Bacterial and Algal Interactions in a Tasmanian Estuary

By

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Declarations

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Abstract

The microbial communities and the physical and chemical environment of the Huon Estuary, Tasmania, Australia, were sampled in an 18 month program. Analysis of field samples, and laboratory experiments were used to examine the characteristics of estuarine and marine bacteria and algae, relationships between bacteria and algae, and their ecological roles. A multidisciplinary approach was used, including morphological, molecular, and chemical techniques. Five new algicidal bacteria strains were isolated from the estuary. The species were identified, based on molecular and phenotypic analyses, as a novel *Pseudoalteromonas sp.*, a novel *Planococcus sp.*, a novel *Cellulophaga sp.*, and two isolates that were closely related to the species *Cellulophaga lytica* and *Bacillus cereus*. The bacteria exhibited a powerful lytic effect on the vegetative lifestage of the toxic dinoflagellate *Gymnodinium catenatum*, an introduced species that blooms intermittently causing shellfish farm closures in the estuary. Excystment or encystment of the cyst lifestage of *G. catenatum* was unaffected by algicidal activity. The bacteria had no observable effect on rotifer, cyanobacteria and diatom species tested. Algicidal ability of the bacteria varied both in culture and in the environment. These variations depend on environmental conditions or may be due to differences in species-specific modes of action. Antimicrobial brominated compounds, unrelated to the algicidal activity of the bacteria, were also identified in one *Pseudoalteromonas* species. A novel *Shewanella* species was isolated that contains the highest proportion of the essential fatty acids 20:5(n-3) reported for a temperate member of this genus. These levels are close to those produced by psychrophilic *Shewanella* species. Fluorescence *in situ* hybridisation (FISH), signature lipid profiling, and morphological data obtained during two *Gymnodinium catenatum* blooms and one diatom bloom, demonstrate that algicidal bacteria can form a major part of the bacterial community. Fatty acid analysis differentiated between different bacterial taxa isolated in this study and between field samples from the estuary. Variation of the branched chain fatty acids (BCFA) reflected the strong association of the *Cytophaga-Flavobacterium-Bacteriodes* (CFB) cluster with microbial degradation of algal blooms in the estuary. Results from fatty acid analyses indicated that the CFB cluster are more

common in the photic zone and during the chlorophyll maximum, while low levels of BCFA indicated that γ proteobacteria may be more abundant in the deeper, marine influenced waters. FISH analysis also showed that the CFB cluster was common in the estuarine community during blooms of the dinoflagellate *G. catenatum*. Member of the genera *Pseudoalteromonas-Colwellia* were a significant component during diatom blooms. Alpha (α) and gamma (γ) proteobacteria were common in the estuary, however, beta (β) proteobacteria were not. Throughout the year in the Huon Estuary, dominance of bacterial genera varied demonstrating distinct and systematic progressions related to the progression of algal blooms.

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Glossary

Abbreviation	Definition
α, β, δ, γ	Alpha, beta, delta, and gamma (proteobacteria)
ACAM	Australian Collection of Antarctic Microorganisms
ACEM	Australian Collection of Estuarine Microorganisms
AHL	Acetylated homoserine lactones
ANOVA	Analysis of variance
BCFA	Branched chain fatty acids
biomarker	An organic compound or compounds that serve as indicators of an individual organism, a group of organisms, or a biochemical process
bloom	High concentration of phytoplankton resulting from increased reproduction as a response to favourable conditions
CDOM	Chromophoric (coloured) dissolved organic matter
CFB	*Cytophaga-Flavobacterium-Bacteriodes* cluster
CFU	Colony forming units (bacterial isolates that form colonies on solid media)
chl	Chlorophyll
cyst	(dinoflagellate cyst) Part of the sexual lifecycle which is dormant for some period of time before reforming an active planktonic cell
diatom	A member of a diverse class of microalgae Bacillariophyceae having siliceous cell walls (generally unicellular)
diel	Involving a 24 hour period (not diurnal which can mean recurring daily or occurring in the day time)
dinoflagellate	A microalgal member of the Dinophyceae, single celled with two flagella
DMSP	Dimethylsulfoniopropionate
DOM	Dissolved organic matter
EPA	Eicosapentaenoic acid (20:5(n-3))
ESI	Electron spray injector
eutrophic	Marine waters containing a high level of nutrients
FAB-MS	Fast atom bombardment mass spectrometry
FAME	Fatty acid methyl ester, derivatives of fatty acids
FFA	Free fatty acids
FISH	Fluorescence *in situ* hybridisation

Abbreviation	Definition
GC	Gas chromatograph
GCMS	Gas chromatograph – mass spectrometer
HAB	Harmful algal blooms
heterotrophs	Organism dependent upon some external source of organic compounds, in this thesis generally referring to copepods, tintinnids etc
HPLC	High performance liquid chromatography
HST	CSIRO Huon Estuary Study Team (2000)
humic	Formed or derived from plants, often refers to humic acids which are complex organics that colour natural water. Subclasses of humic acids are tannins, lignins and fulvic acids. They are derived from peptide, aromatic, lipid, carbohydrate and other precursors. Their formation and digenesis is partially mediated by aquatic bacteria and enzymes. A substantial fraction of the humic mass is in carboxylic acid functional groups. A smaller fraction contains phenolic functional groups. Terrestrial humic acids tend to be more aromatic in nature while marine humic compounds tend to be more aliphatic.
LC	Liquid chromatography
mesotrophic	Between oligotrophic and eutrophic
MUFA	Monounsaturated fatty acids
NMR	Nuclear magnetic resonance
oligotrophic	Marine waters containing a low level of nutrients
PCA	Principal component analysis
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
POM	Particulate organic matter
PSP	Paralytic shellfish poisoning
PUFA	Polyunsaturated fatty acids
pycnocline	Density gradient; in this thesis it refers to the boundary between fresh and saline water masses
SFA	Saturated fatty acids
signature lipid	See biomarker
TFA	Total fatty acids
TG	Triacylglycerol

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1. Introduction: Bacterial interactions in the marine environment

There are dynamic relationships of competition, predation and commensalism among the different trophic levels in the marine microbial loop (Calbet *et al.* 2001, Davidson 1996, Azam *et al.* 1983, Pomeroy 1974). Some of these relationships also apply within individual microbial classes. Bacteria can exist as symbionts, and can be predatory, commensal or competitive with higher trophic organisms. Bacteria also compete with, and prey on each other (Jürgens and Sala 2000, Azam 1998). In many marine environments, bacteria can have a major effect on the dynamics and physiology of microalgal and heterotrophic communities. In particular, they can play an important role in the development and decline of phytoplankton blooms. In these marine and estuarine ecosystems, heterotrophic microorganisms are therefore responsible for a large share of overall carbon-use in the respiration and regeneration of nutrients.

1.1 Microbial relationships

Until recently, marine bacterial studies focussed on the role of bacteria as detritus recyclers and nutrient regenerators. Bacteria recycle nutrients by remineralising phytoplankton exudates and dissolved organic matter (DOM) lost from the grazing chain by inefficient feeding of heterotrophs (Azam *et al.* 1983). More recently, this concept of bacteria has evolved to include a more proactive role. Bacteria recycle nutrients and detritus, but they can also be predatory and aggressive in their role in the food chain (Azam 1998). Bacterial species interact within the microbial community, in ways as diverse as do members of more complex higher trophic levels. These microbial inter- and intra-relationships can be grouped into a number of categories.

1.1.1 Passive recyclers

The traditional understanding of the role of bacteria in the marine environment is that they are relatively passive. They recycle material already dead or dying, thus regenerating nutrients for the benefit of future production. Bacteria produce growth factors that can provide a source of nutrients that are freely available for use within the marine food web (Grossart 1999, Keshtacher-Liebson *et al.* 1995). The contribution of these nutrients aids the production of algae and higher trophic levels. However, it is possible for certain groups of bacteria to discriminate and repress specific algal types yet supply the essential nutrients for others (Fukami *et al.* 1997, Fukami *et al.* 1996, Fukami *et al.* 1992). That is, bacteria can be selective in the algae that they suppress or nurture and can influence the succession of phytoplankton communities that predominate.

