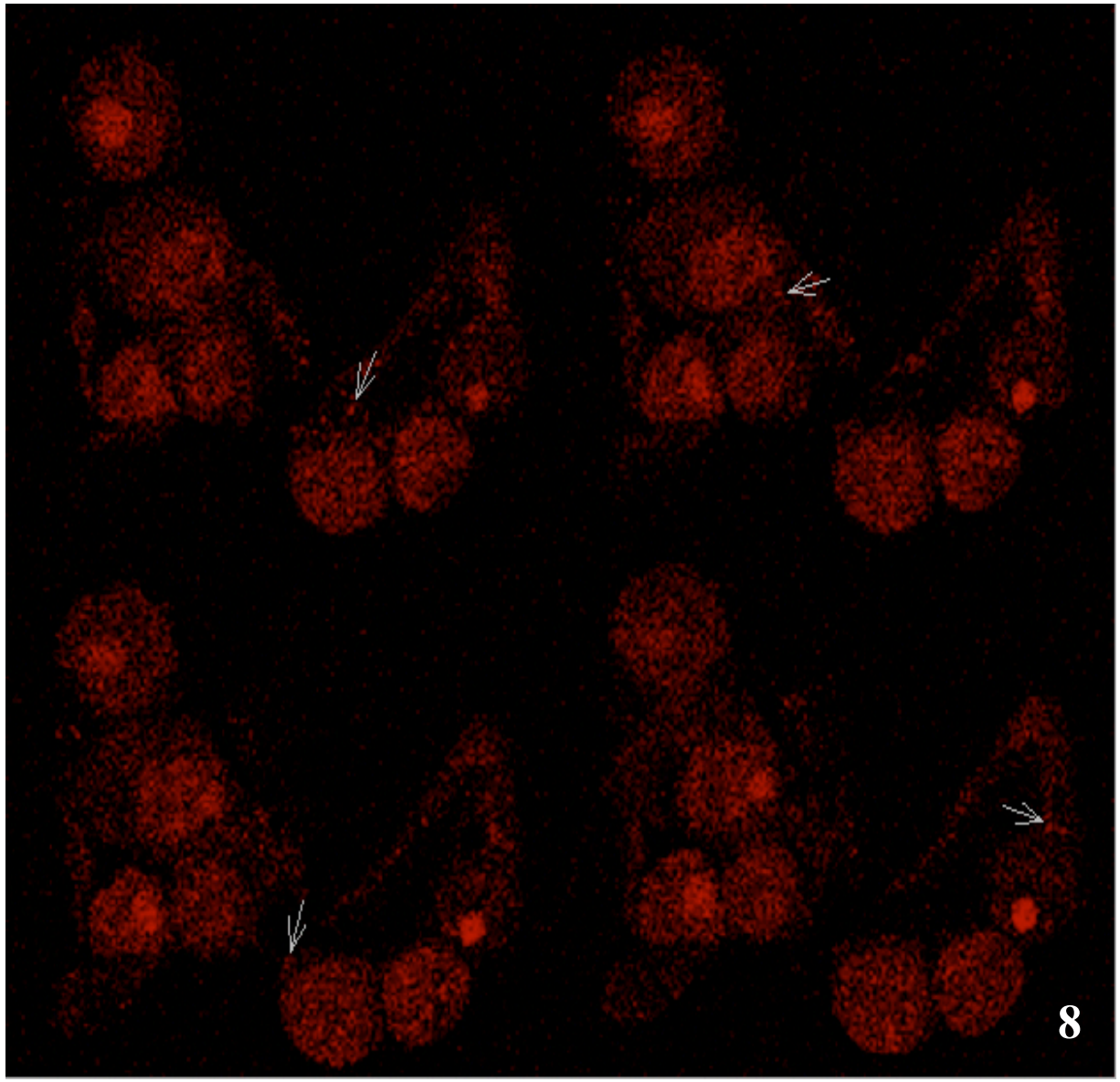

Fig. 8: CLSM optical sections of *A. andersonii,* 0,7 µm thick, 450-490 nm excitation. FISH with *Alteromonas*-clade137R, CY3-labelled. Arrows show hybridized mucilage producing bacteria attached to the cell surface of the dinoflagellate. X 40 enlargement.

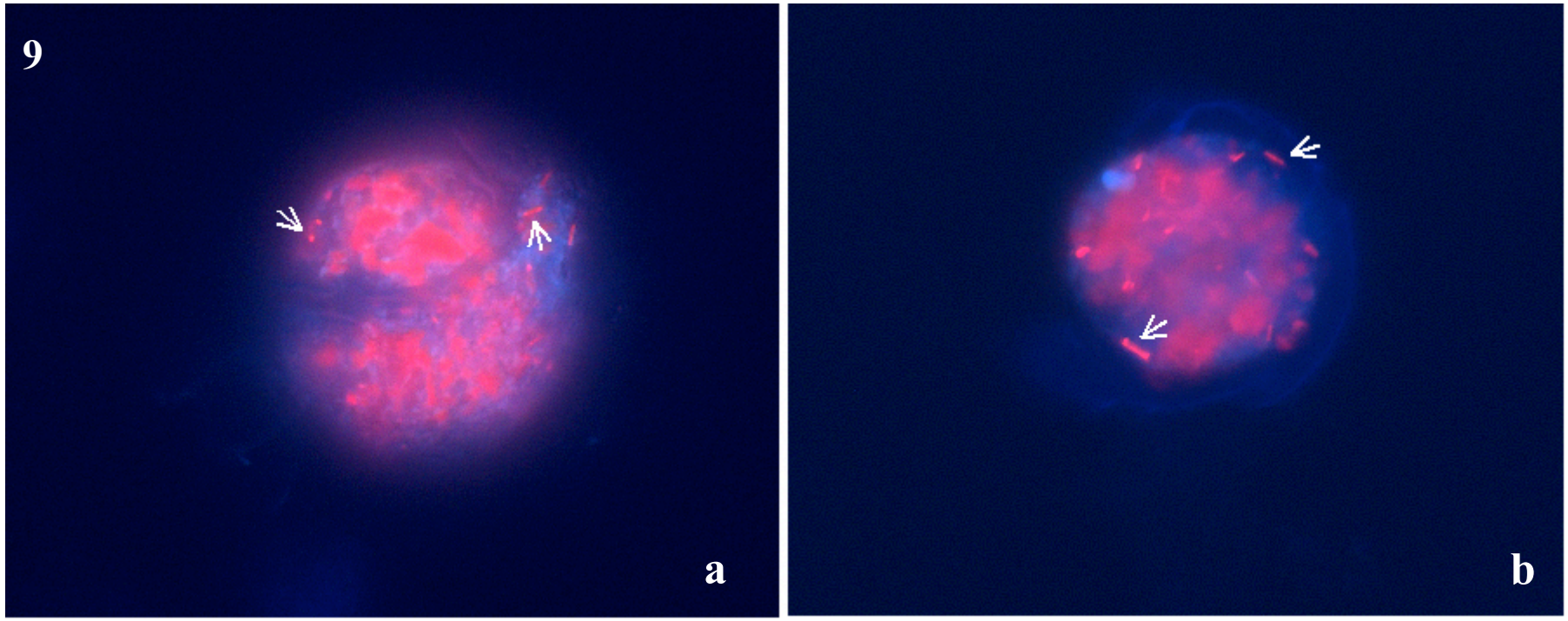

Fig. 9: Fluorescence microscopy of *Alexandrium tamarense* and *Alexandrium lusitanicum.* Excitation: UV G 365 nm. a) *A tamarense.* b) *A. lusitanicum*. The dinoflagellate cells were fixed without killing intracellular bacteria and visualized through treatment with CTC. Arrows show intracellular bacterial like structures which exhibit a pink fluorescence. The dinoflagellates exhibit also a pink fluorescence, because of a residual respiratory activity of the cells. X 100 enlargement.

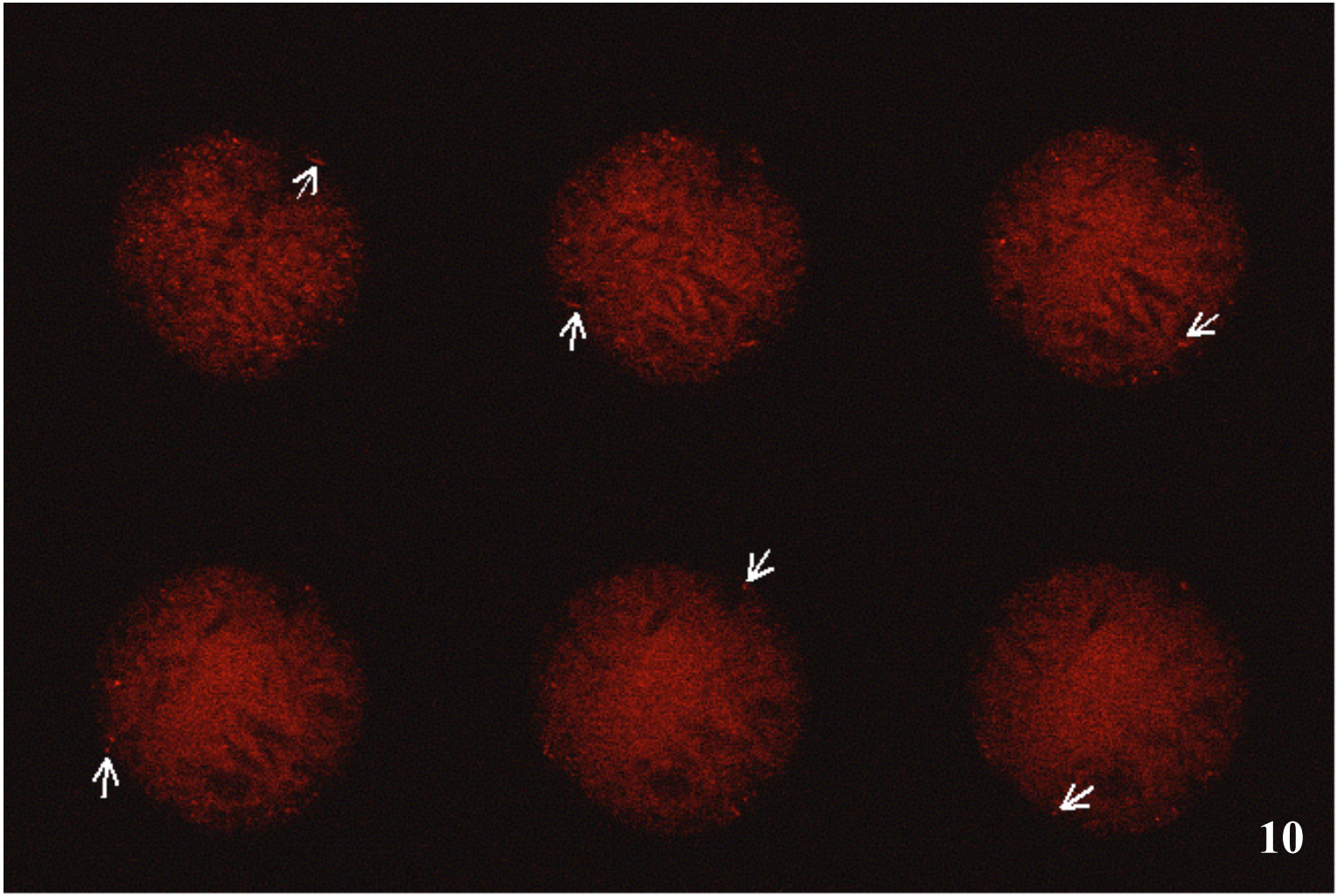

Fig. 10: *Alexandrium tamarense* CLSM optical sections; 0,7 µm thick, 568 nm excitation. The dinoflagellates were fixed without killing intracellular bacteria and visualized through treatment with CTC. Arrows show intracellular bacterial like structures. X 100 enlargement.

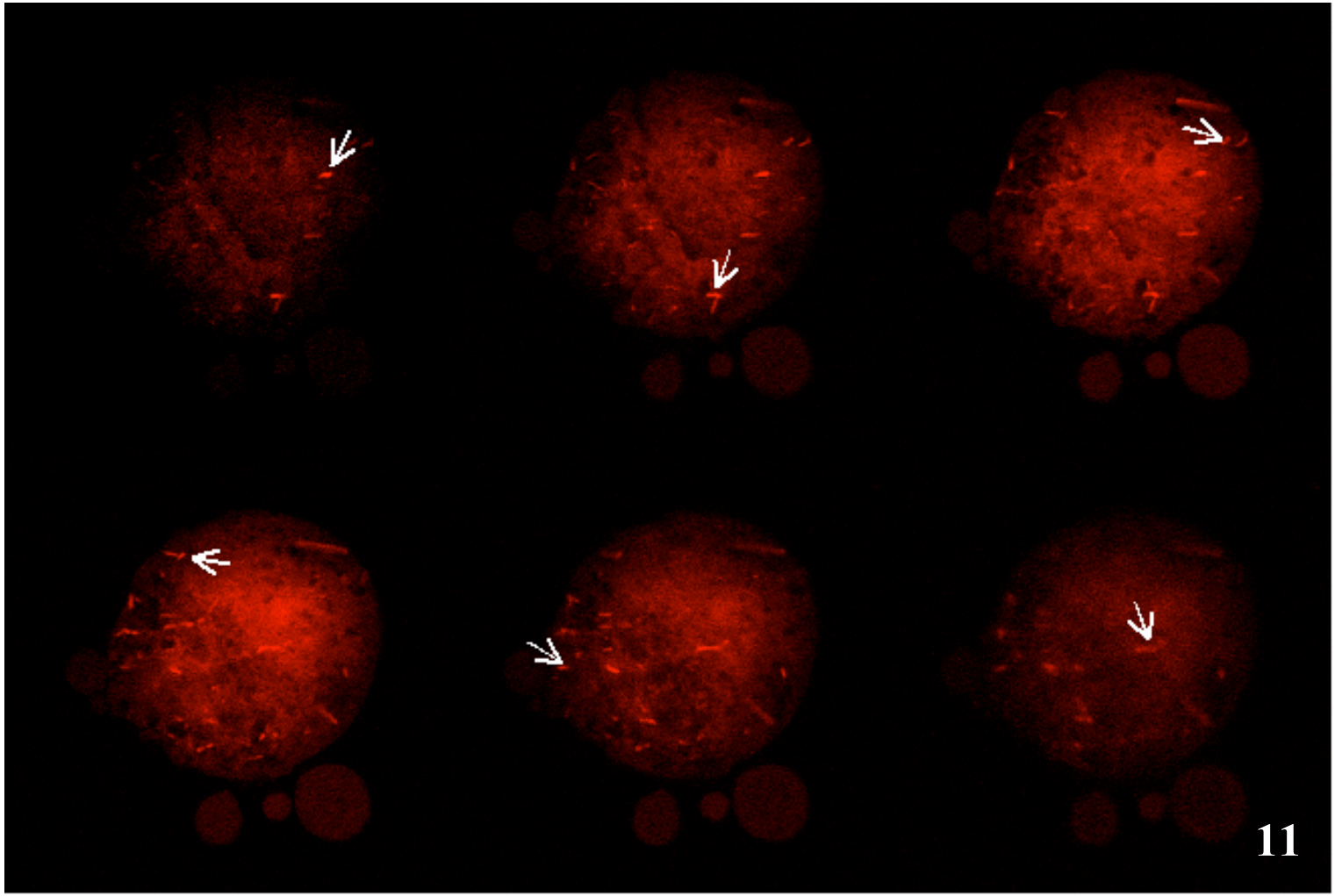

Fig. 11: CLSM optical sections of *Alexandrium lusitanicum*; 0,7 µm thick, 568 nm excitation. The dinoflagellates were fixed without killing intracellular bacteria and visualized through treatment with CTC. Arrows show intracellular bacterial like structures. X 100 enlargement.

2.4 Publication II

SEASONAL OCCURRENCE AT A SCOTTISH PSP MONITORING SITE OF PURPORTEDLY TOXIC BACTERIA ORIGINALLY ISOLATED FROM THE TOXIC DINOFLAGELLATE GENUS *ALEXANDRIUM*

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ABSTRACT

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 toxic 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 α-proteobacterial group of *Roseobacter* and the γ-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 and 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 □-Proteobacteria rather than to *Moraxella*, a □-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 and Soyer-Gobillard 1990). Most recently, Lewis et al. (2000) and Córdova et al. (2002) 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 and Trick 1995; Gallacher and Birkbeck 1995; Levasseur et al. 1996; Gallacher et al. 1997; Gallacher and Smith 1999 and references therein; Córdova et al. 2002).

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 and 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 and 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 and 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. (2002), 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 biotransform PSTs (E. A. Smith, personal communication), although the affect 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 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 α-Proteobacteria (primarily *Roseobacter* spp.), β-Proteobacteria (mainly *Alteromonas* spp.) and to the cyto-subclasses of the Cytophaga among the Eubacteria (Hold et al. 2001a). 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). Visualising individual bacterial cells through *in situ* hybridization of fuorescently labelled, taxon specific ribosomal RNA (rRNA) probes is one way of furthering these investigations. Ribosomal RNA probes are 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. 2001b), including several putatively toxic 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 (Throndsen 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 Flow and The String, whereas four other sites were randomly sampled throughout the 1999 monitoring season (see Fig. 2, Table 2). The phytoplankton-monitoring program of the FRS Marine Laboratory in Aberdeen, UK, analysed these samples in 1999. A modification of normal monitoring procedure was implemented in attempts to maximise 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 program.

Fluorescence 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 µm 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 X phosphate-buffered saline, PBS) and filter sterilized (0.2 µm), then incubated overnight at 4 °C directly in the filter chamber. The samples were rinsed with 10 ml 1 X 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□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. The oligonucleotide probe 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 µl hybridization buffer (20 mM Tris-HCl pH 8.0; 0.9 M NaCl; 0.01% SDS, *x*% formamide) + 50 ng μl^{-1} of each oligonucleotide probe + 50 ng μl^{-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 µl 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 µg 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 with the appropriate filter set (Zeiss filter sets 02, 09 and 14). The entire filter quarter was scanned under 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 X 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 (x 100) 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:

Bacterial numbers ml $^{-1}$= Bacteria per microscope field (average) x (effective filter surface/microscope field surface) / 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 4*a*vs3 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. 2001 b, 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 program (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 X 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*- and *Roseobacter*-clade bacteria and several of the taxon-specific bacteria in these clades were present in the preserved water samples.

With water samples from the two main sites (Scapa Flow and The String), the total bacterial counts as defined using DAPI staining remained relatively stable (average cell count ± SD over the entire sampling period): The String, 2.20 x 10^5 cells ml^{-1} 237 x 10; Scapa Flow, 1.43 x 10^5 and cells ml^{-1} ± 231.5. One example of the stability of cell counts at one site over time is from Scapa Flow, where the average count ± SD over two separate counts taken several months apart was 6.95 x 10^4 ± 500. In contrast the cell counts for the *Alteromonas*-clade using clade-specific probes ranged over the entire sampling period from 0 to 66 x10^3 cells ml^{-1} at The String and from 0 to 63 x 10^3 cells ml^{-1} at Scapa Flow, representing 30-44% of the total bacteria at The String and Scapa Flow. Similarly, for *Roseobacter*-clade bacteria as determined by clade-specific probes, counts ranged from 0 to 48 x 10^3 cells ml^{-1} at The String and from 0 to 54x 10^3 cells ml^{-1} at Scapa Flow 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 The String occurred about 2 weeks after peak densities at Scapa Flow (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 The String where two peaks in *Alteromonas*-clade bacteria

occurred at Scapa Flow: 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 The 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 Flow and The String were randomly sampled during the phytoplankton and shellfish monitoring program (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 program (Figs. 5-8). At Scapa Flow, *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 The 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 Flow, 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 (Fig. 3, Table 4). 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 nonsignificantly correlated (Fig. 6, Table 4).

At The 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 The 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 The 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 4*a*vs3 was negatively correlated with *Alexandrium*

spp. at Scapa Flow. However, the number of times at The String when this strain was detected was too low (only two positive dates) to permit statistical evaluation of the data.

Mussels (*Mytilus edulis*) from the two main water sampling sites were analysed for PSTs. At Scapa Flow, 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 µg 100 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 (4.3x 10^4 cells ml^{-1} and 4.6 x 10^4 cells ml^{-1}), respectively. Three purportedly toxic bacteria, viz., 407-2, 4*a*vs3 and 253-19, also reached their highest numbers on 8 June 1999 at Scapa Flow (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 The 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 Flow, 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 4*a*vs3 was negatively correlated with *Alexandrium* counts but positively correlated with mussel toxicity, both non-significantly.

One month later, on 5 July 1999 (plotted two days later on day 188 in Fig. 8), the highest amount of PSP, 89 µg 100 g^{-1} mussel tissue, was detected at The 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 The String. On 7 July 1999 (day 188), the *Alteromonas*-clade bacterial counts were 5.6 x 10^3 ml^{-1} and the *Roseobacter* bacteria were 1.1 x 10^4 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.

DISCUSSION

Hold et al. (2001b) have shown that bacteria associated with laboratory cultures of PST-producing dinoflagellates belong to the α- and γ-Proteobacteria and the Cytophaga-Flavobacter-Bacteroides. From the Proteobacteria, bacteria of the *Roseobacter*- and *Alteromonas*-clades dominated. This study demonstrates that rRNA probes designed for these bacteria (Brinkmeyer et al. 2000) were successfully used in their detection from Lugol's-fixed water samples obtained from the Orkney Isles, particularly The String and Scapa Flow, during the Scottish phytoplankton monitoring program 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 (TSA) 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*; Elbrächter, personal communication) and, although they do co-occur, only one species of the three will dominate at any one time (Gerdts, 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 (Gerdts, 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 (Macdonald, personal communication). Nevertheless, *Alexandrium* spp. and shellfish contaminated with PSTs (Howard, personal communication) occur yearly in this area, and are why this site was chosen for this study.

Our work utilised data and samples from a monitoring program 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. 2001b).