1.1.2 Symbiosis and commensalism

Symbiotic and commensal relationships between bacteria and plant or animal species are common in the marine environment. Bacteria generally reside within or in close proximity to these species. Species at higher trophic levels may use their natural bacterial community to obtain necessary nutrients required for growth or use specific components as methods of protection or predation. For example, some shellfish species have gut systems unable to obtain certain nutrients required for development without their endemic bacterial population (Klussmann-Kolb and Brodie 1999, Schneider 1998, Distel and Cavanaugh 1994). The symbiosis between *Euprymna scolopes* (squid) and the bacteria *Vibrio fischerii* relies on the exchange of signal molecules, some of which are derived from bacterial cell surface molecules. The squid-*Vibrio* symbiosis is obligate for the squid, but is not nutritionally based. Rather, bacteria produce light, which enables the animal to evade predators (Hirsch and McFall-Ngai, 2000, Ruby 1996, see also section 1.3.5 on bacterial quorum sensing).

There is also the possibility that some marine organisms use bacterial toxins produced by symbiotic bacteria as agents in their own predatory activities. Tetrodotoxins, which are toxins commonly formed by dinoflagellates are also

produced by the symbiotic bacteria of *Nemertean proboscis* (marine worm). The worm is thought to use the tetrodotoxins to subdue prey during capture (McEvoy*et al.* 1998).

Bacteria also provide the nutritional attachment to a surface for most benthic algae and thus their growth on surfaces is the precursor to biofilm formation (van Loosdrecht and Tijhuis 1996, Brisou 1995). In marine waters bacterial attachment to microalgae increases as algae lose viability, however bacterial attachment to microalgae does not always indicate the decline of a bloom. Algal species in logarithmic phase can also have their growth enhanced by bacterial attachment (Mouget *et al.* 1995). Bacteria often supply algal cultures with growth promoting substances such as vitamins or nutrients. The bacterial microflora in non-axenic algal cultures generally contributes to a higher degree of algal health than that observed in their axenic counterparts. However, the same species of bacteria can also cause the phytoplankton to perish due to bacterial secretion of harmful compounds (Delucca and McCracken 1979).

Algae can produce specific extracellular products to stimulate growth of certain beneficial species, deter predators, or inhibit other species in their aquatic equivalent of a rhizosphere. Although the phycosphere is more diffuse and dilute than a rhizosphere, during algal bloom periods the extracellular compounds produced by algae increase in concentration and have the potential to have a significant impact on their surrounding environment (Lovejoy *et al.* 1998). Bacteria can therefore use these extracellular algal products as a source of carbon without harming the alga (Fukami e*t al.* 1997)

1.1.3 Bacterial attack: Direct predation or indirect attack via exudates

The traditional "detritus recycling" role now includes bacteria that are aggressors and/or predators. Bacteria achieve this by three mechanisms: direct attack, indirect attack, or a combination of the two (Doucette *et al.* 1998, Imai 1997). All forms of attack can be beneficial to other bacterial and algal species as they cause the prey to "leak" with the resulting nutrients dispersing into the water column.

Bacteria using direct attack seek out and quickly swarm around the prey. This action can be either species specific or general (Imai *et al.* 1995). Bacteria may bypass certain species in favour of the preferred prey (Doucette *et al.* 1998). Within a short time of contact, lytic enzymes disrupt the cell wall (Imai 1997). The bacteria that directly attack their host generally produce no extracellular products.

Bacteria that exude one or more extracellular algicidal compounds display indirect attack (Imai *et al.* 1995). These compounds kill the prey species by diffusing into the water column and lysing the cell wall of the prey. In some cases, prey may be in an enclosed environment (such as an algal bloom) and then bacteria exude the compounds into the confined area (Imai 1997, Doucette *et al.* 1998). Production of a lytic compound into the water column in many circumstances might be ineffectual because of dilution. However, it has been proposed that during dense algal blooms when there is abundant nitrogen, this would be a realistic method of attack (Lovejoy *et al.* 1998). Higher nitrogen levels caused by dense algal blooms can also increase the lysing efficiency of some bacteria (Doucette *et al.* 1998).

1.1.4 Bacterial size

The main factors underlying the size spectra of bacteria in the marine environment are the same as for algae; grazing and nutrient effects (Jürgens and Güde 1994). Size selective predation is an important factor in controlling bacteria and therefore determines the structure of bacterial communities. Small protists select bacteria by size and preferentially ingest medium to large bacterial cells (Posch *et al.* 1999). In the absence of flagellate grazing, the size structure of the bacterial population shifts to smaller cells (Posch *et al.* 1999, Gin 1996). In one study, larger cells were the most active and these were dominated by dividing and logarithmic bacterial cells in the community (0.2-0.5 μm) (Bernard *et al.*, 2000).

Since large cells are preferentially grazed, being small can make a species less attractive as prey. Bacteria can adapt to nutrient starvation by a reduction in size in order to increase their surface to volume ratio. In doing so, cell size can also be regarded as an adaptation to carbon and nutrient limitation (Jürgens and Güde 1994) as well as predation. Conversely, some of the bacteria found in oligotrophic

environments remain small independent of nutrient availability (Schut *et al.* 1997). In winter and early spring in one study, large cells were abundant in the marine environment as waters were well mixed and nutrient concentrations were high (Gin 1996). Diminishing nutrients, rising temperature and increasing stratification of the water column resulted in an increase in smaller cell sizes. In stratified waters, depth variation of the size spectra demonstrated that larger bacteria and phytoplankton sizes were observed in surface and very deep waters, whereas smallest sizes were typically seen around the thermo/halocline near or at the chlorophyll maximum (Gin 1996).

1.2 Harmful algal blooms

Occurrences of harmful algal blooms are being reported more frequently in Tasmania and around the world. This may be partly explained by an increase in water column observations by government and aquaculture authorities for public health requirements. The detrimental financial and health effects of toxic algal blooms necessitate increased monitoring as harmful algal blooms render shellfish unsaleable because of high toxin levels.

During some summers, *G. catenatum* outbreaks in the Huon Estuary have caused shellfish farms to close for up to a third of the year. The size of the *G. catenatum* bloom is not consistently related to the level of toxin, so the abundance of dinoflagellates cannot always be correlated to toxin level (CSIRO Huon Estuary Study Team 2000 (HST 2000)). De-stratification of the water column was shown as the most effective means to end the blooms (HST 2000). Outbreaks of this alga have also been reported in the nearby Derwent Estuary, although no shellfish farms are situated in this estuary (Coughanowr 1997).

The advantage *G. catenatum* has over other dinoflagellate and diatom species is that it is capable of rapid vertical migration. It also has a higher swimming velocity that would enable it to move against a river current (1.5-6.5 m/h, HST 2000). Diatoms do not move very quickly, so in nutrient depleted periods their blooms rapidly decline as they remain at similar positions in the water column. By

comparison, *G. catenatum* has been shown to undergo large diel vertical migrations to the surface for sunlight during the day before returning to the deep nutrient rich marine waters (20 meters) at night where they sequester bottom water nutrients (HST 2000, Doblin *et al.* 2000). This enables effective use of chlorophyll and nutrients. The swimming ability of *G. catenatum* is also thought to help the dinoflagellate remain in the estuary as the brackish waters flow down stream and the tidal push of the salt wedge keeps the alga within the estuary boundaries (HST 2000). As the estuary has high levels of humic compounds, light penetration can be as little as 1 meter, giving species that are capable of movement in the water column a strong advantage.