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 numbers 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 The String there was also a weak correlation between *Alexandrium* and the *Alteromonas*-clade counts but not the *Roseobacter*-clade. Interestingly, at The 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 and Moran 1997; Acinas et al. 1999), 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 gramnegative bacteria including species from the genera *Pseudomonas*, *Aeromonas*, *Flavobacterium*, *Pasteurella*, *Proteum* and *Moraxella*-like, some of which were shown to produce PSTs (Córdova et al. 2002). 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 and Pierce 1989; Romalde et al. 1990). Some bacterial isolates from the bloom and from algal cultures established from the bloom exhibited cytotoxicity (Buck and Pierce, 1989; Romalde et al. 1990). Evans (1973, cited in Buck and Pierce 1989) postulated a red tide cycle involving (1) initial bacterial growth simulated 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 and 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. Cordóva et al. (2002) 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 program coupled with the fact that the occurrences of PSTs were lower in 1999 than in previous years (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, colourimetric detection as well as conventional FISH detection for detecting *Alteromonas* spp. and *Roseobacter* spp. in paraffin-embedded mussel hepatopancreas (Töbe et al., in prep.) 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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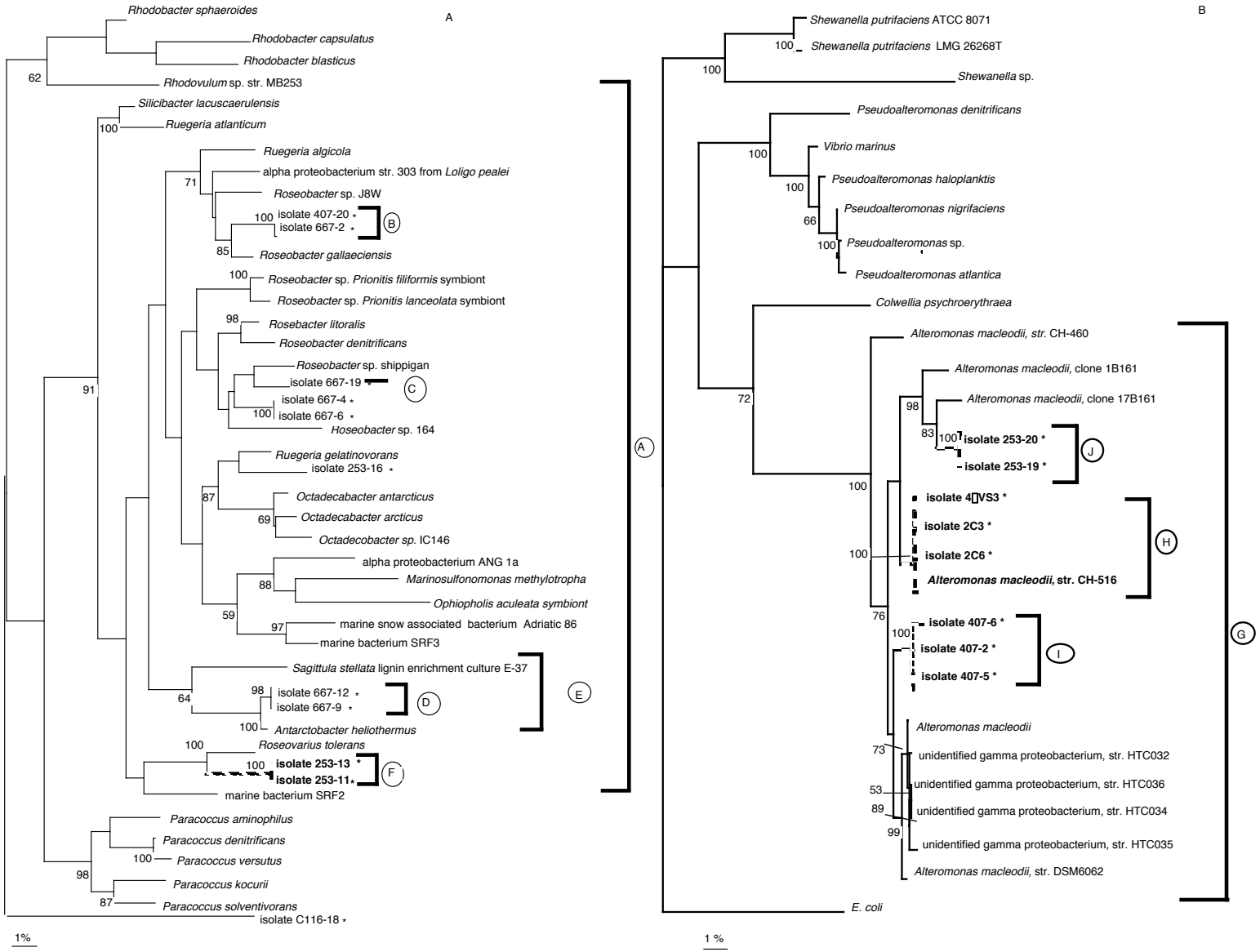


Fig. 1. Phylogenetic reconstructions using the 16S rRNA gene placing the bacteria isolated from various toxic dinoflagellates near their closest neighbours. (*A*) *Roseobacter*; (*B*) *Alteromonas*. The regions/species recognised by the rRNA probes are highlighted in bold on the trees (redrawn from Brinkmeyer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

Table 1. Sequences of the oligonucleotide probes used in this study, with protocol details for detection. Use the letters beside each probe in Fig. 1 to compare the probes listed above with the taxa recognised by each probe. See Brinkmeyer et al. (2000) for details of probes shown in Fig. 1 and not listed here.

Probe	**Sequence**	**Compensating % formamide**	**NaCl (M) in wash buffer**
EUB 338R (Amman et al. 1990)	5'-ACT CCT ACG GGA GGC AGC-3'	20	0.23
A. *Roseobacter*-clade/536R	5'-CAA CGC TAA CCC CCT CCG-3'	18	0.27
Competitor for *Roseobacter*-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. *Alteromonas*-clade/137R	5'-TGT TAT CCC CCT CGC AAA-3'	10	0.45
H. 4□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

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 Flow	Gairsay	String	Cava	Clestrain	Yinstay
24. Feb	55		X				
08. 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	
06. Apr.	96	X, A, M		X, A, M			
16. Apr.	106	X, A		X, A			
19. Apr.	109	M		M			
03. May	123	X, A		X,A			
14. May	134	X, A		X, A			
16. May	136	M		M			
27. May	147	A		X, A			
01. June	152	M		M			
02. June	153	X, A		X, A			
08. June	159	X, A, M		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			
05.July	186	M		M			
07.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					
07.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 and 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	**Date**	**Total counts (cells ml$^{-1)}$**	**Species-specific probes (cells ml^{-1})**		
Alteromonas-clade bacteria			4 □vs3	407-2	253-19
Cava	22. Mar.	2200	0	0	0
	23. June	13300	3	13	2
Clestrain	23. June	2600	1	10	0
Gairsay	30. July	5700	0	0	0
Yinstay	30. July	1200	0	0	0
Roseobacter-clade bacteria			667-9/667-12	667-19	407-
			20/667-2		
Cava	22. Mar.	2300	0	0	0
	23. June	13900	39	4	0
Clestrain	23. June	9200	0	0	0
Gairsay	30. July	11100	0	0	0
Yinstay	30. July	1600	0	0	0

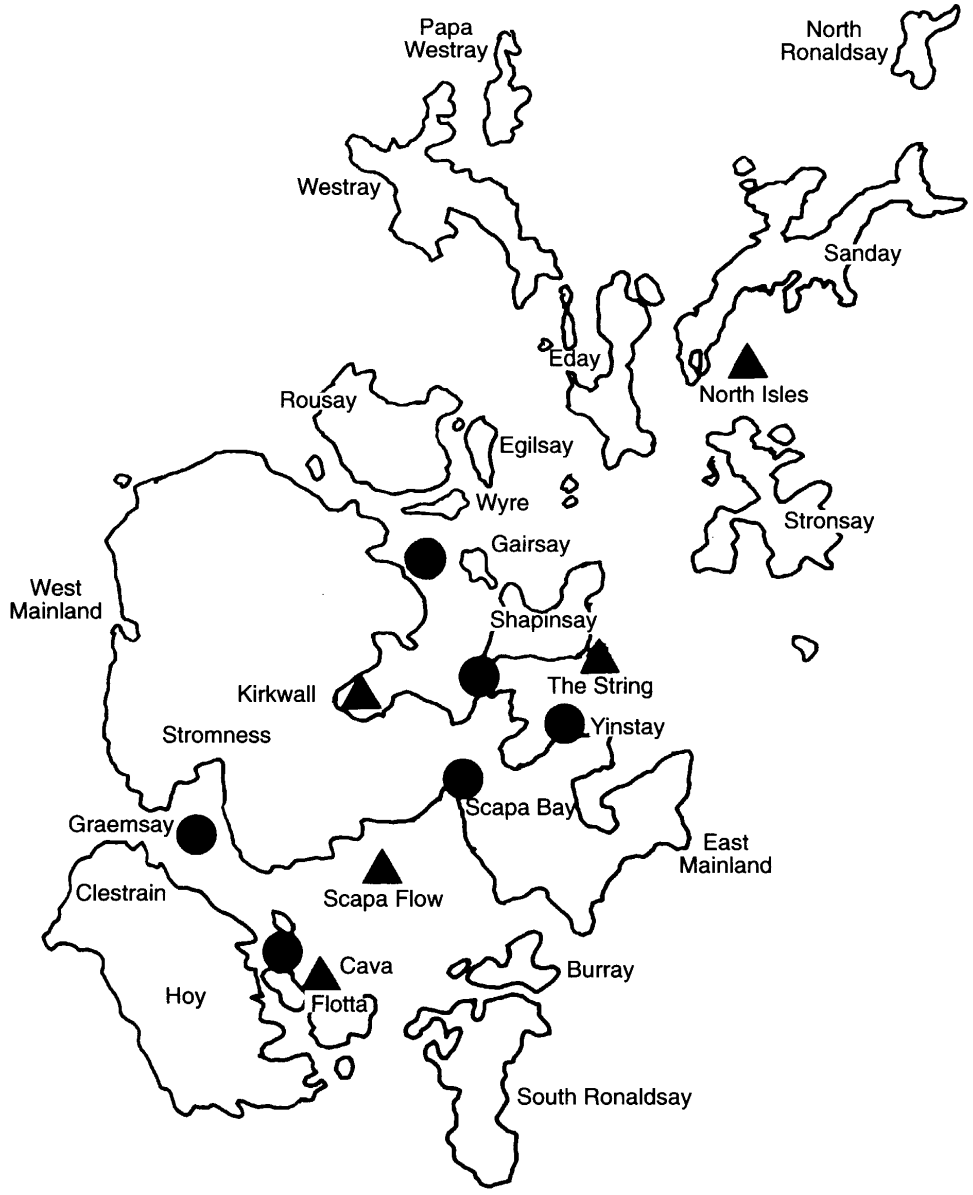
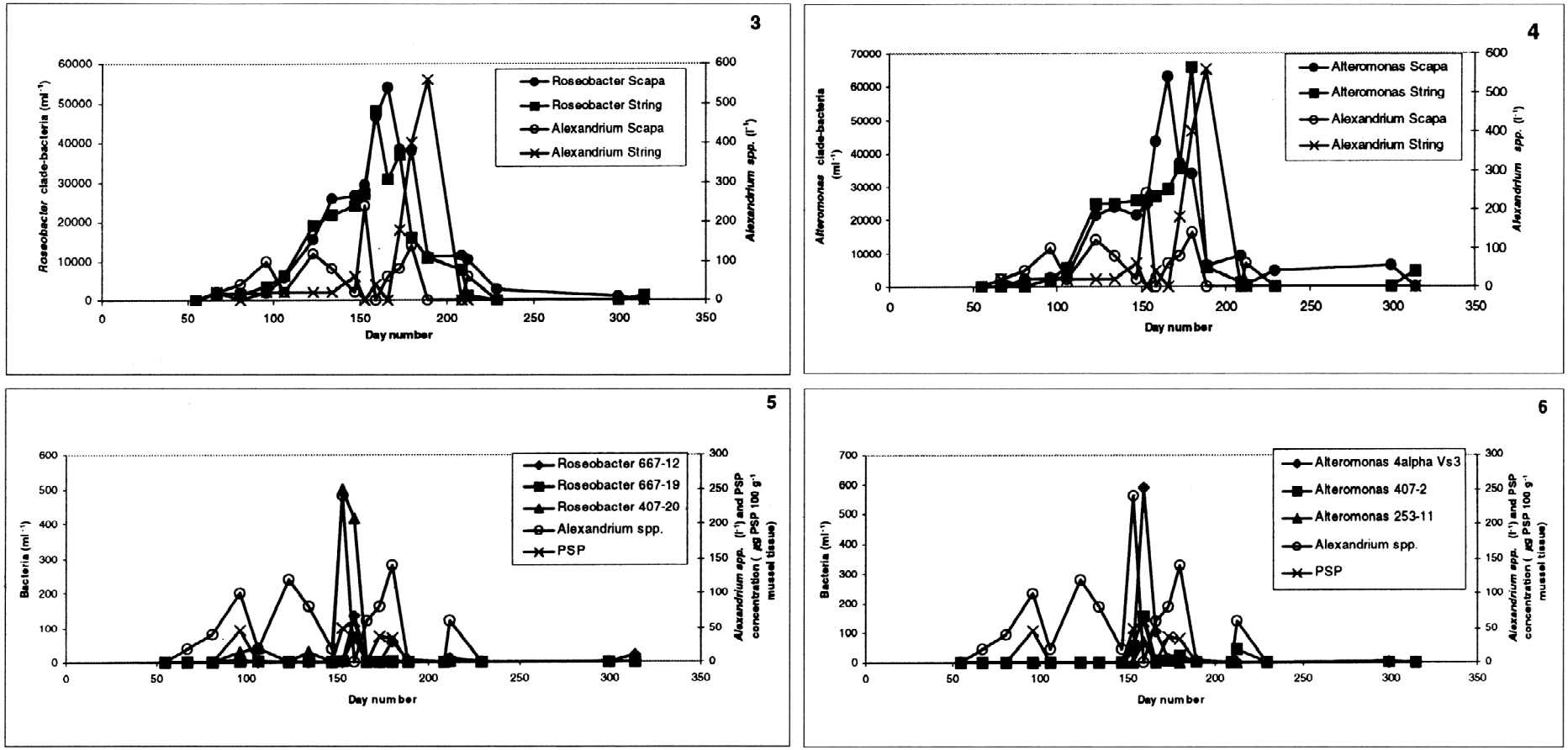
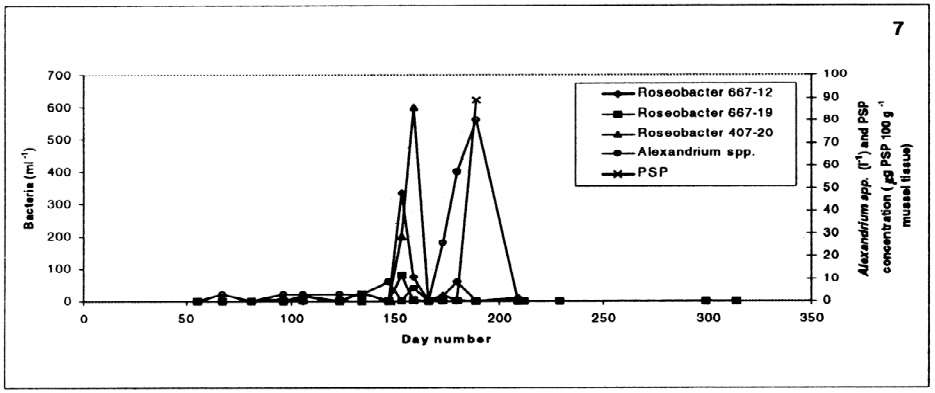
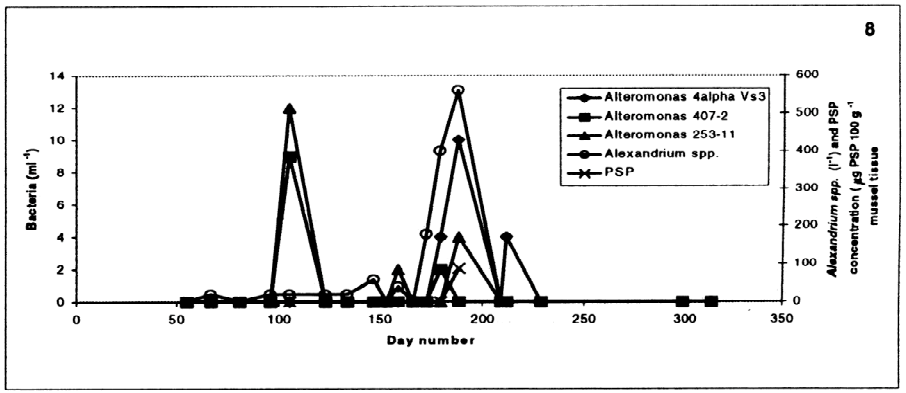


Fig. 2. Detail of the Orkney Islands showing the sites where water samples and mussels were taken and processed as described in the text.

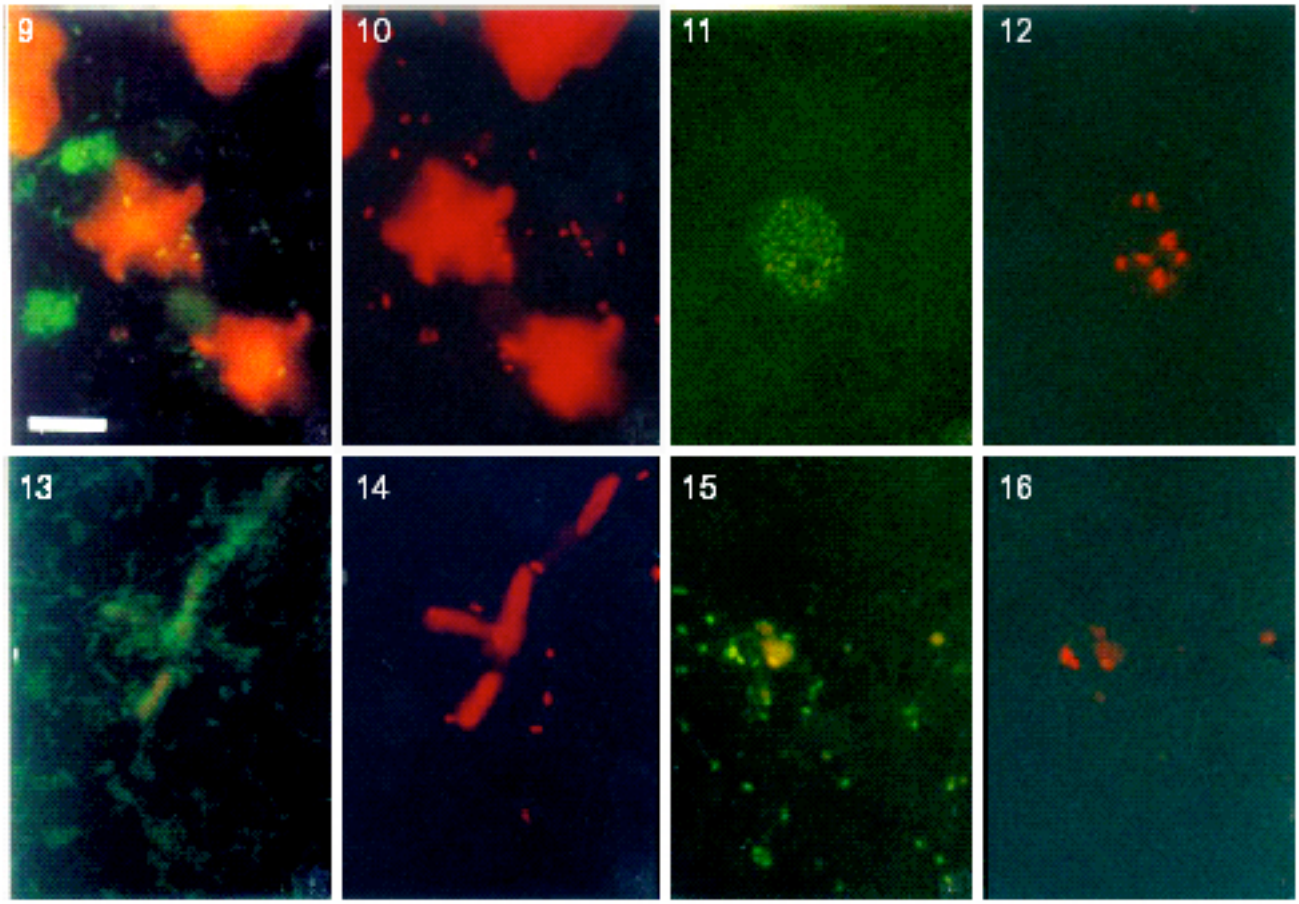


Figs. 3-6. Distribution of putatively toxic bacteria in water samples collected at different sites at the Orkney Islands in 1999 as determined by probe hybridization and compared with total *Alexandrium* counts and mussel toxicity. Fig. 3. Clade specific counts of *Roseobacter*-clade bacteria at both 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 and 667-12, which belong to the *Roseobacter*-clade compared with total counts of *Alexandrium* spp. and with PSP concentration in mussel at Scapa. Fig. 6. total counts of bacterial strains 4□vs3, 407-2 and 253-19, which belong to the *Alteromonas*-clade, 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. 667-19 and 667-12, which belong to the *Roseobacter*-clade, and compared with total counts of *Alexandrium* spp. at and PSP concentrations in mussel tissue at The String. Fig. 8. Total counts of bacterial strains 4□vs3, 407-2 and 253-19, which belong to the *Alteromonas*-clade, compared with total counts of *Alexandrium* spp. and with PSP concentrations in mussel tissue at The String.



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 at Scapa hybridised with the universal eubacterial probe 338R labelled with FITC. Fig. 10. Same cells hybridised with *Roseobacter* clade probe 536R labelled with CY3.

Fig. 11. Water samples from String hybridised with the *Roseobacter*-clade probe 536R labelled with FITC. Fig. 12. Same cells hybridised with the probe 667-12/994R labelled with CY3. Fig. 13.Water samples from String hybridised with the universal eubacterial probe EUB 338R labelled with FITC. Fig. 14. Same cells hybridised with the *Alteromonas*-clade probe 137R labelled with CY3. Fig. 15. Water samples from Cava hybridised with the *Alteromonas*-clade/137R probe labelled with FITC. Fig. 16. Same cells hybridised with the probe 407-2/209R labelled with CY3.

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

	String			***Scapa***		
Correlation tested	R	Probability	No.of observations	R	Probability	No. of observation
Total *Rosebacter* vs. *Alexandrium*	0.354	0.15	18	0.479	0.783	19
Total *Alteromonas* vs. *Alexandrium*	0.477.	0.045*	18	0.458	0.049*	19
Pooled *Roseobacter* taxon-specific counts vs. *Alexandrium*	0.017	0.949	17	0.209	0.391	19
Pooled *Alteromonas* taxon-specific counts vs. *Alexandrium*	0.585	0.017	16	-0.033	0.893	19
Total *Roseobacter* vs. mussel toxicity	n.a.	n.a.	5	0.155	0.770	6
Total *Alteromonas* vs. mussel toxicity	n.a.	n.a.	5	0.174	0.742	6
Pooled *Roseobacter* taxon-specific counts vs. mussel toxicity	n.a.	n.a.	5	0.641	0.170	6
Pooled *Alteromonas* taxon-specific counts vs. mussel toxicity	n.a.	n.a.	5	0.358	0.486	6
4□vs3 vs. *Alexandrium*	n.a.	n.a.	5	-0.068	0.783	19
4□vs3 vs. mussel toxicity	n.a.	n.a.	5	0.291	0.576	6

n.a., not applicable, too few data

*$p < 0.05$

2.5 Appendix to Publication II

MONITORING OF PURPORTEDLY TOXIC BACTERIA AT TWO PSP MONITORING SITES OF THE ORKNEY ISLANDS IN 2000

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INTRODUCTION

The monitoring of the purportedly toxic bacteria at the Orkney Islands was repeated from March to August in 2000, to compare the results of two sequential years. In the second monitoring year field samples were collected from two main sampling sites, Scapa Flow and The String, by the Scottish sampling monitoring program (Table 1). Oligonucleotide probes used in the second monitoring year:

Alteromonas-clade: *Alteromonas*-clade/137R, 4 vs3/210R (bacteria previously isolated from *A. tamarense* UW2C), 407-2/209R (bacteria previously isolated from *A. tamarense* NEPCC 407) and 253-19/175R (bacteria previously isolated from *A. lusitanicum* NEPCC 253). *Roseobacter*-clade: *Roseobacter*-clade/536R, 66-12/191R (bacteria previously isolated from *A. affine* NEPCC 667), 407-20/1446R (bacteria previously isolated from *A. tamarense* NEPCC 407) and 253-11/1318R (bacteria previously isolated from *A. lusitanicum* NEPCC 253).

All oligonucleotides have to have reviewed used in the 1999 survey for their specifity in GenBank databases at regular time periods. The probe 667-19/1241R is no longer species-specific; hence another species level probe (253-11/1318R; Brinkmeyer et al. 2000) of the *Roseobacter*-clade was used in the second monitoring year. The processing of the environmental samples in the second monitoring year and the FISH experiments were carried out as described in Material and Methods of Publication II.

RESULTS

Total Counts. In the year 2000, counts of all DAPI stained bacteria at Scapa Flow remained relatively constant from the end of March until the beginning of June, when they began to increase (Fig. 1a). A maximum was obtained at mid June 2000 (6.31 x 10^5 bacteria ml^{-1}, Fig. 1a), after which time the bacterial numbers decreased. At this time point 3.95 x 10^4 bacteria ml^{-1} of the *Roseobacter*-clade (6.3% of the total bacterial population) were detected in the water column. However, no *Alteromonas*-clade bacteria were detected at this time point. At The String, total bacterial numbers followed a similar pattern with the highest peak in the end of May (9.03 x 10^5 bacteria ml^{-1}, Fig. 1b). At the time of highest total bacterial abundances the percentage of *Alteromonas* and *Roseobacter* bacteria were relatively low. During the times of maximum total bacterial abundances, the second highest peak of *Roseobacter*-clade bacteria (5.47 x 10^4 bacteria ml^{-1}; 6.1% of the total bacterial population) and the third highest peak of *Alteromonas*-clade bacteria (1.81 x 10^4 bacteria ml^{-1}; 2% of the total bacterial population) were observed. However, bacteria reacting to the probes of *Roseobacter* and *Alteromonas* consisted of up to 57% of the total bacterial abundances at The String at the end of May and up to 34% at Scapa Flow at the beginning of June.

Fluorescence in situ *hybridization*. Fluorescently labelled probes were successfully applied to Lugol's fixed field samples in the second monitoring year (Fig. 3). In the year 2000, the *Roseobacter*-clade bacteria reached the highest abundance at Scapa Flow in the beginning of June (1.05 x 10^5 bacteria ml^{-1}), 10 days earlier than in 1999. It was almost the double of the value of the year 1999 (Table 2, Fig. 2a). The highest numbers of *Roseobacter*-clade bacteria at The String in 2000 fluctuated, with each peak increases over the year, with the highest peak in mid July (5.91 x 10^4 bacteria ml^{-1}). The distribution of the *Roseobacter*-clade bacteria at The String in 1999 showed the highest peak at the beginning of June (4.8 x 10^4 bacteria ml^{-1}). In 2000, it was the second highest peak (5.47 x 10^4 bacteria ml^{-1}; Table 3, Fig. 2b). Thus, the highest distribution of these bacteria occurred over a similar time period in 1999 and 2000 at The String and Scapa Flow.

In 2000, the *Alteromonas*-clade bacteria showed at Scapa Flow the highest peak also at the beginning of June (5.19 x 10^4 bacteria ml^{-1}) like the *Roseobacter*-clade bacteria (Table 2, Fig. 2a). But there were also no *Alteromonas*-clade bacteria detectable at other time periods, when *Roseobacter*-clade bacteria were present in the water column. In 1999, the highest peak

of *Alteromonas*-clade bacteria occurred at Scapa Flow at the mid of June (6.03 x 10^4 bacteria ml^{-1}). The *Alteromonas*-clade bacteria at The String in 2000 showed the highest numbers at the end of April (3.91 x 10^4 bacteria ml^{-1}). There the highest peak in 1999 occurred at the end of June (6.6 x 10^4 bacteria ml^{-1}). In this time no *Alteromonas*-clade bacteria were found in the water column in 2000 (Table 3, Fig. 2b). The highest numbers of *Alteromonas* and *Roseobacter*-clade bacteria occurred at Scapa Flow over a similar time period in both years, but at The String a lag of two months in abundance occurred over the two monitored years.

The species-specific bacterial counts for *Roseobacter* and *Alteromonas* bacteria showed very similar patterns for both years and at both sampling sites. In 2000, the highest peak of the species-specific bacteria (Table 2) of the *Roseobacter*-clade occurred in the end of May 2000 (bacteria 407-20 and 667-2) at Scapa Flow and also at The String. In 1999, at Scapa Flow and also at The String bacteria 407-20 and 667-2 also showed the highest bacterial peak. In 2000, the bacterium 253-13 showed also different peaks at The String like the *Roseobacter*-clade bacteria. But in general fewer species-specific bacteria of the *Roseobacter*-clade were detected than in 1999 and even though higher amounts of the *Roseobacte*r-clade bacteria were counted. Therefore, it is likely that other bacteria occurred in the water column in 2000. The highest numbers of species-specific bacteria of the *Alteromonas*-clade detected by applied probes were of the strain 4 vs3 at Scapa Flow and 4 vs3 and 407-2 at The String. In 1999, it was the same for Scapa Flow and for The String, but at the latter sampling site the numbers of both bacteria were very low. The numbers of the specific bacteria at The String in 2000 were higher than in 1999, but at Scapa Flow lower than in 1999, the reason could be also that other bacterial species were dominant in the water column.

Statistical Analysis. As in 1999, the bacterial counts were correlated with the counts for *Alexandrium* spp. and with the values of PST in mussels harvested from the same area. However, in 2000 there were no mussels harvested from The String and thus only comparisons from Scapa Flow for 2000 were made (Table 4). *Roseobacter*- and *Alteromonas*-clade bacteria were not significantly correlated with *Alexandrium* spp. at either site. Species-specific bacteria of both clades were negatively correlated with *Alexandrium* spp. at The String and positively correlated at Scapa Flow, but neither correlation was significant. *Roseobacter* and *Alteromonas*-clade bacteria were not significantly correlated with mussel toxicity nor with species-specific bacteria of either clade.

DISCUSSION

In the second monitoring year rRNA probes designed for purportedly toxic bacteria (Brinkmeyer et al. 2000) were again successfully used to detect these bacteria in Lugol's fixed water samples obtained from the Orkney Islands, at the two main sampling sites, The String and Scapa Flow from March to August 2000.

Alexandrium spp. counts were available for both sampling sites. However, PSP concentrations in mussel tissue were available only for Scapa Flow, because of the adaptive nature of the monitoring program, coupled with the fact that occurrence of PST was lower in 1999 and in 2000 than in previous years (Howard, personal communication), hence fewer mussel samples were collected.

In general, a similar temporal distribution of the putatively toxic bacteria in water samples collected from the Orkney Islands were found in both years, although a time shift in the main abundance of *Alteromonas*-clade bacteria over two month between both years was recognised and also no *Alteromonas*-clade bacteria were detected in the water column, when in the previous year high numbers were counted. However, the temporal distribution of the *Roseobacter*-clade bacteria was similar in both monitoring years, although less putatively toxic bacteria of both clades in 2000 were found. Reasons could be, e.g., the preparation of the samples in that year. Some samples seemed to be not very well fixed and consequently a bacterial loss could have occurred. The species-specific bacterial counts of both *Alteromonas*- and *Roseobacter*-clade bacteria displayed a similar temporal pattern for the two years at both sampling sites.

Additionally, as mentioned above, both years were low PSP years and for this reason the *Alexandrium* associated bacteria could be less abundant in the second monitoring year. It is not known, which *Alexandrium* species co-occur with the putatively toxic bacteria, because the counted dinoflagellate cells of the genus *Alexandrium* were not further identified. At least three *Alexandrium* species have been reported to co-occur in waters of the Orkney Islands: *Alexandrium tamarense*, *A. ostenfeldii* and *A. minutum* (Ellbrächter, unpublished).

Roseobacter and *Alteromonas* spp. were common in the field samples, consisting of up to 57% of the total bacterial counts at The String in the end of May and up to 34% in the beginning of June. The number of bacteria recognised by the species-specific probes amount only approximately 0.5% of the number detected by the clade probes. This means that a large percentage of the bacterioplankton cross reacting with the clade probes could not be

accounted for using the taxon specific probes. For this reason, the vast majority of marine bacterial diversity remains undescribed.

Further investigations should be to determine the unidentified bacterioplankton, which also co-occur with a *Alexandrium* bloom and mussel toxicity in this phytoplankton monitoring area. Additional 16S rRNA oligonucleotides of bacteria originally isolated from shellfish viscera obtained from harvesting areas in West Scotland, and from *A. tamarense* cultures 407-62 and 407-68, respectively were developed (Table 5). The oligonucleotide probes, S9/297R, 407-62/1488R and 407-68/1024R were tested with laboratory cultures employing dot blot and fluorescence *in situ* hybridization formats (Figs. 4-7), for the further use on environment samples. The bacteria isolated from shellfish viscera (strains S9, M12 and QO5) belong to the group of □-Proteobacteria, from which M12 was shown to biotransformate PSTs (Smith et al. 2001). The bacteria isolated from *Alexandrium tamarense* were named 407-62 and 407-68 and are members of the □-Proteobacteria. Therefore, a characterisation of further members of the bacterial community, which co-occur with *Alexandrium* spp. in affected areas, is possible.

Although, this monitoring study is an important first step in studying bacterial/dinoflagellate interactions in terms of PST in the environment, conclusive evidence on the influence of bacteria in relation to the occurrence of PSTs and the environmental parameters is still lacking and needs further investigations. New FISH counting methods are necessary to screen a high quantity of environmental samples faster and easier. One possibility is to use an automated detection system together with the FISH technique. In future a sensitive, rapid and easy use detection method for bacterio- and phytoplankton species will be established. The detection of the organisms will be based on sample filtration and subsequent whole cell *in situ* hybridization. The cells are then detected and enumerated using a solid phase cytometer. In this way, it would be possible to detect whole bacterial or phytoplankton cells, to count them and to evaluate the results by fluorescence microscopy faster than in previous times. Our final target is the adaptation and use of this method for routine monitoring with a high sample throughput.

OVERALL CONCLUSIONS: MONITORING YEARS 1999 AND 2000

In conclusion, data presented in these monitoring studies are the most comprehensive to date with regards to the examination which bacteria, including purportedly toxic bacteria, are present in the water column during periods of shellfish toxicity and when *Alexandrium* spp. were present. The oligonucleotide probes were successfully applied to fresh Lugol's fixed seawater samples. The dinoflagellate-associated bacteria were detected in high numbers

in the water column when *Alexandrium* spp. was both present and absent and during periods when mussels contained PSTs. This study infers a statistically significant association between bacteria belonging to the *Alteromonas*-clade in both years and some specific *Alteromonas* species to *Alexandrium* in 1999.

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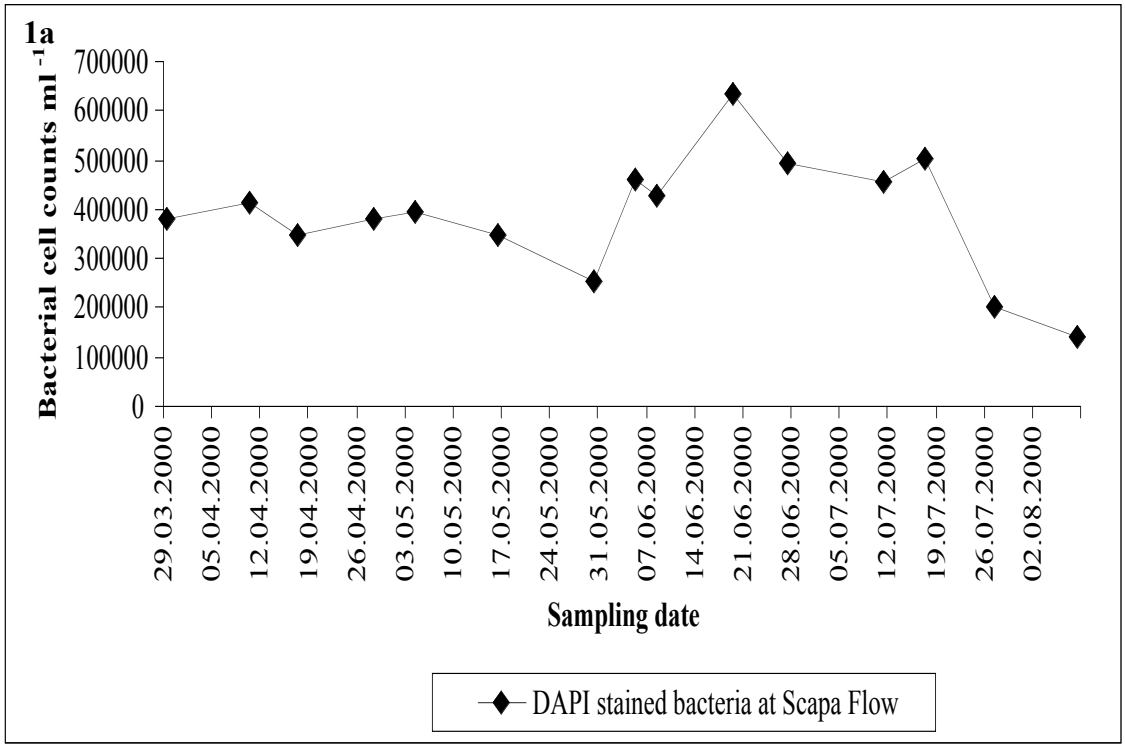


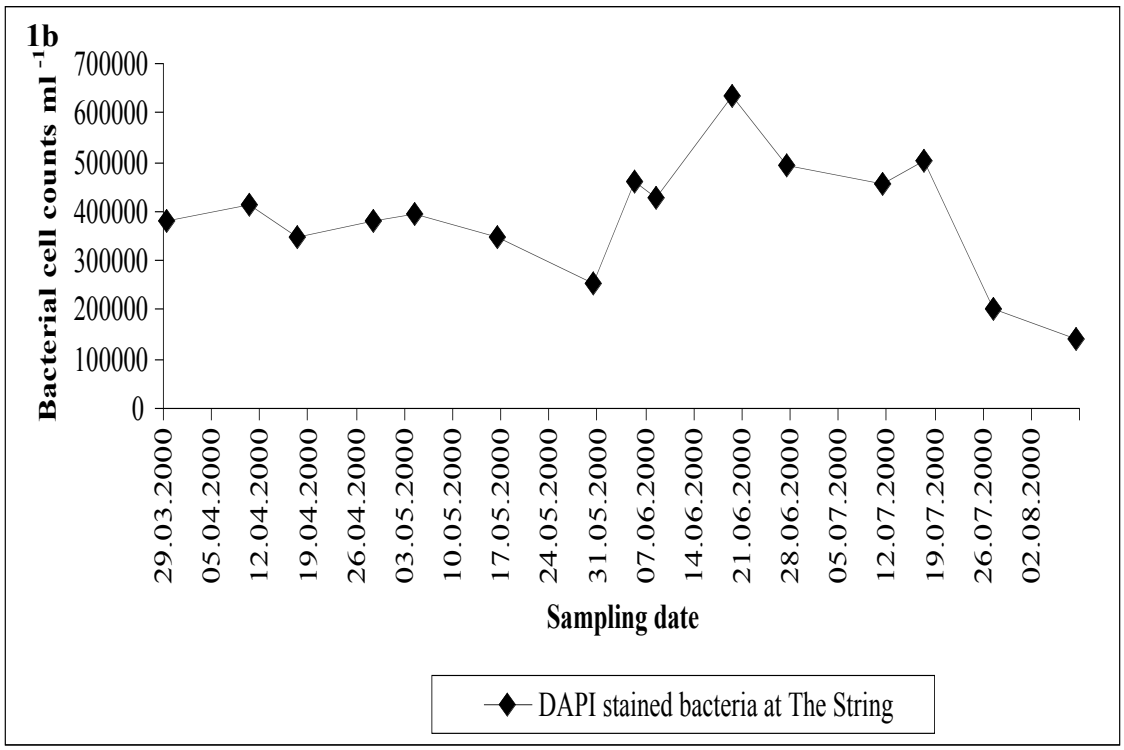
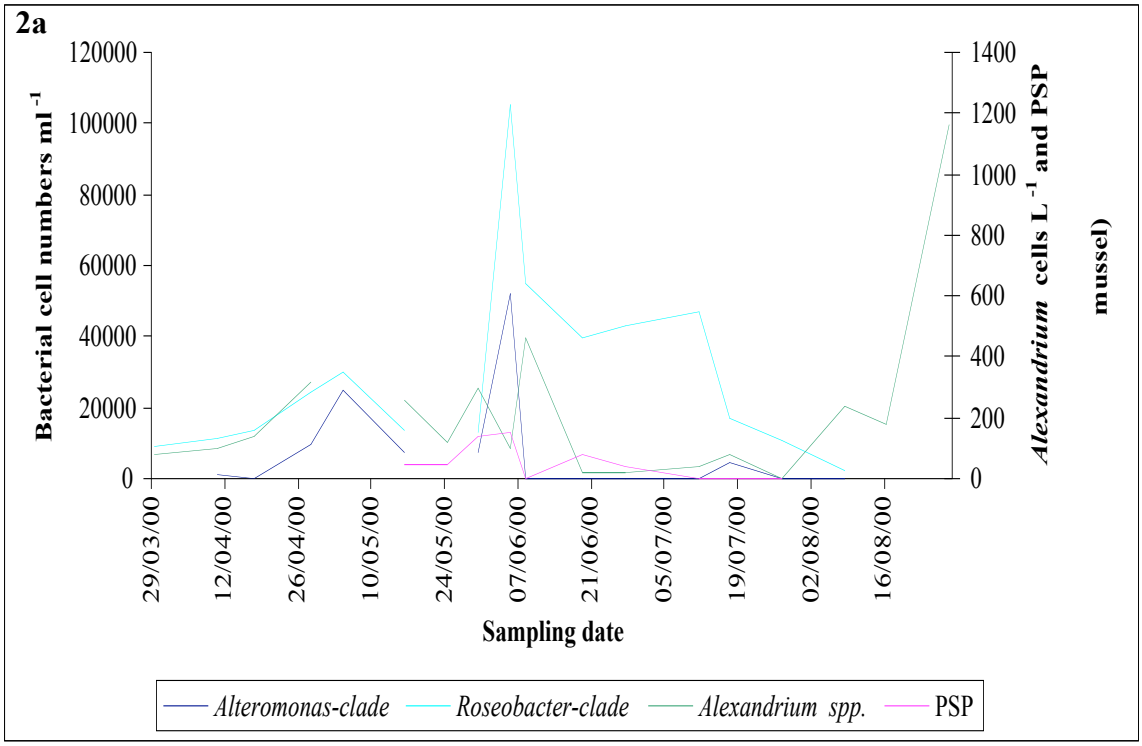


Fig. 1: Total bacterial counts at Scapa Flow (1a) and The String (1b) determined by DAPI staining



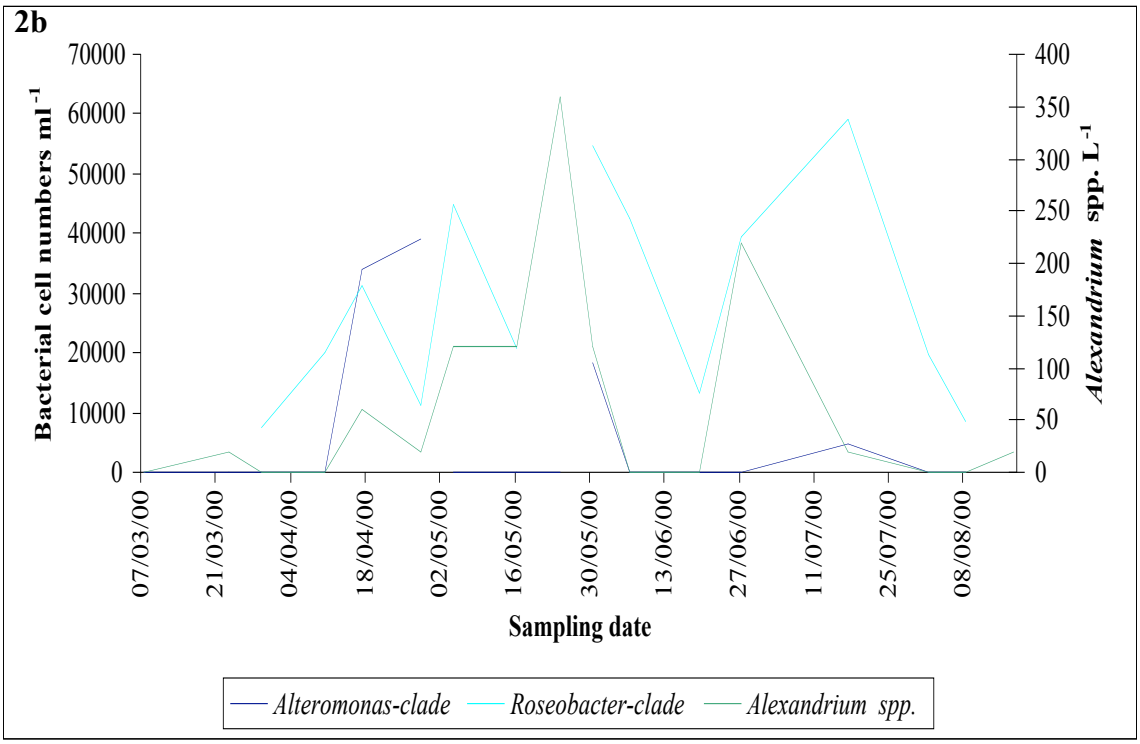


Fig. 2: *Alteromonas*- and *Roseobacter*-clade counts at Scapa Flow (2a) and The String (2b) determined by FISH and compared with total *Alexandrium* counts and mussel toxicity. Breaks of the graph lines were made at times when no samples were available, to show that the cell number did not decrease to zero.

Table 1: Summary of sites and dates in 2000 for which bacterial or algal counts or mussel toxicity were available for this study

Date	Scapa Flow	The String	Date	Scapa Flow	The String
07. Mar		A	**19. Jun**	A, M, X	A, X
23. Mar		A	**27. Jun**	A, M, X	A, X
29. Mar	A, X	A, X	**11. Jul**	A, M, X	
10. Apr	A, X	A, X	**17. Jul**	A, X	A, X
17. Apr	A, X	A, X	**18. Jul**	M	
28. Apr	A, X	A, X	**27. Jul**	A, M, X	
04. May	M	A, X	**01. Aug**		A, X
16. May	A, M, X	A, X	**08. Aug**	A, X	X
24. May	A, M	A	**09. Aug**	M	
30. May	A, M, X	A, X	**16. Aug**	A	
05. Jun	A, M, X	X	**17. Aug**		A
06. Jun		A, X	**28. Aug**	A	
08. Jun	A, X				

Abbreviations: X, Bacterial counts were obtained on that date; A, *Alexandrium* counts were obtained; M, mussels were collected for toxicity measurements.