1.2.1 Bacteria associated with harmful algal blooms

The relationship between the formation of toxins by harmful algal species and their associated bacteria has been reported since the 1960s (Silva 1962). However, research in the area increased significantly in the 1990s (Gallacher and Smith 1999, Gallacher *et al.* 1997, Franca *et al.* 1996, Kodama 1990).

Prokic *et al.* (1998) demonstrated that the bacterium *Ruegenia algicola* (previously *Roseobacter algicolas*, Uchino *et al.* 1998) was closely associated with a toxic dinoflagellate (*Prorocentrum lima*) that produces diarrhetic shellfish poison. The study reported that 83 % of the bacterial population in the dinoflagellate culture was *R. algicola* although it was unclear as to whether the dinoflagellate or the bacteria produced the toxin.

Other bacteria, such as *Pseudoalteromonas,* have the ability to produce tetrodotoxins and saxitoxins (Ivanova 2001, Gallacher *et al.* 1997, Franca *et al.* 1996, Gallacher and Birkbeck 1993). Symbiotic or associated bacteria are thought to contribute to the toxicity of the dinoflagellate *Alexandrium* (Kodama *et al.* 1996, Levasseur *et al.* 1996). However, the relationship between the production of algal toxins and the role bacteria play is not yet fully understood (Doucette *et al.* 1998).

1.3 Algicidal bacteria

Algicidal bacteria are often associated with algal blooms and considerable research centres on their relationship with harmful algal blooms (Doucette *et al.* 1998, Yasumoto *et al.* 1996). Usually algicidal properties are limited to certain bacterial genera, in particular *Pseudoalteromonas* (Egan *et al.* 2001a, Lovejoy *et al.* 1998) and *Cellulophaga* (Johansen *et al.* 1999, Doucette *et al.* 1999). Both these bacterial genera are motile; *Pseudoalteromonas* using flagella motility and *Cellulophaga* by gliding motility.

1.3.1 *Cellulophaga*

Cellulophaga are one of the most prolific and widely studied algicidal genera (Toncheva-Panova and Ivanova 2000, Kondo *et al.* 1999, Yoshinaga 1998, Imai *et al.* 1993, Mitsutani *et al.* 1992, Imai *et al.* 1991, Stewart and Brown 1969). They are the most significant species in causing algal bloom decline and show swarming and predatory abilities. Recent studies using fluorescence *in situ* hybridisation (FISH) and more traditional methods, indicate that *Cellulophaga* species and the *Cytophaga-Flavobacterium- Bacteriodes* (CFB) cluster dominate many marine systems. They are found throughout the water column of temperate and polar marine environments (Fandino *et al.* 2001, Cottrell and Kirchman 2000, Pinhassi and Hagström 2000, Glöckner *et al.* 1999) and are also common in fresh water (Stewart and Brown 1969). They grow easily in oligotrophic conditions as they can utilise a variety of carbon sources for growth. Although capable of growth under nutrient limited conditions, they are mainly associated with marine snow and the decay of algal blooms (Crump *et al.* 1999, Delong *et al.* 1993, Riemann *et al.* 2000), and thrive on nutrient rich media. Pinhassi and Hagström (2000) have shown, using whole-genome DNA hybridisation to community derived DNA, that CFB was the dominant cluster during a coastal phytoplankton decline (43 % of intact cells). Another study by Glöckner *et al.* (1999) demonstrated that members of the family *Flavobacteriaceae* were the most abundant group detected in a number of different marine systems, accounting for an average of 18 % (2 to 72 %) of the 4',6-diamidino-2-phenylindole (DAPI) stained cells. Further work by these

researchers using FISH revealed that the CFB cluster dominated in the North Sea (up to 30 %), although species from the cluster were rarely cultured and no clone related to the CFB cluster was retrieved (Eilers *et al.* 2000).

1.3.2 *Pseudoalteromonas*

Pseudoalteromonas is a highly bioactive genus and has been the source of many different secondary metabolites and antibiotics. Over thirty known bioactive compounds have been described and some are patented for biotechnological uses (see review by Mikhailov and Ivanova *et al.* 1994). The genus is ubiquitous in the marine environment and is part of the γ proteobacteria subdivision. Other common marine genera of this subdivision include *Vibrio, Shewanella, Oceanospirillum,* and *Pseudomonas.* Also included in the γ proteobacteria are many types of symbiotic bacteria found in fish, shellfish and marine worms (Amann *et al.* 1995).

Pseudoalteromonas is the second most abundant and commonly reported genus of algicidal bacteria after *Cellulophaga* (Lee *et al.* 2000, Lovejoy *et al.* 1998, Imai *et al.* 1995, Baker and Herson 1978). They have the ability to lyse cells and swarm using their flagella once the cells are leaking (Doucette *et al.* 1998, 1999, Imai *et al.* 1995). Lysing occurs through the indirect method of extracellular exudation, and they have also shown species-specific algicidal activity (Lovejoy *et al.* 1998). Their algicidal activity is more effective during periods of algal blooms and thus peak nutrient levels. The most highly studied species of this genus to date is *P. tunicata* which produces at least three different bioactive compounds that act independently: antifungal, inhibition of algal spore germination and antifouling (Egan *et al.* 2001a, Holmström and Kjelleberg 1999, Holmström *et al.* 1998).

1.3.3 Other algicidal genera

Dakhama *et al.* (1993) has reported on a marine algicidal species that is not included in the *Cellulophaga* or *Pseudoalteromonas* genera and is similar to *Pseudomonas aeruginosa* (γ subdivision proteobacteria). Isolation and identification of antialgal substances produced by this species inhibited the growth of microalgae and cyanobacteria. Results showed that the inhibition of algal

growth was due to bacterial pigments that were thermo-resistant, low molecular weight compounds (Dakhama *et al.* 1993). These may have been phenazine pigments similar to those found in *Pseudomonas aeruginosa* like pyocyanin, 1-hydroxy-phenazine (a product of degradation of pyocyanin), phenazine-1-carboxylic acid and oxychloraphine (Anjaiah *et al.* 1998, Fernandez and Pizarro 1997).

Another recently identified dinoflagellate-killing bacterial species was closely related to the common marine genus *Oceanospirillum* (Kitaguchi pers. com.). A species closely allied with the genus *Psychroserpens* ([*Flavobacterium*] *sp.* strain 5N-3) was also reported as an algicidal species (Kondo *et al.* 1999).

In freshwater, a number of algicidal genera have been isolated. Algicidal *Lysobacter* (Mitsutani *et al.* 1987), *Myxobacter* (δ subdivision proteobacteria) (Shilo 1970) and *Flexibacter* (Gromov *et al.* 1972) have been isolated from fresh water environments. An *Alcaligenes*-like species was identified as biocidal towards a *Microcystis* bloom (Manage *et al.* 2000). However, the genus of the isolate was only identified phenotypically using BIOLOG. Conversely, in the marine environment, a number of *Alcaligenes* species in a study reporting on algicidal species, produced substances that had a positive effect on microalgal growth (Fukami *et al.* 1997).

1.3.4 Cell mechanics of algicidal species

All algicidal bacteria described to date can survive low nutrient levels. However many can also dominate or take advantage of periods when there is increased nutrient availability (ie *Cellulophaga* spp. and their association with marine particles, section 1.3.1). The cellular characteristics of many algicidal bacteria in liquid media provide information on their predatory behaviours. Some algicidal bacteria adhere to the surface of the culture vessel and produce lace-like growth formation (filiforms) that may be effective as a method of entrapment for algal cell lysis in the natural environment (Doucette *et al.* 1998). Adhesion to surfaces is thought to play a major role in the survival ability of some bacterial groups, with bacterial biofilms being an example of this (Costerton *et al.* 1995). Bacteria that

survive well at low nutrient levels are likely to compete well for low molecular weight energy substrates in the aqueous phase. Small, starved cells can therefore show a greater adhesive ability than normal unstarved cells (Jana *et al.* 2000).