Table 2: Values of clade-bacteria, *Alexandrium* spp. and mussel toxicity at Scapa Flow in 2000

Sampling in 2000 at Scapa	***Alexandrium* spp. cells L^{-1}**	**µg PSP /100 g mussel tissue**	***Alteromonas*-clade bacteria ml^{-1}**	***Roseobacter*-clade bacteria ml^{-1}**
10. Mar	100	n.a	n.a	n.a
29. Mar	80	n.a	652	9163
10. Apr	100	n.a	1296	11473
17. Apr	140	n.a	0	13459
28. Apr	320	n.a	9500	24307
04. May	n.a	0	25000	29728
16. May	260	43	7144	13742
24. May	120	45	0	0
30. May	300	137	7484	13061
05. Jun	100	155	51850	105025
08. Jun	460	n.a	0	55013
19. Jun	20	82	0	39538
27. Jun	20	42	0	43208
11. Jul	40	0	0	46834
17. Jul	80	.n.a	4344	16894
18. Jul	n.a	0	n.a	n.a
27. Jul	0	0	0	11032
08. Aug	40	n.a	0	2412
09. Aug	n.a	49	n.a	n.a
16. Aug	180	n.a	n.a	n.a
28. Aug	1160	n.a	n.a	n.a

n.a: samples not available

Table 3: Values of clade-bacteria and *Alexandrium* spp. at The String in 2000

Sampling in 2000 at String	***Alexandrium* spp. cells L^{-1}**	***Alteromonas*-clade bacteria ml^{-1}**	***Roseobacter*-clade bacteria ml^{-1}**
07. Mar	0	n.a	n.a
23. Mar	20	0	0
29. Mar	0	0	7500
10. Apr	0	0	20163
17. Apr	60	34000	31394
28. Apr	20	39118	11219
04. May	120	0	44872
16. May	120	0	20616
24. May	360	0	0
30. May	120	18194	54792
06. Jun	0	0	42363
19. Jun	0	0	13265
27. Jun	220	0	39474
17. Jul	20	4910	59131
01. Aug	0	0	19602
08. Aug	0	0	8447
17. Aug	20	n.a	n.a

n.a: samples not available

Table 4: Summary of the Pearson correlations (*R*) and their significance obtained between bacterial numbers (square root-transformed), mussel toxicity (M) and *Alexandrium* spp counts (A) for 2000

Correlation of bacterial counts + *Alexandrium* (A) or mussel toxicity	**String-**R of transformed data	Probability (number of observations)	Significance at the 0.02 level	**Scapa-**R of transformed data	Probability (number of observations)	Signifcance at the 0.02 level
Total *Roseobacter* + A	0.516	2.554	yes	0.096	0.411 (15)	no
Total *Alteromonas* + A	0.327	1.467 (20)	no, yes at 20%	0.011	0.049 (15)	no
Pooled *Roseobacter* taxon specific counts + A	-0.052	0.228 (21)	no	0.043	0.159 (16)	no
Pooled *Alteromonas* taxon specific counts + A	-0.173	0.768 (21)	no	0.284	1.058 (16)	no
Total *Roseobacter* + M	n.a	n.a		0.421	1.601 (15)	no, yes at 20%
Total *Alteromonas* + M	n.a	n.a		0.353	1.968 (15)	no, yes at 10%
Pooled *Roseobacter* taxon specific counts + M	n.a	n.a		-0.063	0.055 (16)	no
Pooled *Alteromonas* taxon specific counts + M	n.a	n.a		0.232	0.861 (15)	no
4vs3 + A	n.a	n.a		0.332	1.271(15)	no
4vs3 + M	n.a	n.a		0.311	1.184 (15)	no

n.a: not available because no mussels sampled (M) or all zero values (A)

Table 5: New Probe Sequences

Probe name	**Bacterial Origin**	**5'----------3'**	**Target bacteria**	**Dot Blot Hybridization Temperature**	**FISH Temperature and Formamide concentration**
407-62/1488R	*A.tamarense* laboratory culture 407-62	ctc ggg aaa tct cga tac	407-62	55°C	46°C, 18% FA
407-68/1024R	*A. tamarense* laboratory culture 407-68	gat cgc cag tat gaa agg	407-68	55°C	Not yet tested
S9/297R	Shellfish viscera	cuc acc cgu ccg cgu cgu	S9, M12 and QO5 (all Isolates from shellfish viscera)	63°C	46°C, 34% FA

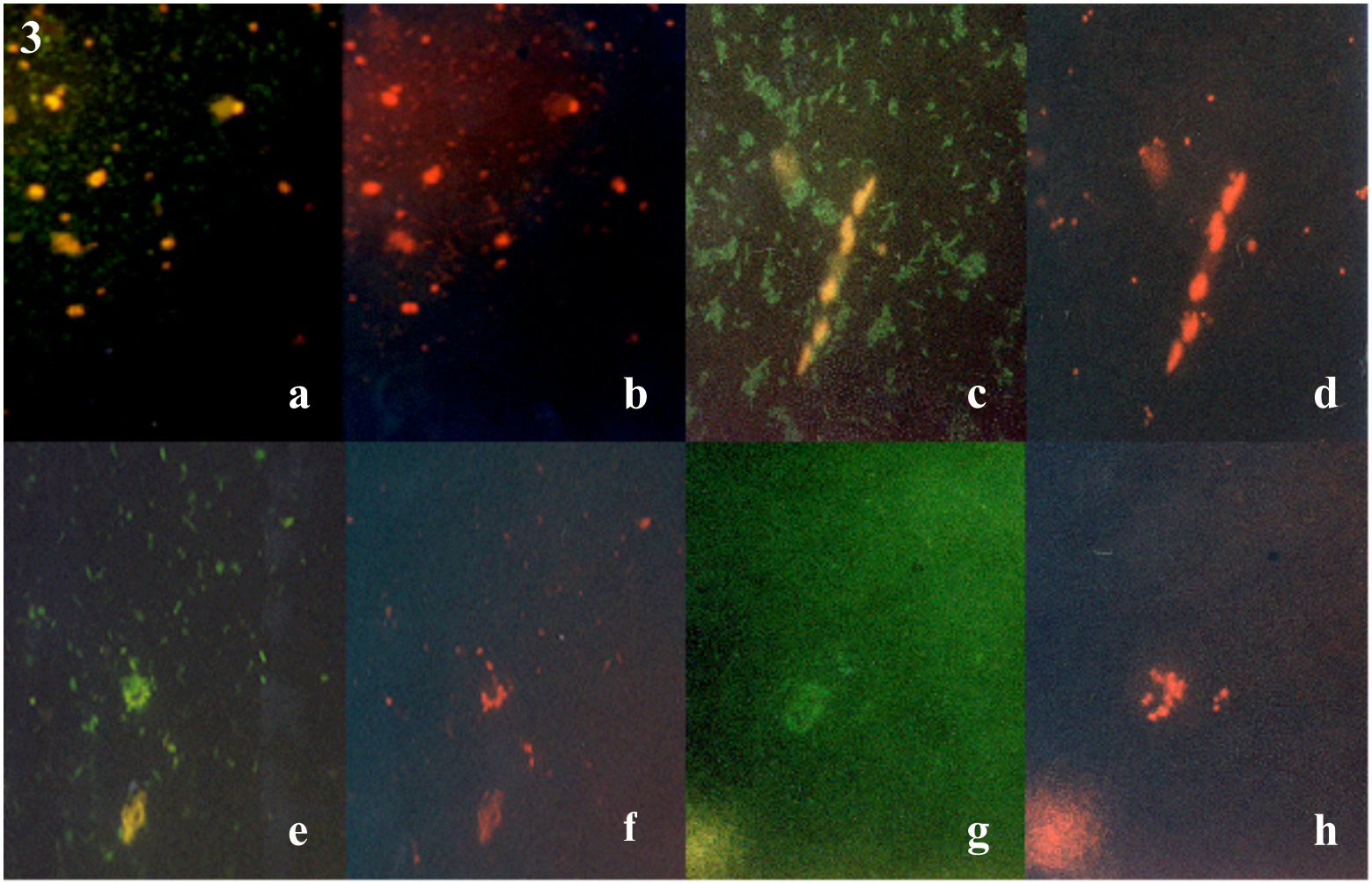


Fig 3. FISH with bacteria taken from water samples at the Orkney Islands, in 2000. a: Water samples from Scapa hybridized with eubacterial probe EUB 338R labelled with FITC. b: Same cells hybridized with the *Alteromonas*-clade probe 137R labelled with CY3. c: Water samples from String hybridized with EUB 338R labelled with FITC. d: Same cells hybridized with the *Roseobacter*-clade probe 536R labelled with CY3. e: Water samples from String hybridized with the *Alteromonas*-clade probe 137R labelled with FITC. f: Same cells hybridized with the probe 407-2/209R labelled with CY3. g: Water samples from Scapa hybridized with the *Roseobacter*-clade probe 536R labelled with FITC. h: Same cells hybridized with the probe 667-12/994R labelled with CY3. X 100 enlargement.

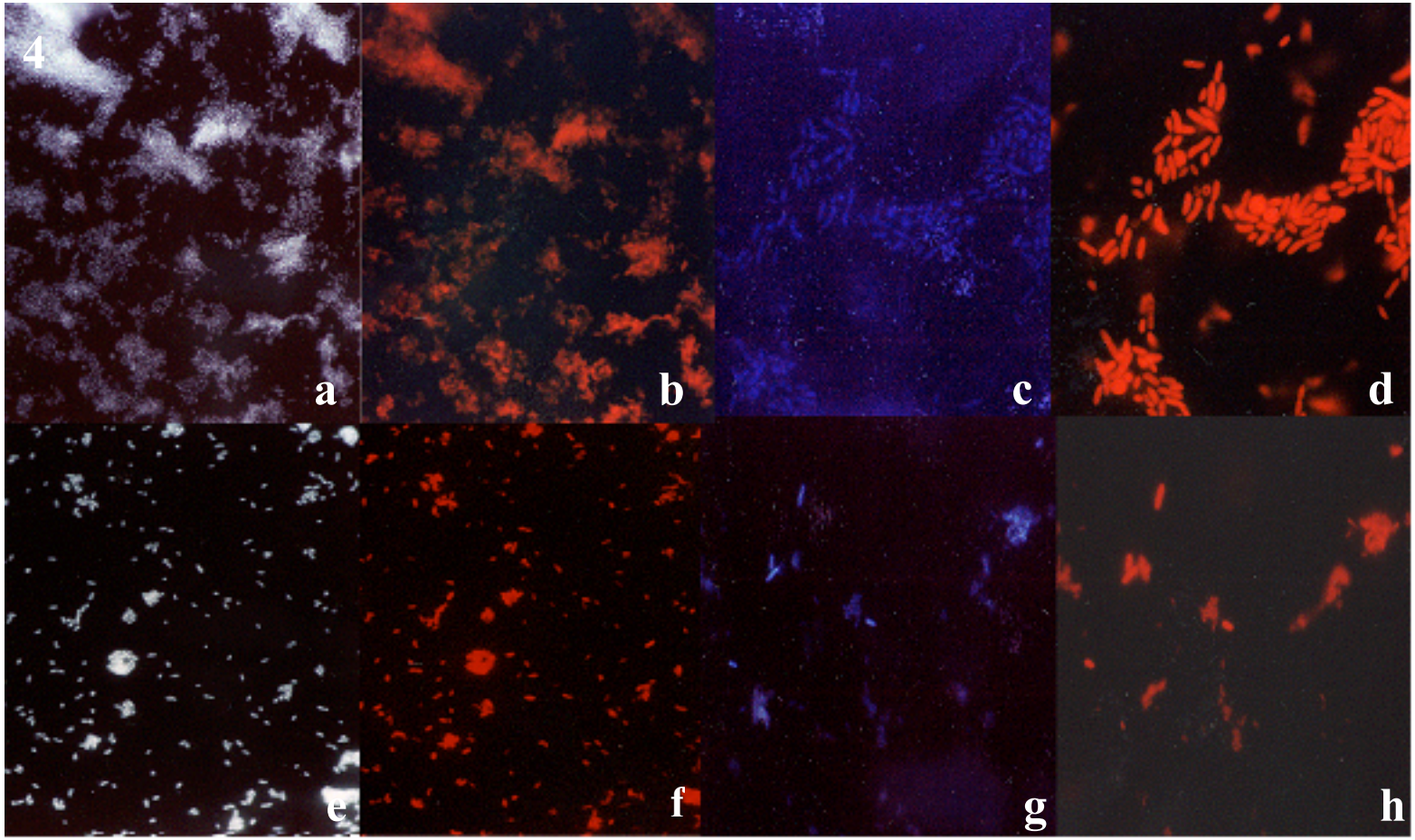


Fig. 4. FISH with newly developed probe S9/297R. a: Bacteria QO5 counterstained with DAPI. b: Same cells hybridized with probe S9/297R labelled with CY3. c: Bacteria M12 counterstained with DAPI. d: Same cells hybridized with probe S9/297R labelled with CY3. e: Bacteria S9 counterstained with DAPI. f: Same cells hybridized with probe S9/297R labelled with CY3. g: Bacteria 407-62 counterstained with DAPI. h: Same cells hybridized with the probe 407-62/1488R labelled with CY3. X 100 enlargement.

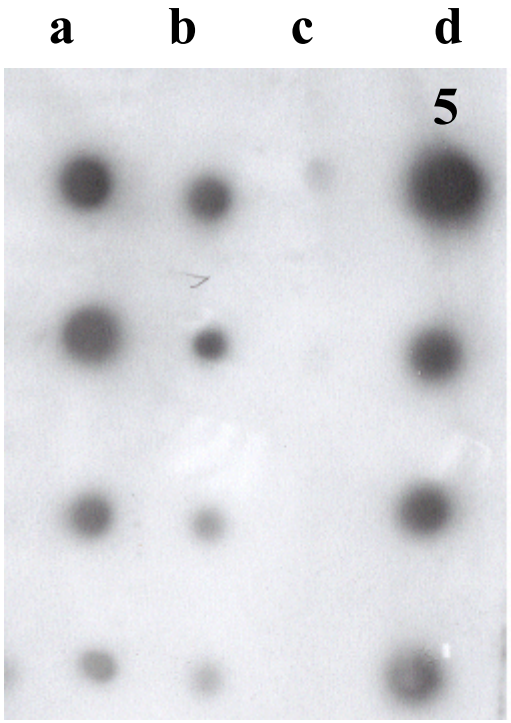


Fig. 5. Specifity of the rRNA probe S9/297R tested by dot-blot hybridization. Different DNA concentrations from bacteria isolated from shellfish viscera harvested in West Scotland were bound to the nylon membrane and hybridized with digoxigenin-labelled 16S rRNA probe S9/297R. 5a: Bacteria QO5 hybridized with probe S9/297R. 5b: Non-target bacteria M11 hybridized with probe S9/297R 5c: Bacteria M12 hybridized with probe S9/297R. 5d: Bacteria S9 hybridized with probe S9/297R.

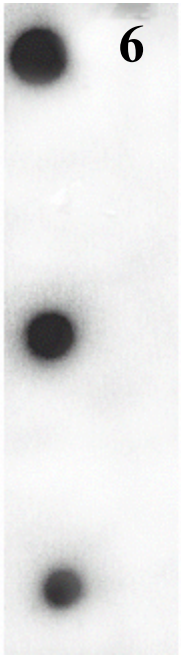


Fig. 5. Specifity of the rRNA probe 407-68/1024R tested by dot-blot hybridization. DNA from bacteria 407-68 isolated from *A. tamarense* cultures was bound to the nylon membrane and hybridized with the digoxigenin-labelled 16S rRNA probe 407-68/1024R.

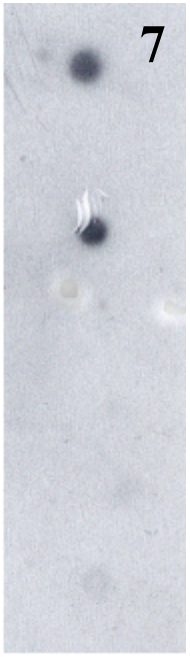


Fig. 5. Specifity of the rRNA probe 407-62/1488R tested by dot-blot hybridization. DNA from bacteria 407-62 isolated from *A. tamarense* cultures was bound to the nylon membrane and hybridized with the digoxigenin-labelled 16S rRNA probe 407-62/1488R.

2.6 PUBLICATION III

Detection of bacteria originally isolated from *Alexandrium* spp. in the midgut diverticula of *Mytilus edulis* after water-borne exposure

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Harmful Algae, in press

Abstract

Bacteria associated with toxic dinoflagellates have been implicated in the production of paralytic shellfish poisoning (PSP) toxins, but it has not been substantiated that bacteria are truly capable of autonomous PSP toxin synthesis or what role bacteria may play in shellfish toxification. In this study, different putatively PSP toxin producing bacteria originally isolated from toxic *Alexandrium* spp. were exposed to the blue mussel *Mytilus edulis*. To document that these bacteria accumulated in the digestive tract of the mussels hybridization techniques that use rRNA targeted oligonuceotides for *in situ* identification of these bacteria were applied. The mussel hepatopancreas was dissected and paraffin and frozen sections were made. The dissected glands were hybridized with digoxigenin-labelled 16S rRNA oligonucleotide probes. Results demonstrate that mussels will readily uptake and accumulate these bacteria in the hepatopancreas. However, the mussels were not rendered toxic by the ingestion of the bacteria as determined by HPLC with UV detection for PSP toxins and determination of sodium channel blocking activity using the mouse neuroblastoma assay. Thus although the role that bacteria play in mussel toxification remains unclear, methods are now available which will aid in further investigation of this relatively unexplored area.

Keywords: Alexandrium, *Mytilus edulis*, bacteria, paralytic shellfish toxins, sodium channel blocking toxins, *in situ* hybridization

1. Introduction

Both wild and cultivated blue mussels *Mytilus edulis* Linné 1758 are exposed to a mixture of particles and microbes and are able to accumulate them in high numbers from the surrounding water (Hernroth et al., 2000a). Thus, the potential for the mussel to become a carrier of food borne diseases is significant (Hernroth et al. 2000b). Paralytic Shellfish Poisoning (PSP) toxins are a group of neurotoxins produced by some dinoflagellates species, such as *Alexandrium* spp. (Cembella 1998). In humans PSP toxins block sodium channels (SCB), which can lead to paralysis and subsequent death. Filter feeding bivalves such as mussels, cockles, oysters, and scallops, can feed on these toxic dinoflagellates, transferring them from the gills to digestive organs where the toxins become concentrated (Maruyama et al. 1983; Lassus et al. 1992; Pillet et al. 1995; Bricelj and Shumway 1998; Shimizu 2000). Some shellfish depurate the toxins relatively quickly, whereas others, e.g., scallops can retain the toxins for months and even years in the digestive glands and the gonad (Whittle and Gallacher 2000).

In recent years, bacteria have also been implicated as a source of PSP toxins. The autonomous production of these toxins by bacteria has been reported (Gallacher et al. 1997), yet their involvement with shellfish toxicity has not been totally elucidated. Evidence for their involvement has been implicated by Kodama and colleagues (1990), who reported that PSP toxins were present in particles of a similar size fraction (0.45-5 μm) to bacteria in seawater from Ofunato Bay, N. Japan, during times when bivalve toxicity increased in the absence of toxic dinoflagellates. Bacteria are also known to metabolise PSP toxins, converting them from one derivative to another (Smith et al. 2001). Thus, bacteria may be involved in both the production and modification of these toxins.

In the marine environment, bacteria can attach to phytoplankton and inorganic particles (Doucette et al. 1998), which can be readily accumulated by animals, such as bivalves. Therefore, in these studies, mussels were fed either a single SCB toxin (a trait associated with PSP toxins) producing bacterium or a mixed assemblage of SCB toxin producing bacteria. Bacteria were exposed

to the mussels in both the presence and absence of particulate matter (silt) in attempts to increase bacterial SCB toxin production via adhesion and attachment (Doucette et al. 1998), increase bacterial uptake rates by the mussels and subsequently increase SCB activity in the mussel flesh. The bacterial strains used in the feeding experiments were originally isolated from toxic *Alexandrium* spp. (Gallacher et al. 1997). Previously, 16S rRNA clade and species-specific oligonucleotide probes have been developed that specifically recognise these bacteria (Brinkmeyer et al. 2000) and have been used to track the abundance and distribution of these bacteria in the water column. (Töbe et al. 2001).

In this study, these probes were used to follow the accumulation of water-borne bacteria in the digestive gland of *Mytilus edulis* as well as the duration of the bacteria in the gut.

2. Material and Methods

2.1 Feeding experimental setup.

Fresh stocks of mussels were maintained in natural seawater in an aerated tank at 12°C and fed daily with *Pavlova lutheri* (Droop) Green 1975. Three days prior to exposing the mussels to sodium channel blocking (SCB) toxin producing bacteria mussels were transferred into autoclaved 1L Kilner jars containing sterile filtered seawater that was aerated using air stones. Seawater was changed daily in the aerated jars in order to reduce any background bacterial flora. Exponentially growing SCB toxin producing bacteria grown at 20°C for 24 h in 200 mL marine broth (Difco, UK) were centrifuged at 8,000 *g* for 10 minutes and re-suspended in sterile seawater. Aliquots (1 mL) adjusted to contain 10^7 cells mL^{-1} were added to six experimental jars each containing ten mussels. Either a single SCB toxin producing bacterium, 407-2 (Experiment A) which belongs to the genus *Alteromonas* (Baumann et al. 1972) or an assemblage of different SCB toxin producing bacteria of the genus *Alteromonas* and *Roseobacter* (Shiba 1991), 407-2 (*Alteromonas*-clade), 2c3 (*Alteromonas*-clade), 4 Vs17 (*Alteromonas*-clade) and 253-13 (*Roseobacter*-clade) previously isolated from dinoflagellate cultures

(Gallacher et al. 1997; Hold et al. 2001) were exposed to mussels, in both the presence or absence of silt particles (< 55 μm) (Experiment B). Control samples consisted of shellfish in seawater not exposed to bacteria and bacteria in seawater in the absence of shellfish.

Seawater (5mL) and three shellfish were collected from individual flasks at 0, 0.5, 1, 2, 3 and 6 h time intervals for bacterial counts in the water column and determination of any SCB activity in the mussel flesh, respectively. At each time point a further mussel was collected and fixed in 4% paraformaldehyde (PFA in 1 X phosphate buffered saline [PBS], pH 7.2 and stored for 24 h at 4°C. The seawater was counted for the presence of bacteria using a Weber-Counting chamber.

2.2 Determination of Mussel toxicity

At each time interval mussel flesh was extracted in 0.1 M HCl, following the method described by the Association of Official Analytical Chemists (AOAC 1990). Sample extracts were analysed using an optimised mouse neuroblastoma assay (Gallacher et al. 1997, Hold 1999) for the detection of SCB activity and by the HPLC method of Franco and Fernández-Vila (1993) for the detection of PSP toxins.

2.3 Determination of bacterial toxicity

Bacterial strains were cultured for 18 h in 30 ml of marine broth (20°C; 120 oscillations min^{-1}). Supernatants and cells were separated by centrifugation at 10000 *g* for 20 min. Supernatant (20 ml) from each isolate was stored at 4 °C until processed by using the mouse neuroblastoma assay as previously detailed (Gallacher et al. 1997).

2.4 Embedding of mussel tissue prior to detection of bacteria

The hepatopancreas of the mussels were dissected under sterile conditions prior to sectioning and analysis with the probes. Two different embedding methods were used to compare which is the most suitable one for this purpose.

Paraffin sections: Dissected hepatopancreas was fixed in 4% PFA, pH 7.2, in 1 X PBS and stored at 4 °C overnight. The digestive glands were embedded in paraffin as described elsewhere (Romeis 1989). The paraffin blocks were cut into 8 µm thin sections with a rotary microtome (model 1512, LEITZ, Germany) and transferred to poly-L-Lysine coated microscope slides (Sigma, USA). Frozen sections: The digestive glands were fixed in 4% PFA, cut into 2 mm thin sections and incubated overnight at room temperature in 30% sucrose solution in 1 X PBS. Subsequently, the sections were stored in Oct compound (Ted Pella, USA), an embedding medium for frozen tissue specimens, at –80°C and warmed to –20°C before use. The specimen block was cut into 10-14 µm thin sections with a cryostat (2800 Frigocut-E, Reichert-Jung, Germany) transferred to Poly-L-Lysine treated glass slides and dried overnight at 40°C. Slides were used immediately or stored at –80°C. In the latter case the cryosections were warmed to room temperature and dried at 40°C for at least 2 h prior to hybridization.

2.5 In situ hybridization on paraffin embedded and frozen tissue sections

Sections were incubated for 30 minutes at 60°C and dewaxed prior to hybridization. The slides were dipped twice for 10 minutes each, in fresh Xylene (Merck, Germany) and rehydrated, by dipping each slide successively for five minutes in 99% EtOH, 96% EtOH and 70% EtOH, then rinsed twice in sterile deionized water. Each section was treated with 20 µl Proteinase K (working solution 100 µg mL^{-1} in TES: 50 mM Tris-HCL, pH 7.4, 10 mM EDTA, 10 mM NaCl) for 30 minutes at 37°C in a humid chamber. The sections were covered with siliconized coverslips (Aquasil, Pierce, USA), post-

fixed in 0.4% formaldehyde for 5 minutes, to preserve the tissue morphology after the proteolytic treatment, immersed in deionised water, dripped and air dried. The tissues were subsequently covered with 5–10 µl hybridization buffer (500 µl hybridization buffer: 10 µl 50 x Denhardt's solution, 50 µl dextran sulfate 50% (w/v), 10 µl sonicated and denaturated salmon sperm DNA (10 mg mL^{-1}), 100 µl 20 x SSC, 10 µl digoxigenin-labeled probe [50 ng μl^{-1}], 70 µl deionised water, 250 µl formamide). Non-labelled oligonucleotides (purchased from MWG, Germany) were DIG-labelled at the 3'-end of the oligonucleotide with the DIG labelling Kit (Roche, Germany). One slide was hybridized with a genus clade-level probe, e.g., *Alteromonas*/137R or *Roseobacter*/536R and a replicate slide with a species-level probe (Table 1). The slides were placed in a humid chamber and incubated at the corresponding hybridization temperature (Table 1) overnight. The slides were washed twice for 5 minutes in 2 x SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) at room temperature and 10 minutes in 0.1 x SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0) at hybridization temperature. Each section was equilibrated in buffer 1 (100 mM Tris-HCl, 150 mM NaCl, pH 7.5). The slides were covered with 40 µl buffer 2 (0.5% (w/v) blocking reagent, Roche, Mannheim, Germany, in buffer 1) incubated at room temperature for 1 h and washed quickly in buffer 1. The anti-digoxigen antibody (Roche, Mannheim, Germany) was diluted 1:500 in buffer 2. Diluted conjugate (10-15 µl) was placed over each section and incubated in a humid chamber at room temperature for 1 h and washed twice for thirty minutes in buffer 1 with gentle shaking. Afterwards the slides were equilibrated in buffer 3 (0.1 M Tris-buffer pH 9.5, 0.05 M $MgCl_2$, 0.1 M NaCl) for 5 minutes. Immediately before use, a fresh NBT (Nitro blue tetrazolium chloride) and BCIP (5-Bromo-4-chloro-3-indolyle phosphate, toluidine salt) working solution were prepared (dilution 1:50 in buffer 3). Twenty µl NBT/BCIP-colour-solution was distributed onto each section, covered with siliconized coverslips and incubated in the dark overnight at room temperature and then washed gently in buffer 3. The tissue sections were counterstained with Neutral Red as described in Romeis (1989).

2.6 Negative controls

Slides of control mussel tissue, which were not exposed to bacteria, were hybridized with different probes. In addition one slide in each hybridization experiment was hybridized with a probe cocktail containing all the ingredients without the labelled probe. Another slide was hybridized with Non-EUB338, which has a sequence complementary to EUB338 and served as a negative control for unspecific binding (Manz et al. 1999).

3. Results

3.1 Determination ob bacterial toxicity

The measured bacterial toxicity was 0.76 x 10^{-4} fmoles STX equivalents per cell for 407-2 and 1.3 x 10^{-4} fmoles of STX equivalents per cell for bacteria 2c3. These measurements were performed within the time framework FAIR.

Both 4alphavs17 and 253-13 have previously shown SCB activity (Gallacher pers comm.).

3.1 Clearance rates of bacteria by Mytilus edulis

Experiment A: Bacteria 407-2. The removal of 407-2 from an initial bacterial cell density of 1.0 x 10^{7} cells mL^{-1} from seawater over 6 h in experimental jars is shown in Figure 1. In the presence of mussels, bacterial levels in the water column fell by 87% within 30 minutes and steadily declined with less than 1% of the initial inoculum remaining after 6 h. In contrast, 96% of the bacterial cell suspension remained after 6 h in control jars containing no shellfish visible signs of pseudofaeces

production by the mussels was not observed in all experimental jars, nor was settling of bacteria apparent. Mussel assimilation of the bacteria was verified using the bacterial probes.

Experiment B: Mixed assemblage of bacteria plus particulate matter (silt). Although no attempts were made to distinguish between individual bacterial isolates in the water column, Figure 2 suggests that *Alteromonas* and *Roseobacter*-clade bacteria were rapidly removed from seawater in experimental jars containing mussels, with the initial cell density of 1.1 x 10^7 cells mL^{-1} being reduced by 99% within 30 minutes. Bacterial cell numbers initially increased in experimental jars in the presence of silt, although a gradual decline in cell numbers was observed over 6 h. In these experiments the bacterial loading slightly proliferated in the absence of mussels between 2 and 6 h.

3.2 In Situ hybridization

In general, both frozen and paraffin sections were suitable for *in situ* hybridization of mussel tissue. However, the paraffin method presents improved tissue morphology than cryosections, but in contrast is more time-consuming and arduous to produce.

Experiment A: Frozen and paraffin sections from mussels fed with the bacterium 407-2 of the genus *Alteromonas* showed that the bacteria were present at each time interval in the digestive tract of the mussels (Fig. 3a). Using the DIG-labelled probes, the examined bacteria are labelled as a blue deposit lining the interior of the midgut diverticula. At time 30', the presence of a 'background' flora of *Roseobacter* was detected in the gut of the mussels, which were not exposed to *Roseobacte*r-clade bacteria (Fig. 4a). After the feeding experiment commenced, this background flora was replaced with the ingested bacteria from the feeding experiment as shown in Figure 4b and c.

Experiment B: Slides prepared from mussels fed with silt and bacteria (407-2, 2c3, 4 □vs17 and 253-13) showed that the fed bacteria could be found in the digestive tract of the mussel at every sampling time (Fig. 3b-c and Fig. 4b-c). In contrast none of the negative controls (mussels not exposed to bacteria) showed a positive signal (Fig. 3d and 4d). Hybridization of the tissue sections

from mussels, which were fed with an assemblage of different bacteria and hybridized with the *Roseobacter*-clade probe were not as strong as the *Alteromonas*-clade signals (Fig. 3b and 4b). The reason is most probably the difference in the proportion of the four bacteria being fed the mussels, the bacteria (253-11) belonging to the *Roseobacter*-clade being only 25% of the total bacterial cells added to the feeding experiment. The probe signal in the gut of those mussels fed silt along with the bacteria and dinoflagellates was also diminished in comparison to that obtained in mussels fed solely on bacteria. This most likely occurred because of the decreased proportion of the bacteria in each liter of water filtered by the mussels.

3.3 Mussel Toxicity

Following exposure of mussels to SCB producing bacteria, the mussel homogenate was examined at 0 h and at time intervals of 0.5, 1, 2, 3 and 6 h for SCB activity using the MNB assay (Gallacher et al. 1997) and for PSP toxins by HPLC (Franco and Fernández-Vila 1993). In shellfish extracts obtained from mussels at all time intervals in both experiments, SCB activity was not detected using the MNB assay with a detection limit of 10 nM STX (ca. 0.3 μg per 100 g of shellfish flesh). HPLC analysis of flesh from mussels fed putatively toxic bacteria also failed to detect any PSP toxins. The detection limit for each toxin was (ng toxin per 100 g of shellfish flesh): GTX 1, 3.73; GTX 2, 4.4; GTX 3, 0.12; GTX 4, 1.6; STX, 7.6 and NEO, 5.02.

4. Discussion

4.1 Clearance rates

The mussels were capable of filtering the SCB bacteria from the seawater as total bacterial numbers were seen to decrease over time compared to controls. Previously, a study of filtration capacity of particles in *Mytilus edulis* (Møhlenberg and Riisgård 1978) showed a marked decline in the uptake of particles smaller than 7 µm, which fell to 20% at 1 µm. Also, it has been shown by Ward and Targett (1989) that microalgal metabolites influence mussel feeding behaviour. This indicates that the mussels have some ability of preingestive selection, presumably on the gills or the labial palps, which is not only related to size but also, to other particle characteristics (Hernroth et al. 2000a).

4.2 In situ hybridization

Using the probes, the presence of these bacteria was recorded in the mussels showing that they were not selectively excluded during filtration. Both embedding techniques are adequate to detect the bacteria in the mussel's gut, whereby the more time-consuming method show a superior tissue morphology. In this study, the mussels did not appear to reject any of the bacteria when fed a mixed assemblage, because our probes detected each of the bacteria in the sections of the mussels gut. In our experiments we could trace the presence of bacteria in the gut of the mussels up to 6 hours after feeding ceased. The signals of the clade specific probes show approximately the proportion of the fed species (Fig. 3c and 4b). The decrease in the signal strength after 6 hours may indicate that the bacteria were being digested by the mussel.

As we have demonstrated the successful application of the 16S rRNA species-specific probes to detect these putatively toxic bacteria in mussels under laboratory cultures, the next step would be to test mussels in nature in areas where mussel beds have been closed because of toxic dinoflagellates to

see if purportedly toxic bacteria, known to be present in the water column during a toxic episode (Töbe et al. 2001), can also be found inside the mussels from the same area. It is still poorly known if bivalves are capable of selectively ingesting particles based on their toxin content. Li and Wang (2001) employed a radiotracer monitoring technique to determine the selective feeding behaviour of the mussel *Perna viridis* Linné 1758 and the clam *Ruditapes philippinarum* (Adams and Reeve 1850) on an algal mixture containing both toxic and non-toxic *Alexandrium tamarense* (Lebour) Balech, 1985. The authors did not find any selective ingestion of toxic and or non-toxic alga, which indicates that the two bivalves were not able to distinguish the particles with different PSP toxin contents.

As probes for different toxic and non-toxic dinoflagellate-clades have now been developed (John 2002) future tracking experiments could include feeding toxic- and non-toxic *Alexandrium tamarense* to *Mytilus edulis*, to determine if a selective ingestion of particles based on their PSP content take place in this shellfish species. Further information about the feeding behaviour and accumulation period of both algae and bacteria of the blue mussel are necessary to clarify the possible role of bacteria on shellfish toxicity.

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Table1.

Oligonucleotide probes used in this study

Probe name	Oligonucleotide Sequence	Target organism	Temperature
Genus/Clade Level Probe			
Alteromonas-clade/137R (Brinkmeyer et al. 2000)	5'-tgt tat ccc cct cgc aaa-3'	*Alteromonas*-clade	50°C
Roseobacter-clade/536R (Brinkmeyer et al. 2000)	5'-caa cgc taa ccc cct ccg-3'	*Roseobacter*-clade	60°C
Species level Probes			
407-2/209R (Brinkmeyer et al. 2000)	5'-ct ttg cgt ggg agc cgg-3'	407-2 bacteria	65°C
4 vs3/210R (Brinkmeyer et al. 2000)	5'-tct ctt tgc gcc aga gct-3'	2 c3 and 4 vs3 bacteria	55°C
253-11/1423R (Brinkmeyer et al. 2000)	5'-acc gtc gtc ggg tag acc-3'	253-11 and 253-13 bacteria	60°C
Non-EUB338 (Manz et al. 1999)	5'-act cct acg gga ggc agc-3'	Serves as negative control	50°C

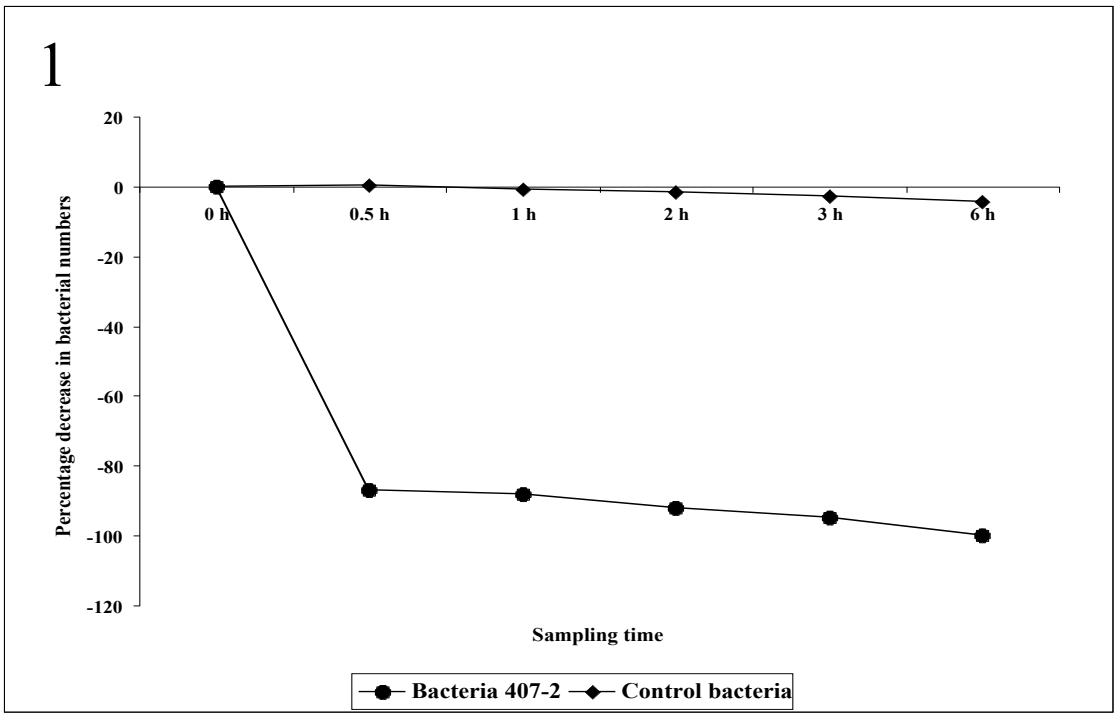

Fig. 1: Percentage decrease of bacteria 407-2 from seawater exposed to mussels and not exposed to mussels (control bacteria)
after different time intervals.

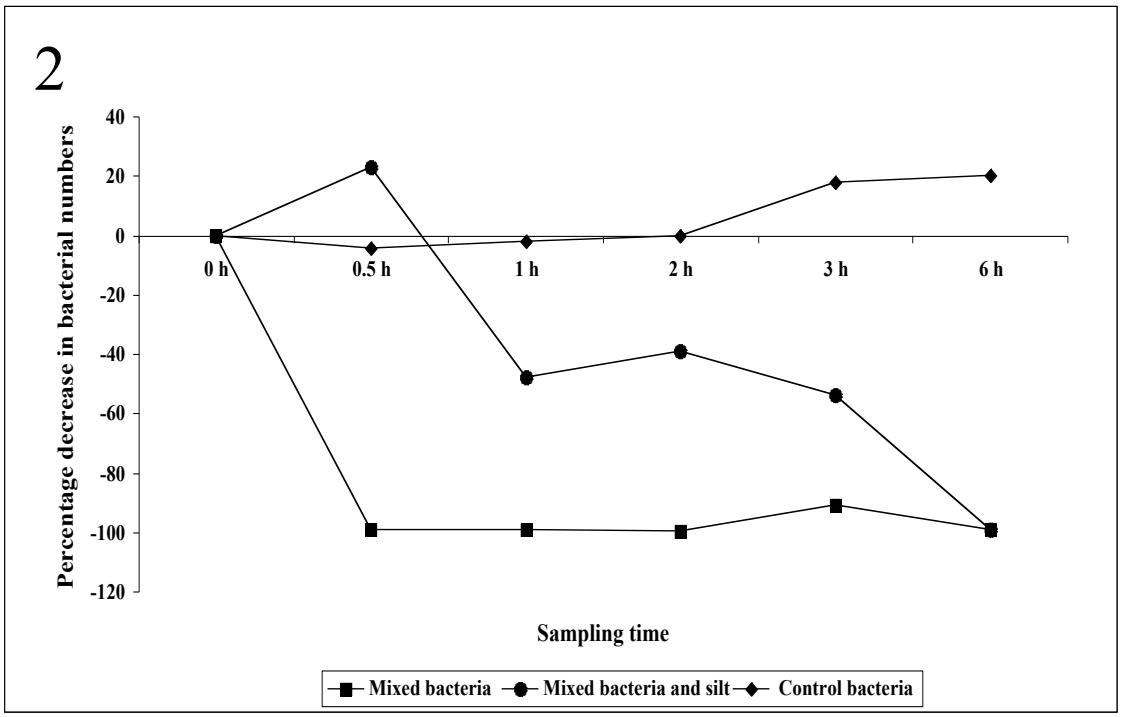

Fig. 2: Percentage decrease of a combination of different bacteria from seawater exposed to mussels and not exposed to mussels (control bacteria)
after different time intervals.

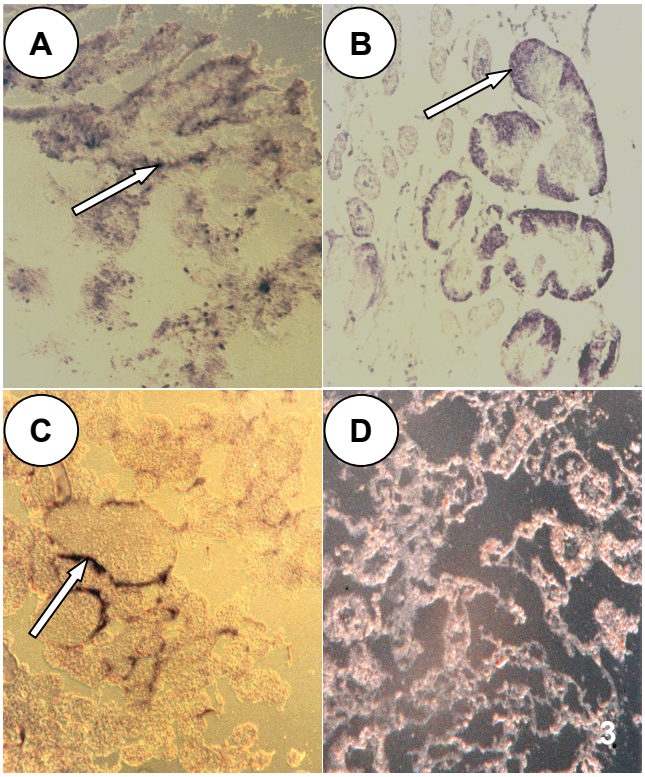

Fig. 3. Light micrographs show *in situ* hybridization of Paraffin (P) and Frozen (F) sections of the hepatopancreas of *M. edulis* after different feeding experiments. The purple/brown colour show positive signals of the hybridized bacteria. All probes were DIG-labelled. (A) Hybridization with species-specific probe 407-2/209R, of the *Alteromonas*-clade, 30‘ sampling time (F, 10 µm). (B) Hybridization with species-specific probe 4VS3/210R of the *Alteromonas*-clade, 3h sampling time (P, 8 µm). (C) Hybridization with *Alteromonas*/137R-clade probe, 6 h sampling time (P, 8 µm). (D) Negative control: Hybridization with NON-338R, 6 h sampling time (P, 8 µm). 10 X magnifications.

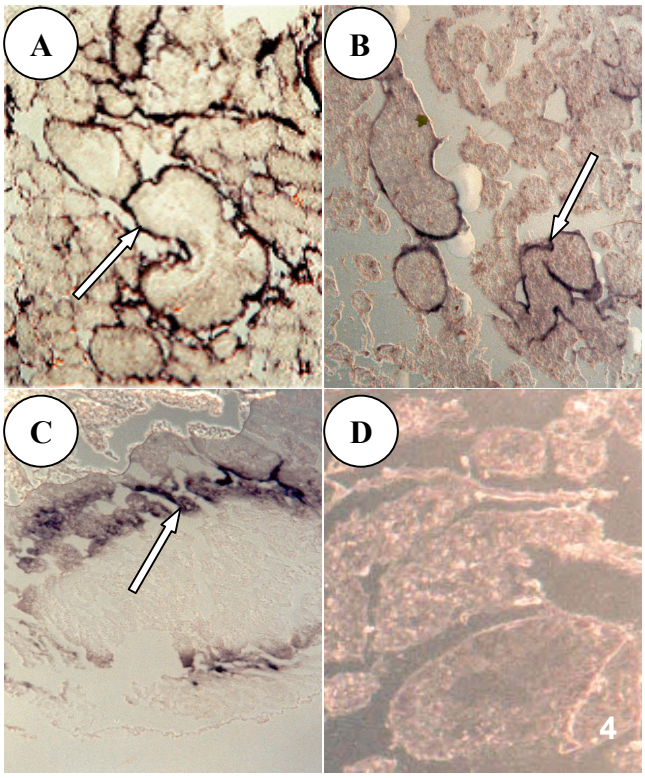

Fig. 4: Light micrographs show *in situ* hybridization of Paraffin sections (8 µm) of the hepatopancreas of *M. edulis* after different feeding experiments. Arrows show the positive signals of the hybridized bacteria. All probes were DIG-labelled. (A). Hybridization with *Roseobacter*-clade/536R probe, showing *Roseobacter* background flora. 30‘ sampling time. (B) Hybridization with *Roseobacter*-clade/536R probe, 3h sampling time. (C) Hybridization with species-specific probe 253-11/1423R of the *Roseobacter*-clade, 6h sampling time. (D) Negative control: Hybridization without a probe, 3h sampling time. X 10 magnifications.

2.7 Publication IV

Reassociation experiments with bacteria into a highly toxic *Alexandrium tamarense* clone

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ABSTRACT

Two bacterial loadings of purportedly toxic bacteria were reintroduced into laboratory cultures of a highly toxic *Alexandrium tamarense* clone (OF 84423-D3) to monitor a possible reassociation of these bacteria with the dinoflagellate cell. One bacterial strain (PTB-1) was originally isolated from the *A. tamarense* clone used in this study and the other bacterial strain (PTB-6) was isolated from another *A. tamarense* clone that is no longer available for study. Taxon and species-specific fluorescently labelled 16S rRNA probes, previously developed to recognise these introduced bacteria were used to detect the bacteria in *Alexandrium tamarense* following a time course of 96 hours. Epifluorescence microscopy and confocal laser scanning microscopy coupled with TSA-FISH showed that none of the reintroduced bacteria became intracellular, the PTB-1 bacteria were found to be attached to the surface of *Alexandrium tamarense*, or free in the culture and seem to form a preferred relationship with this algae, from which the bacteria were originally isolated.