Many algicidal bacteria have flagella or use secretory gliding as their major form of movement. Members of the common algicidal genus, *Pseudoalteromonas*, use flagella for motility. Bacteria with gliding or flagella motility can respond to stimuli and may be able to move toward an algal bloom or nutrient source. The secretory gliding mechanism is often used as a method of algal attack for species belonging to the genus *Cellulophaga*. Gliding bacteria could be already attached to the algae or may be floating in the water column until suitable conditions arise.

The gliding response mechanism of bacteria requires recognition of extracellular components that involves cell-to-cell communication (Youderian 1998). The molecular mechanisms of two types of gliding motility have been described and characterised (Hodgkin and Kaiser 1979). One is a social motility that lets cells hunt in packs and the other is adventurous motility that enables the bacteria to explore the territory of the pack. To disable gliding ability, both these genes have to be disrupted. This form of gliding is as genetically complex as flagella motility.

In the marine environment the use of either flagella motility or secretory gliding by algicidal bacteria, could be a major advantage for sequestering nutrients. The presence of flagella and gliding motility may also benefit these bacteria when highly motile algal species, such as toxic dinoflagellates, predominate.

1.3.5 Bacterial quorum sensing

Predatory and algicidal bacteria may use quorum sensing as a means to collectively detect and kill prey. Bacterial communities can coordinate phenotypic behaviour and gene expressions through signalling molecules called autoinducers. The most well known group of signalling molecules are called acylated homoserine lactones (AHL) (Lilley and Bassler 2000, Bassler 1999, Eberl 1999). AHL accumulate in the external environment and individual bacteria use them to detect total bacterial numbers around them (quorum sensing). Based on the concentration of AHL, bacteria activate or inactivate different functions that can regulate antibiotic

production, conjugation, swarming, toxin production, biofilm formation, luminescence and exoprotease production (Bassler 1999, Eberl 1999). One example of quorum sensing occurs with the pathogenic bacteria *P. aeruginosa* that does not produce virulence factors until quorum density is achieved. Quorum sensing can be seen as bacteria working akin to a multi-cellular organism, enabling the bacteria to coordinate behaviour (Bassler 1999).

An example of AHL in the marine environment is bioluminescence in *Vibrio fischerii,* which is under the control of three or more AHL (Bassler 1999). *Vibrio fischerii* is a marine bacterium found as planktonic cells and as a symbiont in the light organs of squid. In the natural marine environment, this species does not fluoresce as it is at low densities (10^1 cells/ml). However, in light organs the bacterial concentrations are high enough so they bioluminess to attract prey (10^{10} cells/ml). In the light organs the AHL provide a feedback mechanism. When a threshold stimulatory concentration of AHL is achieved, it is translated into bioluminescence (autoinduction). Cell free stationary phase cultures induce bioluminescence in non-luminous mid-log cultures from the marine environment. Thus, the bacteria produce an extracellular substance that induces bioluminescence.

1.4 Techniques used in microbial ecology

It is possible to analyse algal and bacterial communities in the same sample using a variety of techniques. Recent advances in the field of molecular phylogeny have made it possible to study bacterial populations by a culture-independent approach (Amann and Ludwig 2000, Amann *et al.* 1990). Molecular techniques such as fluorescence *in situ* hybridisation (FISH) use flurophores attached to short DNA probes. Probes attach to the rRNA in the bacterial sample and classification of the genera or classes present is achieved using epifluoresence microscopy (Amann 1995).

Signature lipids can identify the physiological status and type of the marine algal blooms as well as identify the biomass and proportion of bacteria present in the same sample. Many species contain a lipid signature that can provide a fingerprint

to differentiate between taxa (Meziane and Tsuchiya 2000, von Keitz *et al.* 1999, Nichols *et al.* 1991, Mancuso *et al.* 1990, Volkman *et al.* 1988, White 1983). For example, many bacteria contain branched chain fatty acids (BCFA) that are not found in algae and other marine organisms. Analysis of mixed bacterial cultures from sediment indicate that BCFA can account for up to 70 % of the total fatty acids (Gillan *et al.* 1983). The bacterial component of field samples can therefore be estimated using BCFA.

Traditional techniques such as plate identification are also useful when working with bacteria that are not growth inhibited by the culture media or that have distinct morphologies. Other methods used in marine microbial ecology include: BIOLOG substrate utilisation profile analysis, amplified ribosomal DNA restriction analysis, cloning and sequencing, terminal restriction fragment length polymorphism analysis (T-RFLP), length heterogeneity PCR analysis (LH-PCR) and denaturing gradient gel electrophoresis (DGGE).

1.5 Estuaries: A microbial perspective

Estuaries are areas of highly variable environmental conditions in terms of temperature, salinity, pH and organic loading. These physical and chemical parameters are often the most important controlling factors of distribution and variability of bacteria (Painchaud *et al.* 1995, 1996). Estuaries are typically highly productive regions and are often more productive than either the ocean or the freshwater systems that enter them (Gayte *et al.* 1999, Saliot *et al.* 1996). Lower current flow, and mixing of organic matter from the fresh and marine systems leads to these specific nutrient and bacterial dynamics. There are complex variations within each estuarine system, including flushing time, tidal flow, estuary shape and sewerage or agricultural inputs. It is noteworthy that the study of many estuarine systems is prompted by a desire to understand anthropogenic contamination.

The nature of the marine or estuarine environment is such that bacterial growth is limited by the availability of carbon and energy sources. The two ways in which bacteria can dominate and survive in these areas are: by growing efficiently in a low nutrient regime or, remaining dormant until a plentiful supply of nutrients

arises. Within an estuary, bacteria can occupy these two different habitats; free-living or attached to particles (Crump and Baross 2000, Revilla *et al.* 2000, Painchaud *et al.* 1995, 1996). Free-living bacteria are largely controlled by hydrodynamic influences and can differ in their importance between and within the transit of estuaries. Some studies have shown that free-living bacteria are responsible for the majority of bacterial productivity, although their presence can be unrelated to chlorophyll levels (Painchaud *et al.* 1995, Griffith *et al.* 1994). Attached and free-living bacteria in San Francisco Bay were demonstrated to be members of river and coastal ocean communities, with a rapid transfer between the two (Hollibaugh *et al.* 2000). In other coastal (east Mediterranean Sea), oceanic (North Pacific and Atlantic central ocean) and estuarine environments (Columbia Estuary, USA), a distinct difference was observed between the free living and particle-attached communities (Moeseneder *et al.* 2001, Crump *et al.* 1999, Delong *et al.* 1993). These findings indicate dissimilarity and limited community interchange occurring between the free living and attached bacteria in these environments.

Particle-attached bacteria in estuarine systems are generally more biologically active and their distribution often correlates with phytoplankton degradation (Grossart and Ploug 2000, Crump *et al.* 1999, Griffith *et al.* 1994). They gain nourishment from the phytoplankton and supply nutrients, resulting in "new" free-living bacteria (Friedrich *et al.* 1999). In contrast, the studies by Painchaud *et al.* (1995) and Griffith *et al.* (1994) found that although attached bacteria may dissolve the particulate organic matter, their growth rates on particles were not always significantly enhanced.

1.5.1 The Huon Estuary

Upper reaches of many estuaries (Delaware Estuary, Hoch and Kirchman 1993), Chesapeake Estuary (Griffith *et al.* 1994), and Schelde Estuary (Goosen *et al.* 1997) have shown the microbial community to be bacterially dominated, and most are affected by areas of urban or agricultural run off. The Huon Estuary in Tasmania (147°E, 43° S) by contrast, has a high natural level of humic compounds from native bushland and rainforest that surrounds the estuary. Land-based

activities and aquaculture have only a minimal localised effect, consequently the estuarine water is of high quality (HST 2000).

The estuary is more productive in summer with two classes of algal blooms, dinoflagellates and diatoms, dominating and alternating in the spring to autumn period of most years (HST 2000). One of the species, *Gymnodinium catenatum,* is a toxin-producing dinoflagellate that was first introduced via ballast waters in the early 1970s (McMinn *et al.* 1997) and has since become a principal alga in the estuary.

Diatom blooms that occur in the estuary are predominantly *Pseudonitzschia* and *Chaetoceros* species. Background levels of small-unidentified flagellates in the estuary also fluctuate over the season. Peak algal biomass ranged from 1-2 mg chlorophyll *a*/m^3 for diatom blooms to 20 mg chlorophyll *a*/m^3 during dinoflagellate blooms (HST 2000). Only one study has reported bacterial concentrations in the water column of the Huon Estuary. In this study, bacterial numbers were around 10^8 cells/l for samples taken over various seasons (Lovejoy *et al.* 1998).

The Derwent Estuary is situated parallel to the Huon Estuary and has a history of urban, industrial, and human impact (Coughanowr 1997). Two major industries in the Derwent catchment have had a key influence on the water quality of the estuary in the past; one is a newspaper print mill and the other is a zinc refinery.

Primary treated sewage from the Tasmanian capital city Hobart was also a major input into the Derwent Estuary until the mid 1990s. Unfortunately, bacterial data from the estuary is related only to pathogens (Coughanowr 1997) rather than total community estimates. Despite the human impact to the Derwent Estuary, the Huon and Derwent estuaries are very similar in their geography and catchment areas and overall, the biological systems are comparable. Tasmanian estuarine bacteria are therefore poorly studied both in relation to their total estimates as well as community variation.

1.6 Thesis outline

The research outlined in this thesis was prompted after a study examined an endemic Huon Estuary bacterium as a nutrient source for the toxic dinoflagellate *G. catenatum*. The bacterial strain was serendipitously found to lyse the cell wall of a number of toxic algae (Lovejoy *et al.* 1998).

A multidisciplinary approach and analysis of the microbial community in the Huon Estuary and the surrounding biological, physical and chemical oceanography of the estuarine sites was undertaken in order to better understand the ecology of algicidal species (chapter 2).

The majority of the research in this thesis concentrates on the four algicidal species isolated from the Huon Estuary. These are species from the genera *Pseudoalteromonas* and *Cellulophaga*. Other algicidal species from the genera *Bacillus* and *Planococcus* were also isolated. Taxonomic characterisations of these novel isolates are presented (chapter 3).

The thesis examines in detail algicidal bacteria and aspects of their algicidal specificity (chapter 4). The concentrations of algicidal components required to lyse the algal cells and their relationship with the different lifestages of *G. catenatum* as well as other toxic and non-toxic algal and microbial species are examined (chapter 4). These results led to a parallel study into the occurrence of algicidal bacteria in the marine environment and investigation of whether algicidal components are produced consistently in the natural setting or if they can be used to control future harmful algal blooms.

Like most of the estuaries and river systems in Tasmania, the marine bacterial community of the Huon Estuary is poorly understood. Using traditional and modern techniques, the thesis aims to increase knowledge and thus allow a broader understanding of the microbial ecology of the estuary. Methods undertaken in this thesis include phenotypic (chapters 5 and 7) and molecular (chapter 7) techniques which allow analyses of the microbial community structure. Investigation of the relationships between algal blooms and bacteria demonstrate how these

communities interact when large blooms of *G. catenatum* or diatoms occur (chapters 5 and 7).

The thesis investigates other compounds, potentially beneficial to humans, produced by algicidal and non-algicidal bacteria isolated from the Huon Estuary. The novel non-algicidal *Shewanella* species produces the essential omega-3 polyunsaturated fatty acids (chapter 6). Novel antibiotic compounds are produced by one of the algicidal bacteria (chapter 8).

Bacterial interaction within the environment is far more complex than simple nutrient regeneration. Bacteria cannot be categorised as a singular community whose members make consistent and similar contributions. Further research of their interactions will continue to demonstrate the same degree of complexity as those involved between higher trophic levels. The thesis aims to examine these microbial interactions with particular reference to novel algicidal bacteria from the estuary, the compounds they produce and their relationship with harmful algal blooms.

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2. Biological, chemical and physical ecology of the Huon Estuary

Summary

The Huon Estuary is an unpolluted estuary with low phosphate and nitrate concentrations. The brown-pigmented surface waters contain high levels of humic compounds that affect the biology of the estuary and are a factor in the availability of light to the deeper layers. A small diatom-dominated bloom occurred in the estuary from Nov. to Dec. 1998. From Dec. 1998 to Jan. 1999 the toxic dinoflagellate *G. catenatum* bloomed. A diatom bloom, dominated by the genus *Pseudonitzschia,* separated the first *G. catenatum* bloom and a second smaller bloom of *G. catenatum* that occurred from April to May 1999. Greatest bacterial diversity was observed in the estuary sediment samples. The estuary side arm contained higher bacterial numbers throughout the year than the main estuary. In the water column, higher bacterial populations occurred in summer than winter for all sites. Knowledge of the environmental parameters and biological ecology of the Huon Estuary is important for understanding the relationship of algicidal bacteria with algal blooms.

Introduction

Physical, chemical and biological data was collected and analysed from the Huon Estuary for the field samples presented in this thesis (Oct. 1998 to Jan. 2000) by Parker (2001) and myself (this thesis). CSIRO Division of Marine Research undertook a larger survey involving the entire estuary from Aug. 1996 to Sept. 1998. I was involved with some of the CSIRO surveys. In this thesis the report from the CSIRO study (CSIRO Huon Estuary Study Team 2000) is referred to as (HST 2000).

The Huon Estuary can be divided into three sections; upper reaches, lower reaches and side arm (Figure 2.1). The annual flow of the river averages 87 m^3/s (summer months: 30-40 m^3/s, winter months: 125-130 m^3/s, (HST 2000)).

Over a two-year study of the Huon Estuary, (Aug. 1996 to Sept. 1998), five biological stations were surveyed (HST 2000). The biological stations were part of a number of larger scale spatial surveys that included 35 sites throughout the estuary during different seasons (Figure 2.1). The biological stations were sampled more frequently to resolve short-term changes and algal bloom behaviour. In the study for this thesis, samples were taken fortnightly at three of the above mentioned biological stations and included sites F1, F3 and X3 (identified as X3b in HST 2000) from Oct. 1998 to Jan. 2000 (Figure 2.1).

The sites F1, F3 and X3 are marine dominated. Two sites (F1 and F3) were situated in the lower-middle reaches of the estuary and one in the sidearm (X3) (Figure 2.1). The lower reach is a marine zone, 40 m deep at the mouth of the estuary and 20 km from the Southern Ocean. The side arm, although shallower, has a lower proportion of fresh water from the two entering rivulets when compared with the main estuary body so is still a strongly marine ecosystem.

Chlorophyll correlations between sites F1 and F3 in the middle estuary and the estuary side arm (X3) were high (r^2, 0.79 to 0.86) compared with correlations between these sites and sites near the estuary mouth (r^2, 0.52 to 0.69) (HST 2000). This was attributable to high algal biomass from dinoflagellate blooms in the lower and middle estuary.

Limited analysis of the bacterial community in the Huon Estuary is presented in this chapter. Greater detail is presented in chapters 5 and 7. The objectives of this chapter were to:

- identify characteristics of the main chemical and physical parameters of the estuary;
- gain an ecological insight into estuarine dynamics in relation to algal blooms.