***Key words**:* Attached bacteria, *Alexandrium tamarense*, putative toxic bacteria, confocal laser scanning microscopy, *Gyrodinium instriatum*, fluorescence *in situ* hybridization, tyramide signal amplification

INTRODUCTION

Bacteria are an integral part of the physical environment of toxic dinoflagellates (Gallacher et al. 1997; Doucette et al. 1998; Prokic et al. 1998) and can be found in the phycosphere, which is a zone around algal cells, in which bacteria are influenced by algae and vice versa (Bell and Mitchell 1972). Bacteria may be in loose or tight associations with phytoplankton (Caldwell 1977; Rothaupt and Güde 1992; Gallacher and Smith 1999), or inside microalgal cells (Cole 1982; Franca et al. 1995; Alverca et al. 2002). The interactions of bacteria and algae range from symbiotic, via commensal, to parasitic interactions (Schäfer et al. 2002) and are highly variable in space and time (Grossart 1999). In the symbiotic relationship bacteria benefit from phytoplankton products, such as exudates (Bell et al. 1974; Cole 1982), whereas phytoplankton profit of bacterial products, such as remineralized nutrients (Golterman 1972), vitamins (Haines and Guillard 1974), and other growth factors (Paerl and Pickney 1996). Bacteria that live commensalic in or around algae benefit from the algae without having any negative effect on it (Barbeyron and Berger 1989). Bacteria may also be parasites of phytoplankton and penetrate into the algae to lead to cell lysis and death (Cole 1982).

Paralytic Shellfish Poisoning (PSP) is a serious illness, which is caused by the consumption of filter feeding bivalves that are contaminated with toxins produced by several dinoflagellates, such as the dinoflagellate *Alexandrium* spp. (Anderson et al. 1990; Kim et al. 1993). It has been suggested that bacteria also produce paralytic shellfish toxins (PSTs) (Kodama et al. 1990a; Gallacher et al. 1997; Hold 1999). This remains controversial, because a unchallengeable proof is still lacking. Bacteria attached to or associated with toxic algae may also produce toxic substances, or influence the toxicity of the algae (Buck and Pierce 1989; Tosteston et al. 1989). Some authors reported higher toxicity in axenic cultures (Singh et al. 1982; Dantzer and Levin 1997), whereas others reported less toxicity (Doucette and Powell 1998). There are also many reports about the association of bacteria with toxic algae in culture (Tosteson 1989; Doucette and Trick 1995; Lafay et al. 1995; Babinchak et al. 1998; Prokic et al. 1998; Hold et al. 2001a; Lewis et al. 2001; Simon et al. 2002), but the role of the bacteria in this relationship has not been resolved. Lewis et al. (2001) assumed that for *Alexandrium* spp., a small number of specialised bacteria are closely associated with the surface of the dinoflagellate and the algae could act as a carbon source for the attached bacteria. They also showed intracellular bacteria in all growth phases and all life-cycle stages of *Alexandrium* spp. (Lewis et al. 2001).

Several putative toxic bacteria have been isolated from toxic and non-toxic cultures of *Alexandrium tamarense*, a dinoflagellate that is associated with PSP outbreaks. The first putatively toxic bacteria, referred to as the PTB strain, isolated from *A. tamarense* was initially identified as a member of the genus *Moraxella* of the β-subclass of Proteobacteria (Kodama et al. 1990b). The putatively toxic bacteria PTB-1, 6, and 7, originally isolated from *Alexandrium tamarense* cultures from different areas in Japan, actually belong to the γ-subclass of Proteobacteria and are likely to be a new genus in that group, clearly unrelated to the genus *Moraxella* (Kopp et al. 1997; Groben et al. 2000).

PTB-1 bacteria were isolated from a highly toxic *A. tamarense* clone, OF84423-D3, Ofunato Bay, N. Japan, PTB-6 bacteria from a weakly toxic *A. tamarense* clone, PT-5, Harimanada Bay, S. Japan (Ogata et al. 1990; Doucette et al. 1998) and PTB-7 bacteria from a non-toxic *A. tamarense* clone (CU-1, Gulf of Thailand). The latter dinoflagellate was later reclassified as *A. affine* (Scholin and Anderson 1994). The PTB-1 strain has been shown to produce sodium channel blocking activity (Ogata et al. 1990). Other authors reported an autonomous production of multiple PSTs derivates by the PTB-1 strain, confirmed by HPLC (Doucette and Trick 1995). The toxicity of the PTB-6 bacteria has not so far been extensively studied. This bacterial strain was also shown to be capable of PST production confirmed by HPLC and a mouse neuroblastoma assay, respectively (Ogata et al. 1990). However, confirmatory mass spectrometer data of the autonomous PST production by marine bacteria are still lacking, therefore these bacteria remain only putatively toxic (Groben et al. 2000).

In this study, a possible physical re-association of putatively toxic PTB-bacteria to a toxic *Alexandrium tamarense* clone was investigated, to gain more information about the physical interactions between algae and bacteria. Group, genus, and species-specific eubacterial probes targeting 16S rRNA were tested on the dinoflagellates in culture to localise and identify the bacteria. Tyramide signal amplification together with fluorescence *in situ* hybridization (TSA-FISH) was applied to amplificate the fluorescence signals of the hybridized rRNA probes. This detection method uses the catalytic activity of horseradish peroxidase (HRP) to generate a strong labelling of the nucleic acid *in situ*. This results in strong signal amplification and enables the detection of rare events, such as the presence of a single bacterium inside an autofluorescence exhibiting dinoflagellate cell. With following confocal scanning microscopy (CLSM) bacteria can be identified, localized, and quantified, either attached to the dinoflagellate or within the alga.

MATERIAL AND METHODS

Organisms. Dinoflagellates *Alexandrium tamarense* (highly toxic strain, OF 84423-D3, Ofunato Bay, N. Japan) and *Gyrodinium instriatum* (non-toxic, LME 176 a, Lisbon, Portugal). The non-thecate dinoflagellate *Gyrodinium instriatum* LME 176a was used as a positive control in this study because it contains intracellular bacteria, both in the nucleus and in the cytoplasm (Silva and Franca 1985; Franca 1994; Alverca et al. 2002).

Bacterial strain PTB-1 (originally isolated from a highly toxic *A. tamarense* clone OF84423-D3, Ofunato Bay, N. Japan) and bacterial strain PTB–6 (originally isolated from a weakly toxic *A. tamarense* clone, PT-5, Harimanada Bay, S. Japan)

Maintenance and growth of organisms**.** *The dinoflagellates were cultured in F/2 medium (Guillard 1975)* The dinoflagellates were maintained in IMR/2 medium (Eppley et al. 1967) at a photon flux density of 100 µmol m^{-2} s^{-1} at 15°C and a 14:10 h light/dark photon cycle. Relative fluorescence of the dinoflagellate cells was measured at the day of sampling in a Turner Model 10-AU Flurometer as an indication of relative growth rate per day. Aliquots of bacterial glycerol stocks hold at –80°C were plated on a complete seawater medium based agar (SWC, Haygood and Nealson 1985) and incubated at 23°C. Colonies were picked and inoculated in SWC medium, incubated overnight at 23°C while shaking at 180 rpm and their density was photometrically determined.

Fixation and filtration of Alexandrium tamarense *and PTB-bacteria*. The non-axenic *Alexandrium tamarense* culture were filtered through a 0.5 µm filter (Millipore, Bedford, MA, USA) and repeatedly washed, to reduce the bacterial background of the culture. An antibacterial treatment of the culture would have reduced the viability of this *Alexandrium tamarense* strain, as determined in preliminary studies and therefore this approach was abandoned. Two bacterial loadings of PTB-1 and PTB-6 bacteria, respectively, were used. One inoculum at 10^3 and the second at 10^5, to investigate the effect of different bacterial loadings on the growth of the dinoflagellates.

At a time 5 ml of the dinoflagellate/bacteria mixture was fixed in freshly made 4% paraformaldehyde (pH 7.2, in 1 X Phosphate buffered saline, PBS, 0.2 µm filter sterilised) in solution for 1 h at 4°C. The dinoflagellate/bacteria mixture was sampled at 0 h, 48 h, and 72 h and after 96 h. At a time one ml fixed dinoflagellate/bacteria mixture was filtered onto white 0.2 µm pore size, 25 mm diameter (Millipore, Bedford, MA, USA), or onto black

polycarbonate filters (Osmonics, Minnetonka, USA). The filtration on white or black filters was made in triplicate for each experiment and sampling day. The filters were dehydrated in a graded ethanol series (50, 80, 100%) for 5 minutes each.

Fixation and filtration of Gyrodinium instriatum *and PTB-bacteria*. One bacterial loading of 10^5 PTB-1 and PTB-6 bacteria, was inoculated into the *Gyrodinium instriatum* culture. At each sampling time 5 ml of the dinoflagellate/bacteria mixture was filtered onto white polycarbonate filter. *G. instriatum* is very sensitive to PFA fixation and tended to burst, therefore a saline ethanol fixation was conducted (Scholin et al. 1996) prior to PFA-fixation. The filters were fixed with 3 ml freshly prepared saline EtOH (25 ml Ethanol, 2 ml dH_2O, 3 ml 25X SET [3.75 M NaCl, 25 mM EDTA, 0.5 M Tris, pH 7.8, 0.2 µm filter-sterilised]), which was added directly to the filter and incubated for 1 h at room temperature and filtered down. Three ml hybridization buffer (189.0 ml dH_2O, 48.0 ml 25X SET, 2.4 ml 10% IGEPAL-CA630, filter-sterilised, + 0.75 ml poly A [stock 10 mg/ml]) was given onto the filter and incubated for 5 minutes at room temperature and filtered down. After this initial saline/ethanol fixation, the dinoflagellate/bacterial mixture was fixed in 4% PFA for 1 h at 4°C and further processed as described for the *A. tamarense*/bacteria samples. Sampling times were at 0 h, 48 h and after 96 h.

Fluorescence in situ *hybridization.* The filters were cut into four pieces with a sterile scalpel. For each filter piece varying probes (Table 1, Fig. 1) were used for hybridization. To ensure a proper probe penetration into the dinoflagellate cells, the algae cells were treated with lysozyme. One ml of a lysozyme solution (5 mg ml^{-1} in sterile Milli-Q) was applied to each filter and incubated for 30 minutes at 37°C in a humid chamber. To stop the enzymatic reaction, the filters were rinsed three times for 1 minute in 5 ml sterile Milli-Q-water and dehydrated again by a graded ethanol series (50, 80, 100%) for 5 minutes each (Amann et al. 1995). Twenty µl hybridization buffer (20mM Tris/HCL, [pH 8.0], 0.9 M NaCl, 10% deionized formamide, 0.01% SDS) + 2 µl labelled probe (50 ng μl^{-1}) were added to each filter piece. Hybridizations were performed at 46°C for 2-3 h in the dark. The filter pieces were washed (wash buffer: 10% formamide: 20 mM Tris /HCl [pH 8.0], 0.45 M NaCl, 5 mM EDTA, 0.01% SDS, 20% formamide: 20 mM Tris /HCl [pH 8.0], 0.23 M NaCl, 5 mM EDTA, 0.01% SDS) two times for 10 minutes at 48°C, while shaking (Amann et al. 1990) and separated in those for TSA-FISH and in those without an enhancement of the fluorescence signal. The filters without a signal enhancement were washed for a few seconds in sterile Milli-Q and air dried. Cells were mounted in Citifluor (Citifluor products, London, United

Kingdom) and counterstained with DAPI by applying a Citifluor/DAPI mixture (1 ml Citifluor + 0.5 ml sterile water + 1.5 µl DAPI [stock 1 µg μl^{-1}]) directly onto the filter and incubated for 10 minutes in the dark. The filters were washed in sterile Milli-Q for five minutes and air dried. Coverslips were placed over the dry filters and the slides were sealed with nail varnish. For each experiments and each sampling day three filters were hybridized. All hybridization steps were performed in the dark.

TSA-FISH: 1. Horseradish Peroxidase (HRP)-labelled probes. After the last washing step, the filter pieces, which were hybridized with Horseradish Peroxidase (HRP)-labelled probes were rinsed in sterile Milli-Q and equilibrated for 15 minutes in TNT-Buffer (0.1 M Tris-HCL, [pH 7.5], 0.15M NaCl, 0.05% Tween 20). Forty per cent dextran sulfate (w/v in sterile Milli-Q water) was mixed 1:1 in 2 X Amplification Diluent of the TSA-direct Kit (NEN Life Science Product Inc., Boston, MA, USA). Dextran sulfate reduces the unspecific staining of non-target cells during long-term incubation (Schönhuber et al. 1999). One µl fluorescein tyramide (FT [TSA-Direct Kit]) was given to 50 µl of this mixture, to create the FT-working solution. Twenty-five µl of this FT-working solution per filter quarter was applied and the filter quarters were incubated for 30-45 minutes at room temperature in the dark. To remove non-implemented FT and to stop the enzyme reaction, the filter quarters were washed twice in TNT-Buffer for 15 minutes at 55°C (Schönhuber et al. 1997 and 1999), rinsed in sterile Milli-Q water, air-dried and mounted and counterstained with Citifluor and DAPI as mentioned before.

2. FITC labelled oligonucleotide probes. After the last washing step in the standard FISH procedure, filters with FITC-labelled probes were rinsed in sterile Milli-Q and equilibrated for 15 minutes in TNT-Buffer and air dried. The dry filter was blocked with 40 µl TNB-Buffer (0.1M Tris-HCl, pH 7.5, 0.15M NaCl, 0.5% blocking reagent [Roche, Mannheim, Germany]) per filter piece for 30 minutes at room temperature to prevent unspecific binding of the antibody and then drained off carefully. Twenty-five µl anti-fluorescein-HRP diluted 1:100 in TNB were added to the filter quarters, incubated for 30 minutes at room temperature and washed three times for five minutes in TNT-buffer at room temperature, while shaking. The FT stock solution (in dimethyl sulfoxide) has to be diluted 1:50 in 1 X Amplification Diluent (NEN life science products, USA) to set up the FT-working solution. Twenty-five µl FT working solution was applied to each filter piece and incubated for 5 minutes at room temperature. Afterwards the filters were washed two times

for 15 minutes in TNT-Buffer at room temperature while shaking, air dried, mounted and counterstained as described before.

Negative controls. On filter quarter of each filter was cut again in half pieces. One piece was hybridized with FITC labelled NON-338R which has a sequence complementary to EUB338 and served as a negative control for unspecific binding (Manz et al. 1999). TSA-FISH without a probe was made with the other piece to verify the received hybridization signals. Additionally, FISH was made with the unwashed *A. tamarense* clone OF 84423-D3, without inoculated bacteria to determine, if a natural background floor of PTB-1 or PTB-6, respectively, was present in the culture. The used probes and hybridization pattern were the same as in the hybridization of the reassociation experiments (see Table 1 and Fig. 1).

Counts of Alexandrium tamarense *by epifluorescence microscopy and confocal scanning microscopy.* Slides were viewed with a Zeiss Microscope (Axioskop 2 plus, Oberkochen, Germany) fitted for epifluorescence microscopy under oil immersion (Leica immersion oil, Oberkochen, Germany) with the appropriate filter set (Zeiss filter sets, 03: UV G 365; 04: blue BP 450-490 nm; 05: green BP 510-560 nm). The epifluorescence images were aquired with a X 63 objective (Plan-Apochromat, numerical aperture 1,40 Oil DIC) and analysed with the Leica program Axio vision 3.1.

All dinoflagellate-cells were counted with visible bacteria attached to the dinoflagellates and those without PTB-bacteria. Three filters were analysed for every experiment and sampling day. To locate possible intracellular bacteria the cells were scanned by confocal scanning microscopy. Optical sections were acquired with a Leica confocal laser scanning microscope (TSNT, 165081, Leica, Oberkochen, Germany) equipped with an argon-krypton laser. The dinoflagellates were observed using excitation/ emission lines of the krypton argon laser: blue light (excitation 488 nm, emission 522/32 nm) to visualise the fluorescence of FITC and HRP-hybridized bacteria and green light (excitation 568 nm, emission 605/32 nm) to visualise CY3. The confocal images were acquired with a X 40 objective (NA Oil Planapo numerical aperture 1,25 x 0,75 Leica). The confocal images were analysed using the Leica-TCSNT program.

RESULTS AND DISCUSSION

So far, only little is known about the attachment behaviour of bacteria to algal cells (Kogure et al. 1982; Vaqué et al. 1990; Worm and Sondergaard 1998). Hold et al. (2001b) have shown from isolation and culture experiments that a number of different bacterial species are associated with dinoflagellates, some of which are common to each of the dinoflagellate cultures examined, whereas others appear to be unique to a particular dinoflagellate. Our study showed a difference in the re-association of the PTB-1 bacteria originally isolated from the dinoflagellate used in this study and PTB-6 bacteria originally isolated from another related weakly toxic *Alexandrium* clone (PT-5).

Alexandrium tamarense cultures to which a PTB-1 inoculum of 10^3 and of 10^5, respectively, was introduced increased similarly in numbers up to 72 h after inoculation. Thereafter, dinoflagellate cell growth decreased. *A. tamarense* inoculated with PTB-6 (inoculum 10^3) showed an increased cell growth up to 96 h when sampling stopped. The *Alexandrium* culture inoculated with PTB-6 (inoculum 10^5) increased in numbers until 72 h after inoculation and then began to decrease (Fig. 2), probably because of a depletion of nutrients in the culture media.

PTB-1 bacteria were found attached to *A. tamarense* cells and free in the media. TSA-FISH produced very bright fluorescence signals, therefore had any of the introduced bacteria reassociated intracellularly, it would have been possible to detect them because a positive fluorescence signal would have overcome the dinoflagellate's autofluorescence. The cell numbers of attached PTB-1 bacteria to *A. tamarense* numbered from 1 to 12 bacteria per dinoflagellate cell (inoculum 10^3) and from 3 to 13 (inoculum 10^5; Fig. 3).

PTB-6 bacteria were found free in the culture media, but rarely found attached to *A. tamarense* and when found, cell numbers of associated PTB-6 bacteria to the dinoflagellate surface ranged from 3-12 bacteria per dinoflagellate cell (inoculum 10^3) to 5 to 15 (inoculum 10^5; Fig. 3).

The numbers of attached bacteria to algae cells in culture found by other authors are in the same range like our findings. Rausch de Trauenberg and Soyer-Gobillard (1990) found less than ten bacterial cells on cultured cells of *Prorocentrum micans* during exponential and stationary phases. Simon et al. (2002) showed that putatively toxic bacteria are able to attach to *A. tamarense* in culture, especially as the culture entered stationary growth phase. The authors monitored attachment of putative toxic bacteria to phytoplankton over a time period of 35 days and reported from less than ten bacteria per dinoflagellate until Day 10 after their

experiment started, and an average number of 9, 16 and 30 attached bacteria at Day 34. The authors suggested that the physiological state of dinoflagellates influenced the ability of the investigated putative toxic bacteria to become attached to the dinoflagellate. However, attachment of these bacteria did not induce toxin production by originally non-toxic dinoflagellates (Simon et al. 2002).

In this study, the number of attached bacteria range in the same manner over the course of the experiment, and did not change in the monitored incubation time. This bacterial association to the dinoflagellate cells are probably stimulated by organic exudates produced by the algae, and the numbers of attached bacteria could represent the optimal numbers, e.g., for the exchange of nutrients between both organisms.

No PTB-1 or PTB-6 bacteria were found intracellularly in *A. tamarense*. However, intracellular bacteria of the □-subclass of were found intracellularly located within *A. tamarense* BAHME182 by FISH and CLSM (Töbe et al. unpublished). In contrast to these findings no intracellular bacteria were found in other *A. tamarense* strains (PLY173a and NEPCC 407) after FISH by Biegala and co-authors (2002). The authors suggested that these strains did not contain any intracellular bacteria at the time of sampling, although in *Alexandrium*-strain NEPCC 407 bacteria were detected by transmission electron microscopy (TEM), although in low abundance (Lewis et al. 2001). Thus, great differences in the intracellular bacterial population exist between different strains of *Alexandrium tamarense* and this should be investigated further to get more deep insights in the relationship between algae and their associated bacteria.

The positive control *Gyrodinium instriatum* clearly showed endocytic and extracellular attached bacteria in the cytoplasm detected by hybridization with the FITC labelled eubacterial probe 338R, following TSA-FISH combined with CLSM. No PTB-bacteria were found attached to the dinoflagellate or intracellularly in *G. instriatum* (Fig. 4-5).

The negative controls showed no unspecific binding of NON-338 to bacterial cells. However, some dinoflagellate cells were coloured green generated by TSA alone. No PTB-bacteria were found as a natural background flora in the washed and filtered *Alexandrium* culture without inoculated bacteria, hence the detected bacteria were indeed newly-associated bacteria.

Although the number of associated bacteria with an individual cell of *A. tamarense* was in the same range in both cultures, PTB-1 bacteria re-attached more frequently to this dinoflagellate than did PTB-6 bacteria. The cultures with the PTB-1 reintroduced bacteria also seemed to grow slower than the culture with the reintroduced PTB-6 bacteria. This

observation needs to be investigated further to determine if in some manner the bacteria influenced the general health of the dinoflagellate cell.

With FISH in combination with TSA and confocal scanning microscopy we have shown a specific attachment of the inoculated putatively toxic PTB strains to a highly toxic *Alexandrium tamarense* culture on the cellular level without destroying the morphological features of the organisms. However, further investigations on this subject are necessary to unravel the real meaning of the physical interactions between bacteria and microalgae, especially in environmental studies.

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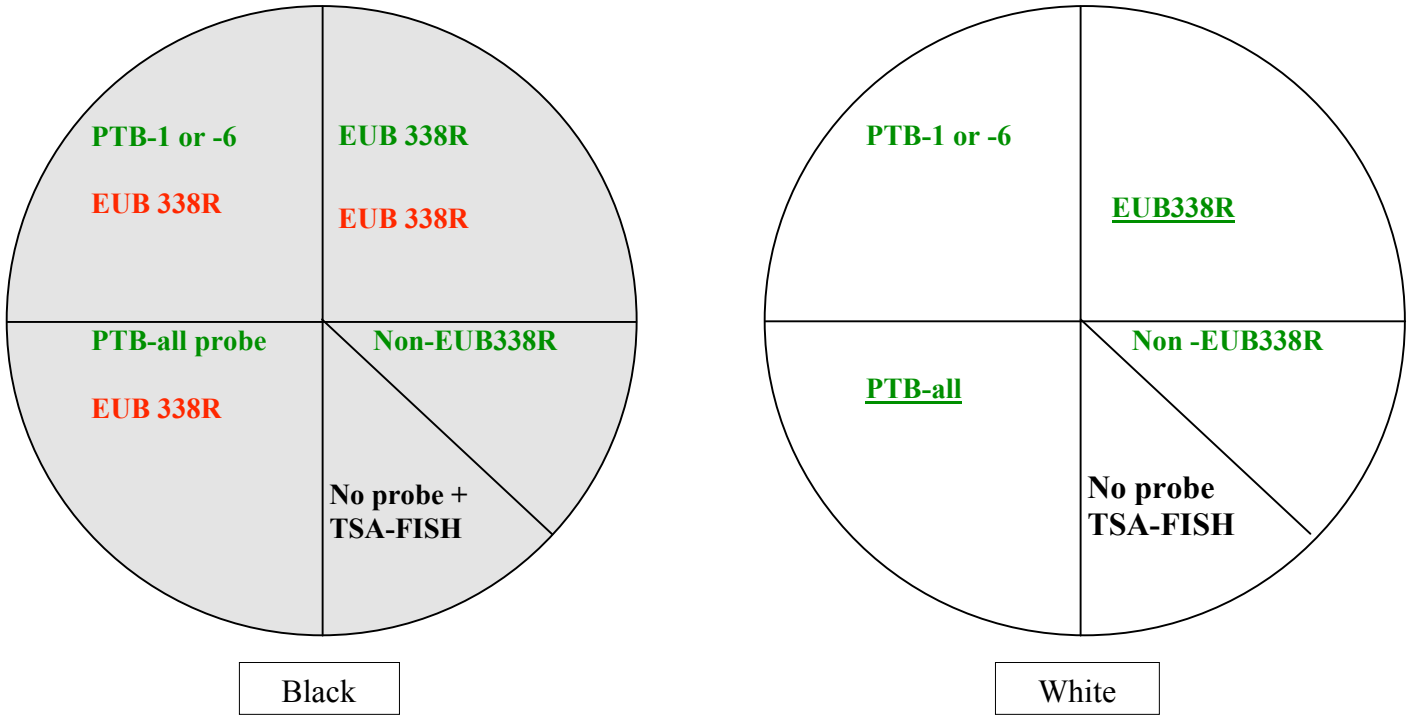

Fig. 1: FISH pattern: FITC-labelled oligonucleotide probes marked in green; HRP labelled probes marked in green, underlined; CY3-labelled oligonucleotide probes marked in red.