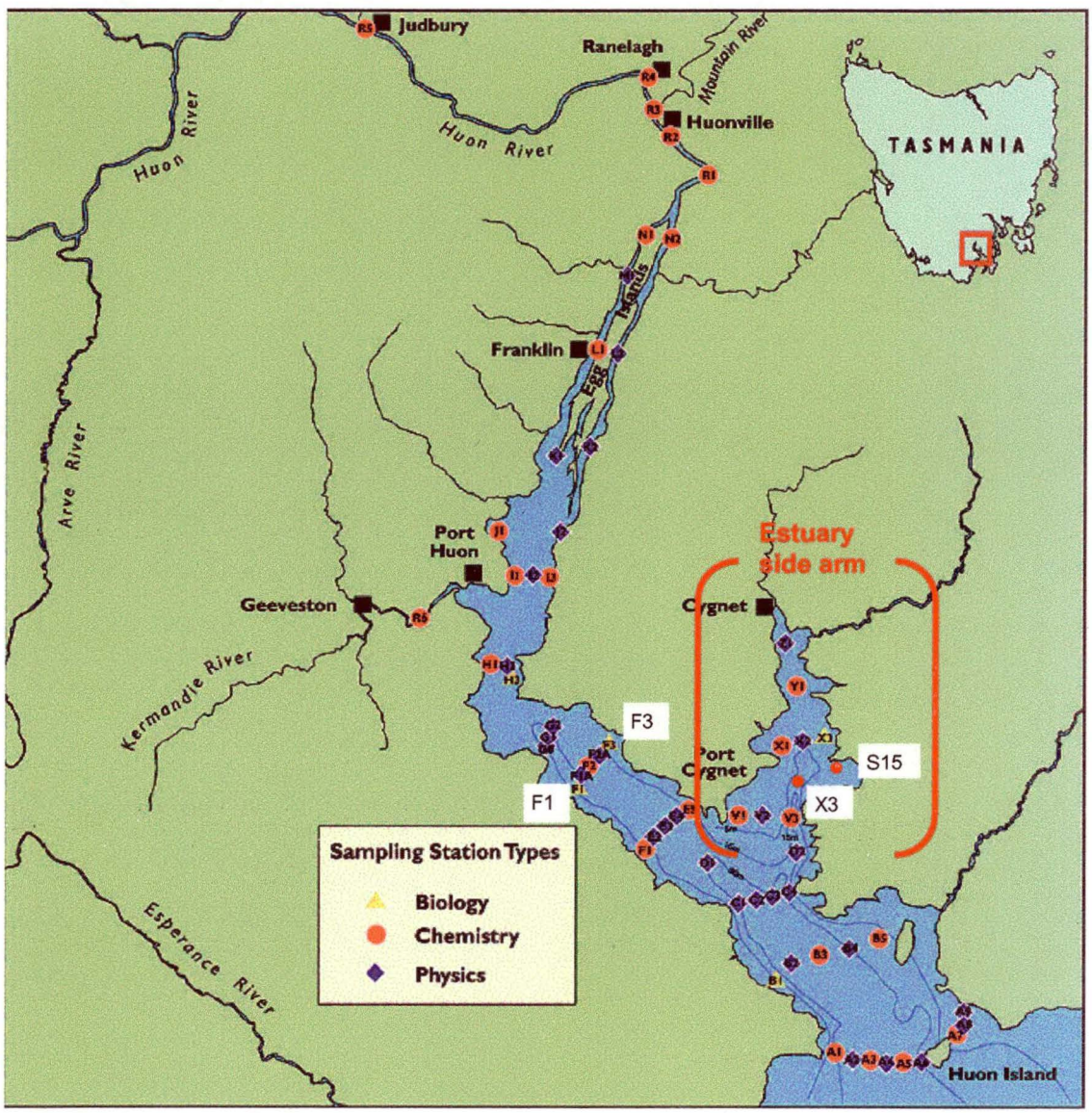

Figure 2.1 Sampling sites for the 1996 to 1998 study (HST 2000) highlighting site S15 and the three main sites sampled from 1998 to 1999; sites, F1, F3 and X3.

Methods

2.1 Huon Estuary study sites and sampling

The Oct. 1998 to Jan. 2000 survey completed for this thesis was confined to three sampling areas in the Huon Estuary on a fortnightly basis: sites F1, F3 and X3. The sites were the same locations as those sampled in the CSIRO study (HST 2000) from Aug. 1996 to Sep. 1998.

Bacterial estimates of the water column for two surveys involving the entire estuary (spatial surveys) were completed during the 1998 summer and winter (see Figure 2.1 for site details). Water temperatures were at their respective maximum or minimum for these two surveys. Bacterial estimates involving the sediments of the entire estuary were completed during 1998. All samples taken for bacterial analysis from Aug. 1996 to Sept. 1998 were primarily for isolation of bacterial species.

Water depths at the three sampling sites were site F1, 26 m, site F3, 18 m and site X3, 30 m. Sites F1 and F3 were on opposite sides of the estuary, mid way down the main estuary (Figure 2.1). Site F1 had the greatest river flow and was situated on the main estuary straight. Site F3 was close to a protected cove and was the shallowest site with the slowest river flow.

All three sites were situated near shellfish or finfish farms. Corner markers for farm boundaries were normally 50 m away from the cages. Only the estuary side-arm contained shellfish farms and site X3 was on the corner marker of one of these small mussel farms. Site location near fin and shell fish farms was unimportant in contributing to phytoplankton growth because of the high flushing time of the estuary and the time scales required for phytoplankton growth (HST 2000). Samples taken included: surface waters, pycnocline (typically a depth of 2 m for all 3 sites), an integrated sample from 0 to 12 m and a 20 μm plankton net sample (Figure 2.2, Table 2.1).

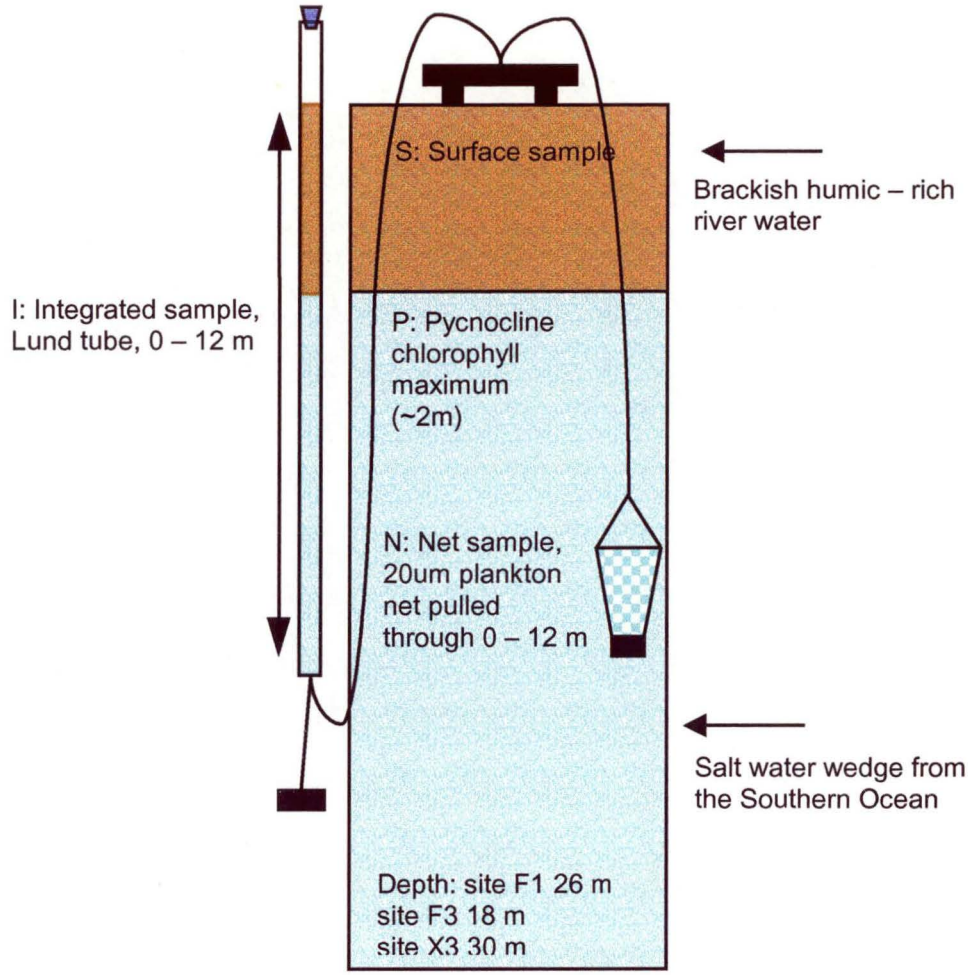

Figure 2.2 Schematic representation of the sample types taken during the field study and depths of the three sites. Stratification of the brackish and salt water is indicated.

Table 2.1 Sampling methods for collection of samples from sites F1, F3 and X3.

Depth	Sample type	Collection device	Depth	Sampling procedure
I	Integrated	Lund tube (weighted 2.5 cm x 12 m silicone tubing ~ 5 l)	0 – 12 m	Water collected in tube, emptied into plastic container, mixed and subsampled
S	Surface	5 l Niskin	0 – 0.2 m	Niskin inverted 3 times
P	Pycnocline /Mid depth	5 l Niskin	2 m or at the pycnocline which was calculated with a salinometer	Niskin inverted 3 times
N	Net	20 µm net	0 – 12 m (site F3 0 – 9 m)	Plankton net sampled at ~1 m/sec

2.2 Physical and chemical analyses

Precision salinity was determined by conductivity (Cowley 1999). A Secchi disc was used to measure light penetration depth. Standard flow-analysis procedures (Plaschke 1999) were modified slightly for the determination of the nutrients: nitrate, nitrite, phosphorus and silica. In this thesis, 'dissolved' refers to analysis of the filtrate after passing water samples though a 0.45 µm filter membrane. Nitrate and nitrite (or NO_x) were separately determined. In other studies nitrate is sometimes used as an abbreviation for nitrate and nitrite.

Phosphate and silicate were measured using the variants of the 'phospho-Molybdenum blue' method, which are selective for orthophosphate or orthosilicic acid and its ionic forms and measure other reactive condensed phosphates and silicates (dimer only). Some organic fractions of phosphorus and some colloidal silicic acid may also be included in the analytical measurements (HST 2000, Koroleff 1983, Robards *et al.* 1994).

2.3 Chlorophyll analysis

Phytoplankton abundances were measured using two methods: microscopic examination with emphasis on bloom forming species and by spectraphotometric absorbance to measure concentrations of chlorophyll *a, b* and *c*.

Chlorophyll *a* is the major pigment of all marine plants and microalgae and is commonly used as a routine measure of phytoplankton biomass (Jeffrey *et al.* 1997). Samples for chlorophyll analysis were collected and analysed for all three depths as described in the HST 2000 Supplementary Section (2000). Briefly, samples were transferred to clean 2 l plastic bottles in the field and stored in the dark on ice. Once in the laboratory, samples were filtered though a 47 mm Whatman GF/F glass fibre filter and the volume of the filtrate was recorded. The filters were stored frozen in liquid nitrogen until analysis was undertaken. Filters were cut up, placed in 90% acetone, agitated ultrasonically for 5 minutes, stored overnight at -20°C and then resonicated and centrifuged. Absorbance of the

resulting solution was measured using a spectrophotometer with 90% aqueous acetone in the reference beam.

2.4 Bacterial enumeration

Detailed analysis of bacterial numbers and classifications is presented in chapter 7. A brief description is provided in this chapter on bacterial numbers in the water column throughout the estuary during the 1998 summer and winter and for site X3, which was studied independently from site F1 and F3 during the CSIRO study. Bacteria were analysed using both plate and direct counting. The fluorescent stain 4'6'-diamidino-2-phenylindole (DAPI, Sigma) was used to compare with colony forming units (CFU) using the method of Lovejoy *et al.* (1998) at site F3 (chapter 7). Bacterial isolations and enumeration at the site in the estuary side arm (X3) were undertaken in order to isolate algicidal bacteria and ascertain their numbers. Integrated samples (0-12 m) were taken fortnightly from sites X3 and F3 (bacterial numbers for site F3 are reported in chapter 7).

Duplicate sediment samples (0.5 g x 2) for bacterial enumeration using DAPI were collected and diluted with particle-free (0.2 mm-filtered) artificial seawater (Sigma). Subsamples were alternately vortexed, sonicated and vortexed (1 min:1 min: 1 min) in 10 ml of filtered autoclaved artificial seawater to help detach bacteria from sediment grains before staining with 5 µg/ml DAPI for 15 min. Enumeration of bacterial cells was completed after filtration onto 0.2 µm black polycarbonate filters (Millipore). The duplicate sediment suspensions (each 50 µl) were also cultured in duplicate (i.e. 4 plates per sample site) on Marine Agar (1 g Yeast Extract (Oxoid), 4 g Bacteriological peptone (Oxoid), and 1 l filtered Huon Estuary river water adjusted to a salinity of 28 psu with artificial sea salts and incubated at 22 °C for 10 days). Bacterial numbers were related to wet sediment mass as these have been reported as a more consistent measure (i.e. wet volumes rather than dry) (Schmidt 1998). Bacterial estimates of water column samples were ascertained by a dilution series on the above media (see also chapter 7, methods).

Results

2.5 Light penetration

The Secchi disc is used as an indicator of light penetration and could not be observed past 1 m for some sampling dates (Figure 2.3). These dates corresponded to high rainfall episodes (rainfall data not shown). Site F1, had the highest light penetration depth of the three sites for all four seasons

During the summer and autumn *G. catenatum* blooms (Figure 2.3, section 2.8), there was an increase in Secchi depth at sites F1 and F3 signifying lower marine layers mixing with upper waters. This was followed by a period of stratification. At the end of both *G. catenatum* blooms, an increase in light penetration was observed in the water column (Figure 2.3).

2.6 Salinity

There was distinct salinity stratification during episodes when influx of fresh water high was high (rainfall data not shown) and light penetration was low (see section 2.5). Stratification of salinity was strongly delineated and occurred at approximately 2 m. The vertical distance of stratification from brackish water to saltwater was 5-10 cm for most of the year. Greater stratification of salinity between the surface samples and the pycnocline samples was noticeable for the two sites in the main estuary (sites F1 and F3) when compared to site X3 in the estuary side arm (Figure 2.3).

2.7 Temperature

In late summer, the brackish surface layer of the Huon Estuary reached higher temperatures than the lower marine layer (Figure 2.4). In winter, lowest temperatures were observed in the surface samples. Snowmelt contributed to river discharge at this time.