Table.1: Sequences of the used oligonucleotide probes

Oligonucleotide probes	**Probe sequence**	**Target strain**	**Hybridization temperature and Formamide concentration**
PTB-all/646 (Groben et al. 2000)	5'-tct cgg act caa gac ttc-3'	PTB-clades 1 and 2	46°C, 10%
PTB-1/1014 (Groben et al. 2000)	5'-cga agg gaa aaa cga cat ct-3'	PTB-1 cluster	46°C, 10%
PTB-6 and 7/997 (Groben et al. 2000)	5'-ctc tgg aag tag cac caa a-3'	PTB-6 and 7	46°C, 10%
EUB338 (Amann et al. 1990)	5'-gct gcc tcc cgt agg agt-3'	Eubacteria	46°C, 20%
Non-EUB338 (Manz et al. 1999)	5'-act cct acg gga ggc agc-3'	Serves as a negative control	46°C, 20%

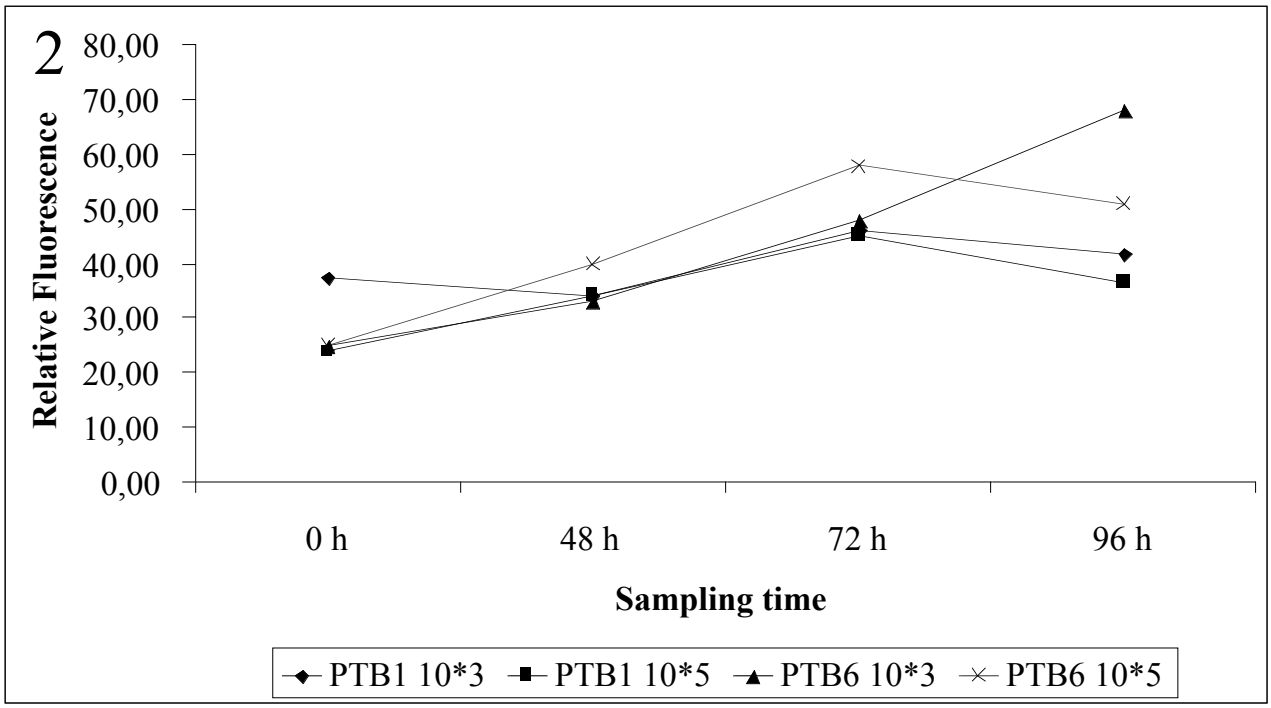

Fig. 2 Relative fluorescence of *Alexandrium tamarense* and reintroduced bacteria as an indication of relative growth rate at each sampling time.

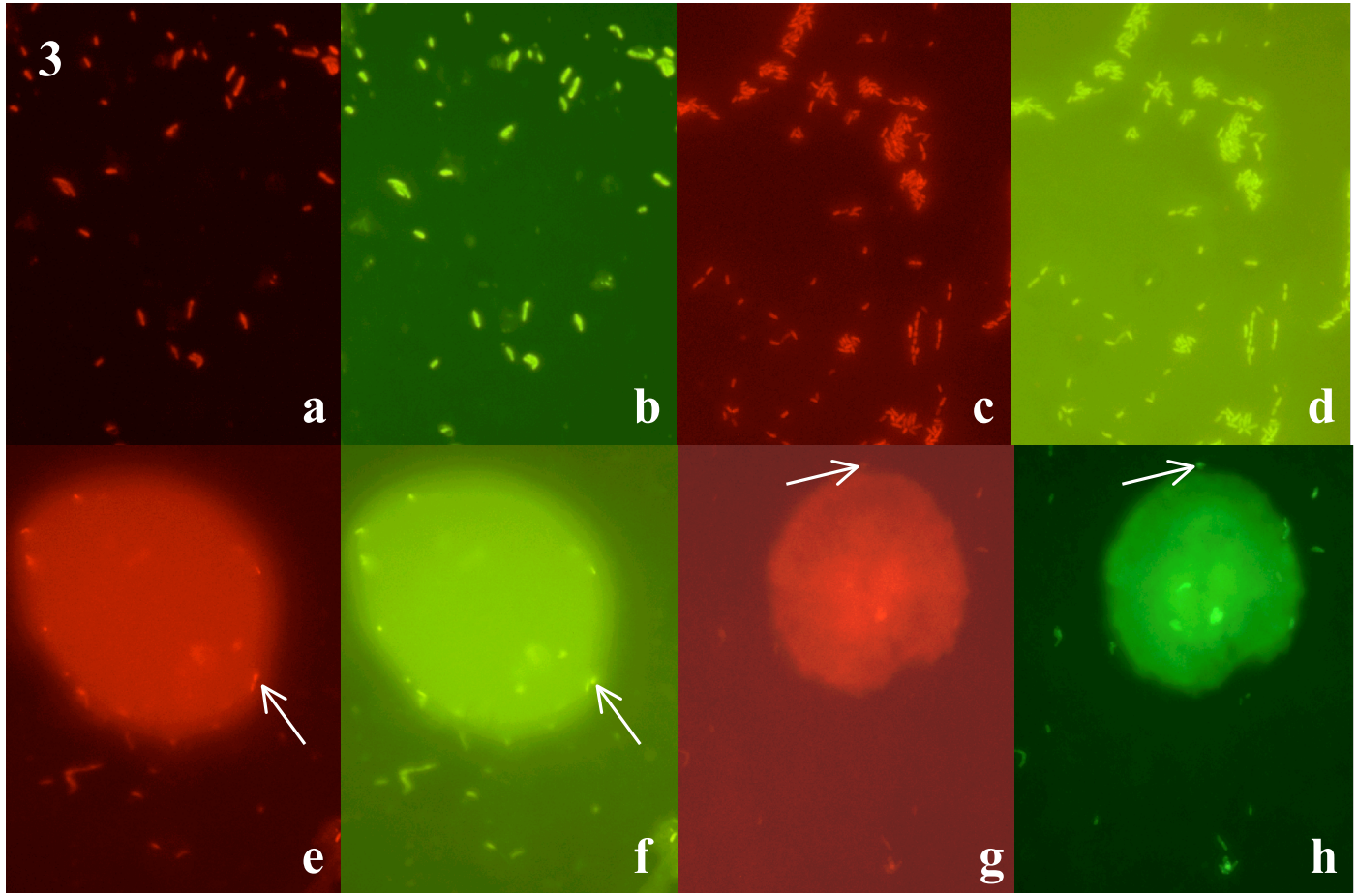

Fig. 3: a) PTB-1 bacteria hybridised with EUB 338R, CY3-labelled; b) Same cells hybridised with PTB-1 probe, FITC-labelled; c) PTB-6 bacteria hybridised with EUB 338R, CY3-labelled; d) Same cells hybridised with PTB-6 probe, FITC-labelled; e) PTB-1 bacteria attached to *A. tamarense* hybridised with EUB 338R, CY3-labelled; f) Same cells hybridised with PTB-1 probe, FITC-labelled; g) PTB-6 bacteria attached to *A. tamarense* hybridised with EUB 338R, CY3-labelled; h) Same cells hybridised with PTB-6 probe, FITC-labelled. X 63 enlargement

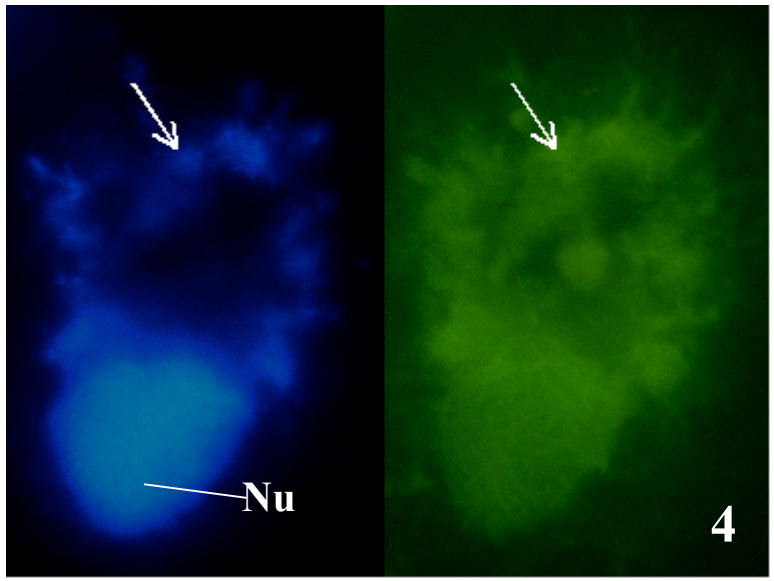

Fig. 4: Epifluorescence microscopy of *Gyrodinium instriatum.* a) DAPI staining of *G. instriatum*. b) Same cell hybridised with EUB 338R, FITC labelled and TSA enhancement. Arrows show endocytoplasmatic and endonuclear bacteria. Nu: Nucleus. X 63 enlargement.

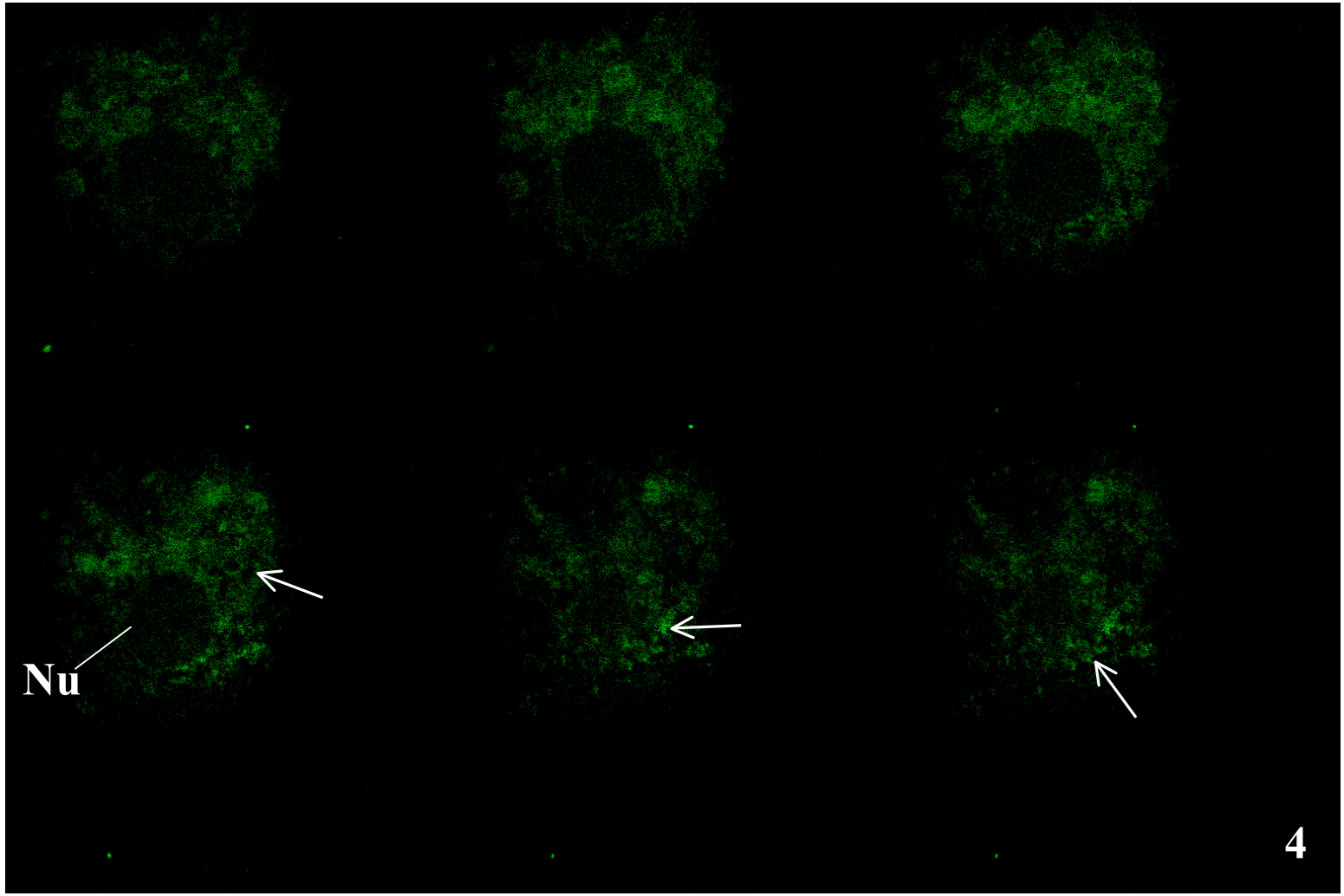

Fig. 4: CLSM Optical sections of *Gyrodinium instriatum,* 0,7 µm thick, 450-490 nm excitation. TSA-FISH with EUB 338R , FITC labelled. Arrows show hybridised endocytoplasmatic and endonuclear bacteria. Nu: Nucleus. X 40 enlargement.

3. SYNTHESIS

3.1 Bacterial flora of cultured dinoflagellates

Bacteria are an integral part of the dinoflagellates physical environment and can occur free in the medium, attached to the dinoflagellate cell, or located within the algae cell (Silva 1978; Gordon et al. 1994; Rausch de Trauenberg et al. 1995; Lewis et al. 2001; Seibold et al. 2001; Alverca et al. 2002). These physical interactions between bacteria and algae are highly variable in space and time (Grossart 1999). It has been shown that marine bacteria are dominated by the subclass of □- and □□Proteobacteria and the Cytophaga-Flavobacterium-Bacteroides (Glöckner et al. 1999), and members of these bacterial classes have been found in association with different species of dinoflagellate (Lafay et al. 1995).

In **Publication I** the bacterial community from laboratory cultures of toxic and non-toxic dinoflagellates of the genus *Alexandrium* by FISH and confocal scanning microscopy (CLSM) were identified, and localised. Besides FISH, cyanoditolyltetrazolium chloride (CTC) was used in combination with CLSM to detect metabolically bacteria inside the dinoflagellate cells. The CTC detection method of metabolically active bacteria does not discriminate among different bacterial groups, but this was achieved with the use of rRNA probes. FISH with 16S- and 23S ribosomal RNA (rRNA) oligodeoxynucleotide probes detected Eubacteria, the □-subclass of Proteobacteria and bacteria of the genus *Roseobacter* attached to the cell surface of *A. tamarense* and *A. lusitanicum*. Bacteria of the □-subclass of Proteobacteria were found in limited numbers intracellularly in *A. tamarense*. *Alteromonas*-clade bacteria were found attached to the cell surface of *A. andersonii* and free in the culture medium. *Alteromonas*-clade and *Roseobacter*-clade bacteria are common in marine environments and are often found associated with microalgae (Lafay et al. 1995; Doucette and Trick 1995; Gallacher et al. 1997; Hold et al. 2001a; Alavi et al. 2001). A dominance of *Roseobacter*-clade bacteria with the occurrence of toxic and non-toxic dinoflagellates has also been described in studies investigating bacteria in field material from HAB areas (Gerdts et al. 2000; **Publication II** and **Appendix to Publication II**). Gerdts et al. (2000) reported a domination in the water column by a □-subclass Proteobacteria during toxic *A. tamarense* blooms in the area of the Orkney Islands and the Firth of Forth in 1998. Their results indicated, that Proteobacteria of the □-subclass were closely associated with the blooms of toxic *A. tamarense*.

In **Publication I**, FISH experiments revealed bacteria attached to several but not all the dinoflagellates investigated. Bacteria were found attached to *A. tamarense, A. lusitanicum* and *A. andersonii.* Additionally, intracellular bacteria were found within *A. tamarense*. Hybridization with group level probes failed to detect any bacteria inside or attached to the surface of *A taylorii* and *A. ostenfeldii.* However, the autofluorescence of *A. taylorii* is very high and could have covered possible hybridization signals. It is also possible that bacteria of *A taylorii* and *A. ostenfeldii* were not in active growth period and therefore did not have a sufficiently high number of ribosomes, which are the targets for the binding of the labelled oligonucleotide (Lee et al. 1993). Another reason for the different hybridization results could be that the probes failed to penetrate the cell wall of the dinoflagellate, because these cells could be less sensitive to lysozyme digestion, than others of the same species.

CTC serves as an indicator for the detection of metabolically active bacteria within microalage, because it can only be reduced to the water insoluble red fluorescent formazan, when metabolically active cells, such as bacteria, reduce it by capturing electrons derived from the respiratory chain and therefore preventing its release from the cell (Rodriguez et al. 1992; Córdova et al. 2002). Metabolically active bacteria were found in *A. tamarense* and *A. lusitanicum*. However, no intracellular bacteria were found in *A. lusitanicum* by FISH. No metabolically active bacteria were found within *A. andersonii, A. taylorii* and *A. ostenfeldii*. CTC caused sometimes a background coloration of the dinoflagellates probably because of a residual respiratory activity of the algae (Córdova et al. 2002), so that small amounts of CTC were reduced and remain associated to the cytoplasm. The FISH technique and the CTC treatment of dinoflagellates cannot be performed concurrently and therefore on the same dinoflagellate cell, because of the different sample preparation. Therefore, the results of both methods can only be compared among different dinoflagellate cells of one species.

In this study, using rRNA probes it was possible to identify the genus/species of the bacteria inside the dinoflagellate and attached to the dinoflagellates cell surface. Moreover, metabolically active bacteria were shown within dinoflagellates of the genus *Alexandrium* by the use of the compound CTC. This data provides important information about the association between bacteria and algae and their bacterial community.

In summary, different members of the genus *Alexandrium* were screened for intracellularly located and/or extracellularly attached bacteria by application of group or clade specific fluorescently-labelled 16S and 23S rRNA probes. Fluorescence *in situ* hybridization combined with confocal laser scanning microscopy (CLSM) revealed extracellularly attached bacteria to the cell surface of *Alexandrium tamarense*, *A. lusitanicum* and *A. andersonii*. Intracellularly associated bacteria were found in *A. tamarense.* Moreover, the presence of metabolically active intracellular bacteria within log and stationary phase-fixed *Alexandrium tamarense* and *A. lusitanicum* was documented with the use of cyanoditolyltetrazolium chloride in combination with epifluorescence microscopy and CLSM.

3.2 Monitoring of a bacterial contribution to saxitoxin in the environment

It has been reported that bacteria play an important role in the production of Paralytic Shellfish Toxins (PSTs), although the precise mechanisms remain unclear. However, if both bacteria and the dinoflagellate play necessary roles in toxin production, it cannot be explained how such an association co-evolved. It has been suggested that the 'saxitoxin genes' may have evolved once, probably in a prokaryote, and were subsequently transferred by gene transfer to other aquatic microorganisms (Plumley and Wei 1996; Plumley 1997 and 2001). Nevertheless, cyanobacteria from which some species are reported to produce saxitoxins are prokaryotes and other bacteria produce the chemically close relative tetrodotoxin, so it is likely that the toxin is of bacterial origin.

So far, definitive evidence for PST production by marine bacteria by the provision of spectral data and the isolation and purification of PSTs from bacteria are lacking. Quantities of PSTs for bacteria range between 0.004 to 3.71 x 10^{-4} pg STX equiv. $cell^{-1}$ (Gallacher and Smith 1999). This is considerably lower than the 0.12 pg STX equiv. $cell^{-1}$ reported for the cyanobacterium *Anabaena circinalis* (Negri et al. 1997) and the 6-58 pg STX equiv. $cell^{-1}$ reported for a range of isolated *Alexandrium fundyens*e and *A. tamarense* (Anderson et al. 1994).

Interactions between algae and bacteria are commonly observed in the marine environment, and bacteria are increasingly postulated as potentially important regulators in processes of algal bloom initiation, maintenance and decline (Doucette 1995). Up to now, the interactions of bacteria and HABs have not been investigated to a great extent (Doucette et al.

1999), although coupling of both organisms through the microbial loop is well-documented (Azam et al. 1983).

Only little information is available with regard to the identity of bacterial populations occurring with toxic *Alexandrium* species in the environment. The results of **Publication II** and **Appendix to Publication II** 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. In this studies, rRNA probes designed for purportedly toxic bacteria (Brinkmeyer et al. 2000) were successfully applied to Lugol's-fixed water samples obtained from the Orkney Islands in two monitoring years. The successful application of the FISH technique 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. Bacteria were successfully detected using FISH in samples from as far back as 1997, but the signal was considerably lower as compared to that obtained in fresh samples from 1999 and 2000. One possible alternative approach is the use of dot blot hybridizations, because they give stronger signals than FISH in older samples. It may also be possible to improve the FISH signal with a tyramide signal amplification (TSA) method used in **Publication IV**. Nevertheless, bacteria cross-reacting with the probes were easily detected, which would suggest that they were actively growing members of the bacterioplankton.

Bacteria reacting to probes for the *Roseobacter* and *Alteromonas*-clades in samples of the year 1999 were common, consisting of up to 46% of the total bacterial population and the number of bacteria cross-reacting to the species-specific probes was approximately 1% of the number detected by the clade probes. In 2000, *Roseobacter* and *Alteromonas* spp. consisting of up to 57% of the total bacterial counts at The String in the end of May and up to 34% in the beginning of June at Scapa Flow. However, the number of bacteria recognised by the species-specific bacteria was only up to 0.5% of the number detected by the clade probes. This indicates that there is a large percentage of the bacterial community belonging to these two clades that could not be accounted for using the species-specific probes. The vast majority of marine bacterial diversity remains undescribed, and it is possible that our probes may have even targeted as yet unknown, perhaps closely related bacteria with the same target sequence.

In the samples of the year 1999, a weak significant positive relationship between *Alteromonas*- and *Roseobacter*-clade counts and those of *Alexandrium* spp. at Scapa Flow was obtained. At The String there was also a weak correlation between *Alexandrium* and the *Alteromonas*-clade counts but not the *Roseobacter*-clade. Data were not available on the rest

of the phytoplankton community and therefore it was not possible to determine whether any relationship existed between the bacteria and other phytoplankton species. In 2000, the bacterial counts were again correlated against the counts for *Alexandrium* and with values of PST in mussels harvested from the same area. However, in 2000 no mussels were harvested from The String and thus only comparisons from Scapa Flow could be made. *Roseobacter*- and *Alteromonas*-clade bacteria were not significantly correlated with *Alexandrium* spp. at either site. Species-specific bacteria of both clades were negatively correlated with *Alexandrium* spp. at The String and positively correlated at Scapa Flow, but neither correlation was significant. *Roseobacter* and *Alteromonas*-clade bacteria were not significantly correlated with mussel toxicity nor were species-specific bacteria of either clade. These environment studies on Lugol's fixed seawater samples showed that there was no significant correlation between the number of putatively toxic bacteria cross-reacted with the used oligonucleotide probes, and PST in mussels sampled at the same time. However, neither year was considered to be particularly noteworthy in terms of PSP toxification.

It has been reported that PST were present in particles of a similar size fraction (0.45-5 μm) to bacteria in seawater from Ofunto Bay, Japan during times when bivalve toxicity increased in the absence of toxic dinoflagellates. Therefore, it was hypothesised that bacteria could be the source of the toxins detected in these shellfish (Kodama et al. 1990). However, the presence of PSTs in shellfish in times when no toxic phytoplankton were detected in surrounding waters is more probable generated by a different toxin source. Toxic dinoflagellate algae can form cysts that rest in the sediment as the bloom declines (Vale and Sampayo 2001). *Alexandrium* cysts have been detected in the area of the Orkney Islands, thereby providing potential seed beds for the vegetative dinoflagellate cells (Macdonald, personal communication). These cysts could be as toxic as the suspended vegetative forms that are present during a toxic bloom. Shellfish being bottom dwelling filter feeders, can continue to consume cysts during non-bloom periods and accumulate PSP toxin in this way (Vale and Sampayo 2001). This might explain why after a bloom certain shellfish species maintain toxicity for long periods and also why shellfish show toxicity when no *Alexandrium* spp. were detected in the water column (Vale and Sampayo 2001). Additionally, some shellfish take longer to depurinate than others and thus can remain toxic long after the dinoflagellate cells disappear from the water column.

From this study there does not seem to be evidence to support the suggestion that bacteria could be responsible for PST in mussels at the concentrations frequently observed in monitoring programs.

In summary, data presented in these studies are the most comprehensive to date with regards to examine the bacterial community, including purportedly toxic bacteria, occurring during periods of shellfish toxicity and when *Alexandrium* spp. were present in the water column. The successful application of fluorescently labelled oligonucleotide probes to Lugol's fixed seawater samples were described for the first time in this study. The toxigenic bacteria were detected in high numbers in the water column whether *Alexandrium* spp. were present or absent and during periods when mussels contained Paralytic Shellfish Toxins. A statistically significant association were found between bacteria belonging to the *Alteromonas*-clade in both years and some specific *Alteromonas* species to numbers of *Alexandrium* cells in 1999.

3.3 Effect of putatively toxic bacteria on mussels toxicity

Almost virtually nothing is known about the effect of putatively toxic bacteria on shellfish toxicity. In **Publication III** the presence of bacteria in the gut of *Mytilus edulis* up to 6 hours after feeding ceased was demonstrated. The mussels did not appear to reject any of the bacteria when fed a mixed assemblage, because the used probes detected each of the bacteria in the sections of the mussel's gut. The strength of the signals of the clade specific probes show approximately the proportion of the fed species. The decrease in the signal strength after 6 hours may indicate that the bacteria were being digested by the mussel.

In this study the bacteria did not invoke toxicity in the mussels. However, in preliminary investigations a known sodium channel blocking toxin producing bacterium (407-2) render mussels toxic after feeding experiments. The concentration of this bacterium in seawater fell by 73% in the first hour, compared to only 2% in control jars. The rapid removal of this bacterial strain correspond to the detection of 0.84 µg STX equiv./100 g mussel flesh, with the highest level of 1.275 µg STX equiv./100 g mussel flesh detected after 4 h determined by the use of the Mouse Neuroblastoma Assay. Toxicity levels subsequently dropped to 0.4 µg STX equiv./100 g mussel flesh after 6 h, with a further rise in toxicity after 24 h.

Although the bacteria in this study did not render the mussels toxic, we still can not conclusively eliminate any influence that the bacteria may have on the dinoflagellate toxicity and further investigations are needed. As the successful application of the 16S rRNA species-specific probes to detect these putatively toxic bacteria in mussels under laboratory cultures

was demonstrated, the next step would be to test mussels in nature in areas where mussel beds have been closed because of toxic dinoflagellates to see if purportedly toxic bacteria, known to be present in the water column during a toxic episode, can also be found inside the mussels from the same area.

In summary, putative toxic bacteria were readily filtered from the water column by *Mytilus edulis*. The bacteria were detected in the hepatopancreas of the mussels up to six hours after feeding ceased by *in situ* hybridization. Mussels were not rendered toxic by the ingestion of these bacteria as determined by HPLC with UV detection for Paralytic Shellfish Toxins and determination of sodium channel blocking activity using the Mouse Neuroblastoma Assay. However, preliminary studies showed a toxificatio
n of mussels by a single sodium channel blocking producing bacterium (407-2).

3.4 Physical interactions between bacteria and algae

Another question in bacteria/dinoflagellate interactions is, whether bacteria are specifically associated with the dinoflagellate, or whether the associations among the organisms occur randomly. Hold et al. (2001b) showed that a number of different bacterial species are associated with *Alexandrium* spp. and *Scripsiella* sp. Some of these bacteria are common to each of the investigated dinoflagellate cultures, whereas others appear to be unique to a single dinoflagellate species. The authors suggested a species-specific association between some bacteria and certain algal species and postulated differences in the microflora between toxic and non-toxic dinoflagellates (Hold et al. 2001a).

In **Publication IV,** PTB-1 bacteria originally isolated from the *Alexandrium tamarense* strain used in this study showed a preference for attachment to this dinoflagellate when reintroduced to a culture of this dinoflagellate than did PTB-6 bacteria, which were originally isolated from another *A. tamarense* clone. These findings also agree with the findings of Simon et al. (2002) who showed that putative toxic reintroduced bacteria are able to re-attach to *Alexandrium tamarense* in culture. Simon et al. (2002) further suggested that it depended on the physiological state of dinoflagellates, if their investigated purportedly toxic bacteria did or did not attach to the dinoflagellate. Until now the ecological meaning of these association between both organisms has not been fully clarified and most of the involved bacterial have not been identified (Hold et al. 2001a). It has been shown that interactions between bacteria and microalgae in the marine environment play an important role in processes, such as carbon fluxes and nutrient regeneration (Cole et al. 1988; Azam 1998).

Bacteria are also increasingly named as potentially important regulators in processes of algal bloom initiation, maintenance and decline (Doucette 1995). It has also been shown that several bacteria are capable of killing various HAB species and consequently are involved in the termination of HABs and the regulation of population dynamics of marine phytoplankton (Doucette et al. 1998). Bacteria have also been postulated to influence the algal toxin production, either extracellularly or intracellularly. Some authors reported an influence of attached putatively toxic bacteria on the toxicity of dinoflagellates. Some authors report higher toxicity in axenic cultures (Singh et al. 1982; Dantzer and Levin 1997), whereas others reported a lesser toxicity in axenic cultures (Doucette and Powell 1998). In contrast to the findings of Doucette et al. (1998), who suggested that putatively toxic bacteria could control toxin production by attachment to the surface of microalgae, Simon et al. (2002) showed that putatively toxic bacteria did not necessarily induce toxin production in *A. tamarense*. The authors suggested that attachment could influence toxin production by bacteria and/or dinoflagellates in the environment.

In **Publication IV** the specific attachment of the inoculated putatively toxic PTB strains to a highly toxic *Alexandrium tamarense* culture on the cellular level was shown by FISH in combination with TSA and confocal laser scanning microscopy. Further investigations on this subject are necessary to unravel the real meaning of the physical interactions between bacteria and microalgae, especially in environmental studies.

In summary, two bacterial loadings of purportedly toxic bacteria were reintroduced into laboratory cultures of a highly toxic *Alexandrium tamarense* clone to examine a possible reassociation of these bacteria with the dinoflagellate cell. One bacterial strain (PTB-1) was originally isolated from the *A. tamarense* clone used in this study and the other bacterial strain (PTB-6) was isolated from another *A. tamarense* clone. Oligonucleotide probes, previously developed to recognise these introduced bacteria were used to detect the bacteria in and around the dinoflagellate. Confocal laser scanning microscopy coupled with TSA-FISH showed that none of the reintroduced bacteria became intracellular, the PTB-1 bacteria were found to be attached to the surface of *Alexandrium tamarense*, and seem to form a preferred relationship with this algae, in comparsion to the attachment behaviour of PTB-6 bacteria.

3.5 Future research

So far, the exact ecological role the dinoflagellate-associated bacteria play, e.g., in their contribution to dinoflagellate toxicity or Harmful Algal Blooms, has not been fully clarified. Such close interactions or symbioses between bacteria and microalgae need further investigations. Up to now a conclusive answer cannot be given as to whether or not the bacteria that live as commensals or symbionts in or attached to at least some toxic dinoflagellates are able to synthesize PSP toxins autonomously; or if their role is to enhance the toxicity of the algae through the supply of, e.g., precursor molecules.

Data from the two monitoring years described in **Publication II and Appendix to Publication II**, suggests that there may be a relationship between *Alteromonas*-clade related species and *Alexandrium* spp. However, these experiments are the first of their kind in relation to toxic dinoflagellates and the results should be considered preliminary. Further research, particularly studies that take other members of the phyto-and bacterioplankton into account are required. New FISH counting methods are necessary to screen the high quantity of environmental samples faster and easier. A possibility is the use of an automated detection system together with the FISH technique. A sensitive, rapid and easy use detection method for bacterio- and phytoplankton species should be established. The detection will be based on sample filtration and subsequent fluorescence *in situ* hybridization. The cells will then be detected and enumerated using a solid phase cytometer. Thus, it would be possible to detect whole bacterial or phytoplankton cells, to count them and to evaluate the results by fluorescence microscopy faster than previously. The final target is the adaptation and use of this method for routine monitoring with a high sample throughput.

The successful application of the 16S rRNA species-specific probes to detect these putatively toxic bacteria in mussels under laboratory cultures was demonstrated. Thus, the next step will be to test mussels from areas where mussel beds have been closed because of toxic dinoflagellates, to investigate if purportedly toxic bacteria, known to be present in the water column during a toxic episode (**Publication II**), can also be found inside the mussels from the same area. Another important experiment would be to feed toxic- and non-toxic *Alexandrium tamarense* to *Mytilus edulis*, to determine if the mussel can selectively ingest particles based on their PSP content. The genus *Alexandrium* is the dominant source of PSP in contaminated bivalves (Li and Wang 2001). For this approach, probes for different toxic and non-toxic *Alexandrium*-clades have been developed (e.g., John et al. 2003) and thus it would be possible to discriminate between toxic and non-toxic strains inside the mussel gut. Further

information about the feeding behaviour and accumulation period of algae and bacteria of the blue mussel are important to clarify the possible role of bacteria on shellfish toxicity.

Also, further characterisation of the bacterial flora of dinoflagellates is necessary to show single bacterial species, which are associated with dinoflagellates and whether the bacteria are located extra- or intracellular by the application of species-specific molecular probes. Additionally, other strains of the used dinoflagellates in **Publication I** should be screened for attached or intracellular bacteria to compare the bacterial flora between different strains of one dinoflagellate species. Subcultures of the same dinoflagellate strain need to be screened for bacteria to confirm the findings achieved in this study. Also, further re-association experiments with different dinoflagellate strains as performed in **Publication IV** should be performed.

Many questions still remain open. It cannot be concluded that bacteria are able to produce autonomous saxitoxin and if they do, how it is possible that the bacteria produce such large amounts detected, when in laboratory culture only minute quantities are produced. Additionally, it has not been totally clarified, if dinoflagellates are able to synthesize saxitoxins in the absence of bacteria. With the continuing evolution of more sophisticated molecular tools the potential exists for considerable further advancement in our understanding of the mechanisms involved in PST production and accumulation in shellfish. This study has added to this data and can be used as a basis for further research in this scientific area.

4. Summary

This doctoral thesis aimed to investigate the interactions of purportedly toxic bacteria with toxic and non-toxic dinoflagellates in the occurrence of Paralytic Shellfish Poisoning (PSP). The putative toxic bacteria used in this study were originally isolated from toxic and non-toxic dinoflagellates of the genus *Alexandrium*. The toxins causing the symptoms of PSP are termed collectively as saxitoxins. Dinoflagellates especially of the genus *Alexandrium* produce saxitoxins and also cyanobacteria were shown to produce these neurotoxins. Additionally, marine bacteria have been postulated to produce saxitoxins. The possible synthesis of saxitoxins by bacteria is controversially discussed, because only minor quantities of saxitoxins have been shown to be produced by these bacteria and confirmatory mass spectrometer data are still lacking. Therefore, the investigated bacteria remain only putatively toxic.

To gain more information about bacteria/dinoflagellate associations laboratory cultures of the dinoflagellate species *Alexandrium tamarense*, *A. lusitanicum*, *A. taylorii*, *A. andersonii* and *A. ostenfeldii* were examined to determine their bacterial population. 16S and 23S ribosomal probes (rRNA) were applied to determine extracellular associated bacteria, intracellular bacteria and bacteria free in the dinoflagellate culture. Extracellular associated bacteria of the α-subclass of Proteobacteria and of the genus *Roseobacter* were shown to be associated with *A. tamarense* and *A. lusitanicum* and also found in high numbers free in culture. Bacteria of the β-subclass of Proteobacteria were found intracellularly in *A. tamarense*. Bacteria of the *Alteromonas*-clade were found attached to the surface of *A. andersonii* and free in culture. Moreover, Cyanoditolyltetrazolium chloride (CTC) was used to detect endocytic metabolically active bacteria in these dinoflagellates. Active respiring intracellular bacteria were detected in *A. tamarense* and *A. lusitanicum.*

In field studies, the occurrence of putatively toxic bacteria together with *Alexandrium* spp. and PSP in mussels was investigated. Lugol's fixed field samples from the Orkney Islands (Scotland) were screened for these bacteria in two sequential years. In this area, toxic phytoplankton blooms do occur on a yearly basis. Fluorescently-labelled 16S rRNA probes were applied to show the putatively toxic bacteria *in situ* in field samples. The used probes recognise two clades of related bacteria and single bacterial species within these clades. For the first time, fluorescently labelled probes were successfully applied to Lugol's fixed environmental samples. The purportedly toxic bacteria were detected in high numbers in the

samples, when both *Alexandrium* spp. were present and absent in the water column and when mussels contained Paralytic Shellfish Toxins (PSTs).

The direct effect of purportedly toxic bacteria on mussel toxicity was examined with feeding experiments. The bacteria were fed to the mussel *Mytilus edulis*, to clarify if bacteria were accumulated in the digestive system of the mussels and if the mussels contained PSTs after feeding ceased. The bacteria were detected *in situ* by the use of digoxigenin-labelled 16S rRNA probes in tissue sections of the mussel hepatopancreas, where PSTs become concentrated in mussels. Measurements of mussel flesh did not show toxification after feeding upon the putatively toxic bacteria, although the blue mussels have filtered the bacteria in high numbers. However, it had been shown in preliminary experiments, that sodium channel blocking toxins were present after feeding putatively toxic bacteria to blue mussels.

The physical interactions between bacteria and dinoflagellates were investigated by conducting reassociation experiments. The study aimed to identify, if purportedly toxic bacteria which were originally isolated from the *Alexandrium tamarense* clone (PTB-1bacteria) used in this experiments, or isolated from another related *A. tamarense* clone (PTB-6 bacteria), respectively, physically reassociate again with this dinoflagellate. Different concentrations of the bacteria PTB-1 and PTB-6 were inoculated into the cultivated toxic *A. tamarense* clone, and a possible reassociation was investigated by the use of fluorescently labelled 16S rRNA probes. No intracellular reassociated PTB-bacteria were detected in the dinoflagellates. However, a preferred extracellular attachment of the PTB-1 bacterial strain to the *A. tamarense* strain was observed.

5. Zusammenfassung

In dieser Dissertation wurden die Interaktionen von potentiell toxischen Bakterien mit toxischen und nicht-toxischen Dinoflagellaten untersucht, um eine mögliche Beteiligung dieser Bakterien an der Entstehung der paralytischen Muschelvergiftung zu ermitteln.
Die potentiell toxischen Bakterien wurden von toxischen und nicht-toxischen Dinoflagellaten der Gattung *Alexandrium* isoliert. Die Symptome der paralytischen Muschelvergiftung werden durch Gifte hervorgerufen, die kollektiv als Saxitoxine bezeichnet werden und die von Dinoflagellaten, hauptsächlich der Gattung *Alexandrium* spp., sowie von drei Cyanobakterien-Arten synthetisiert werden. Es wird zudem postuliert, dass diese Gifte auch von marinen Bakterien produziert werden. Die potentielle Synthese von Saxitoxinen durch marine Bakterien wird kontrovers diskutiert, da bisher nur sehr geringe Mengen an bakteriell produzierten paralytischen Muschelgiften gemessen wurden und überzeugende massenspektrometrische Daten fehlen. Aus diesem Grund werden diese Bakterien nur als potentiell toxisch bezeichnet.

Um Erkenntnisse über die mit Dinoflagellaten vergesellschafteten Bakteriengemeinschaften zu gewinnen, wurde die Mikroflora von Laborkulturen der Dinoflagellaten *Alexandrium tamarense*, *A. lusitanicum*, *A. taylorii*, *A. andersonii* und *A. ostenfeldii* untersucht. Fluoreszenz *in situ* hybridisierung (FISH) mit 16S und 23S ribosomalen Sonden (rRNA) wurde angewendet, um extrazellulär angeheftete, intrazelluläre und frei in der Kultur vorkommende bakterielle Populationen nachzuweisen. Bakterien der □-Proteobakterien Subklasse und der Gattung *Roseobacter* wurden in Assoziation mit der Zelloberfläche von *A. tamarense* und *A. lusitanicum*, sowie auch frei in der Kultur nachgewiesen. Zudem wurden Bakterien der □□Subklasse der Proteobakterien intrazellulär in *A. tamarense* lokalisiert. *Alteromonas* spp. wurde angeheftet an die Zelloberfläche von *A. andersonii* und frei in der Kultur detektiert. Zusätzlich wurde Cyanoditolyltetrazoliumchlorid (CTC) eingesetzt, mit der aktiv atmende Bakterien in der Dinoflagellaten Zelle nachgewiesen werden können. Es konnte gezeigt werden, dass *A. tamarense* and *A. lusitanicum*, intrazelluläre, metabolisch aktive Bakterien besitzen.

Darüber hinaus wurde das Auftreten potentiell toxischer Bakterien zusammen mit *Alexandrium* spp. und der Akkumulation von paralytischen Muschelgiften in Muscheln untersucht. Mit Lugol‘sche Lösung fixierte Wasserproben von den schottischen Orkney Inseln wurden in zwei aufeinander folgenden Jahren auf das Vorhandensein dieser potentiell toxischen Bakterien hin getestet. In den Gewässern vor den Orkney Inseln treten jährlich

toxische Algenblüten auf. Fluoreszenz markierte 16S rRNA Sonden wurden eingesetzt, um *in situ* die Bakterien in den Feldproben zu identifizieren. Mittels der eingesetzten Sonden können Gruppen verwandter Bakterien und einzelne Bakterienstämme nachgewiesen werden. Erstmalig konnte hier gezeigt werden, dass die Fluoreszenz markierten Sonden erfolgreich auch in mit Lugol'sche Lösung fixierten Feldproben angewendet werden können. Die als toxisch postulierten Bakterien wurden in großer Anzahl in den Feldproben identifiziert, wenn *Alexandrium* spp. in der Wassersäule vorhanden und nicht vorhanden war und wenn Muscheln paralytische Muschelgifte enthielten.

Zudem wurden die direkten Auswirkungen putativ toxischer Bakterien auf Muscheln der Spezies *Mytilus edulis* untersucht, indem Fütterungsexperimente durchgeführt wurden. Nach der Filtration von Bakterien durch *M. edulis*, wurde untersucht, ob sich die Bakterien im Verdauungskanal dieser Muscheln anreichern und ob die Mollusken danach paralytische Muschelgifte enthielten. Die Bakterien konnten in Gewebedünnschnitten der Mitteldarmdrüse der Muscheln *in situ* mittels gegen die Bakterien gerichteter digoxigenin markierter16S rRNA Sonden nachgewiesen werden. In dieser Verdauungsdrüse reichern sich die paralytischen Muschelgifte in *M. edulis* an. Messungen des Muschelgewebes ergaben jedoch keine Toxifizierung der Muscheln, obwohl die Miesmuscheln die Bakterien in hoher Anzahl filtrierten.

Des Weiteren wurden die physischen Interaktionen zwischen Bakterien und Dinoflagellaten untersucht, indem Reassoziationsexperimente mit toxigenen Bakterien in einen toxischen *Alexandrium tamarense* Stamm durchgeführt wurden. Es wurde untersucht, ob Bakterien, die ehemals von diesem in der Studie verwendeten *A. tamarense* Stamm (PTB-1), bzw. von einem verwandten *A. tamarense* Stamm (PTB-6) isoliert wurden, erneut eine Assoziation mit den Dinoflagellaten eingehen. Dazu wurden verschiedene Konzentrationen der potentiell toxischen Bakterienstämme PTB-1 und PTB-6 in die *A. tamarense* Kultur inokuliert und eine mögliche Reassoziation mittels Fluoreszenz markierter 16S rRNA Sonden ermittelt. Eine intrazelluläre Assoziation der PTB-Bakterien konnte in diesem *A. tamarense* Stamm nicht nachgewiesen werden. Es zeigte sich jedoch, dass eine spezifische Anheftung der PTB-1 Bakterien an die Algenzellen stattgefunden hat.

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Hiermit erkläre ich, dass ich die vorliegende Dissertation selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Die entnommenen Stellen aus benutzten Werken wurden wörtlich oder inhaltlich als solche kenntlich gemacht.

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