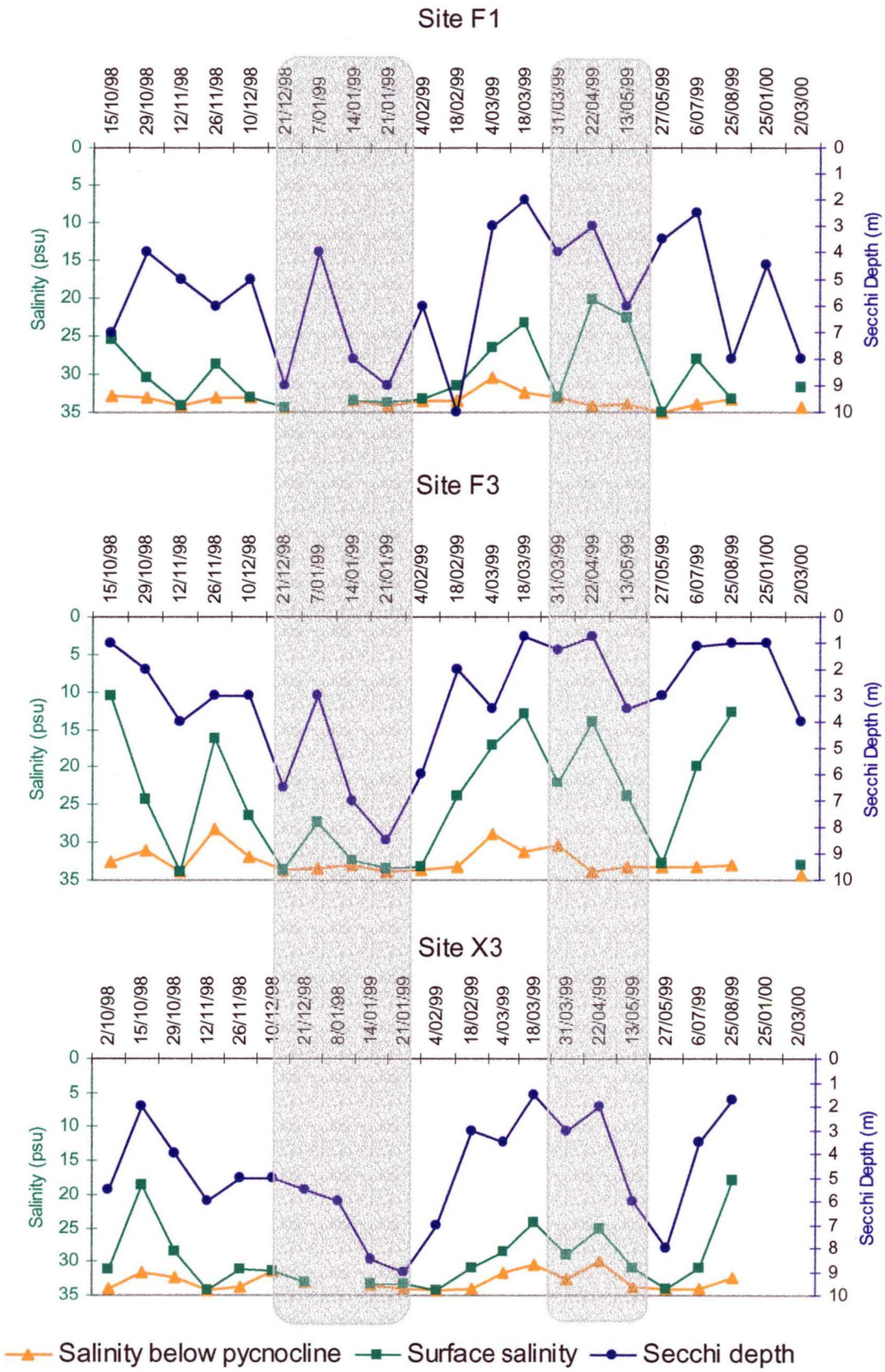

Figure 2.3 Variation in salinity (psu) above and below the pycnocline at sites F1, F3 and X3 for samples taken from 1998 to 2000. Secchi depth (m) indicates level of light penetration and is shown on the 2nd Y axis. represents approximate duration of the summer and autumn *G. catenatum* bloom.

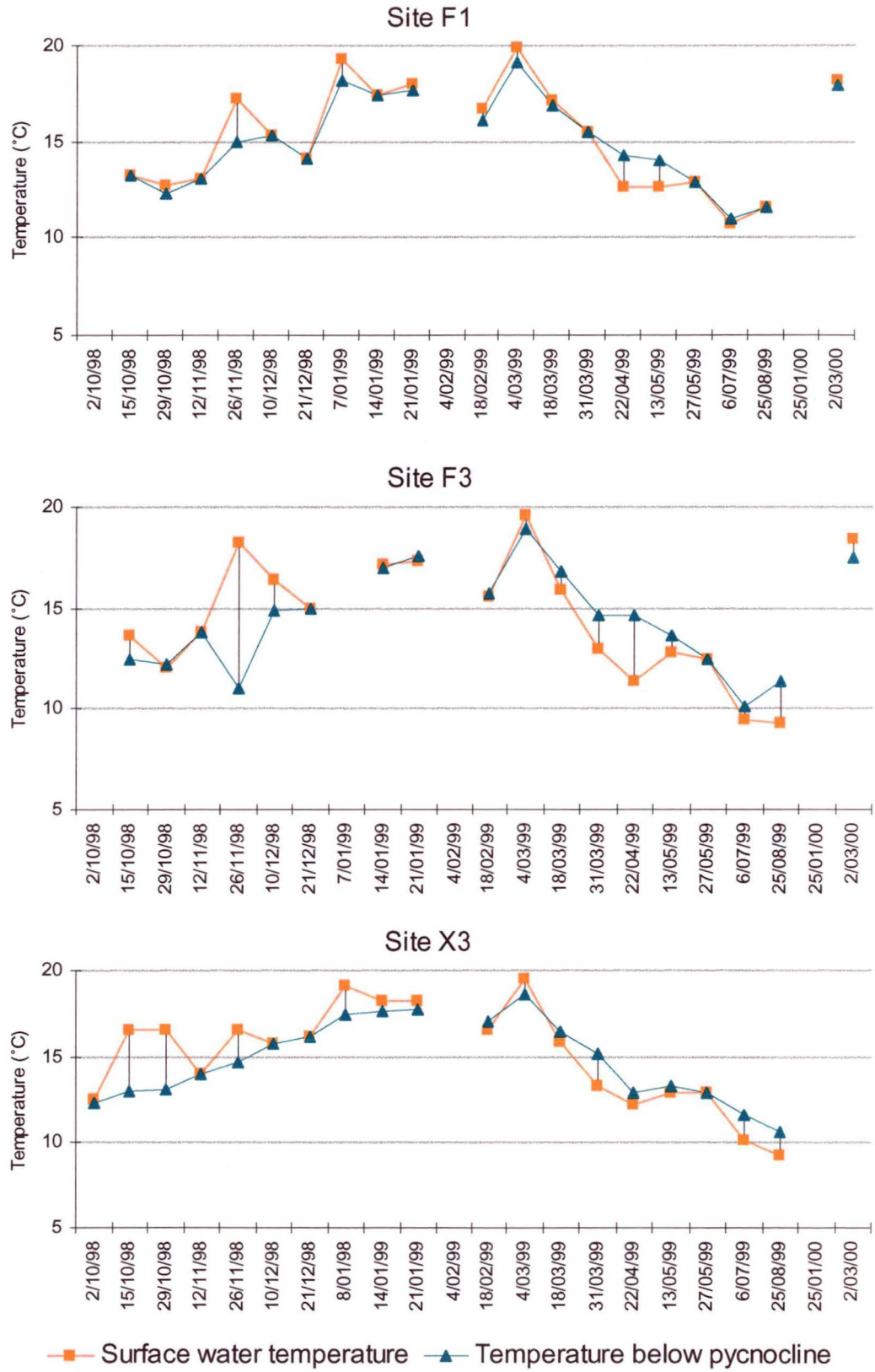

Figure 2.4 Variation in temperature (°C) of surface and pycnocline samples for the sites, F1, F3 and X3 from Oct. 1998 to Jan. 2000.

Highest and lowest temperatures were recorded in the surface samples. Maximums were observed in Feb. and minimums in Aug. Temperatures below the pycnocline from May to Jan. were warmer than surface waters for all three sites. From April to June the reverse applied, where surface waters were warmer than the underlying marine layer. Temperatures were similar for both the surface and marine layers from Jan. to March 1999. Variation between temperatures at the surface and pycnocline from 1998 to 1999 was greatest at the sheltered site in the main estuary (site F3).

2.8 Algal community structure

During sampling in 1999, four major changes in algal species occurred in the estuary (Figure 2.5). A small diatom-dominated bloom (flagellates and dinoflagellates were also observed) occurred in Nov. and Dec. 1998. A large bloom of the diatom *Pseudonitzschia* spp. dominated in late summer (Feb. to March 1999). This bloom was largely composed of *P. pseudodelicatissima* species (Parker pers. comm.). Two blooms of *G. catenatum* were also observed. One occurred in mid-summer (Dec. 1998 to Jan. 1999) and a second smaller bloom, in late autumn (April to May 1999) (Figure 2.6). This repeated the sequence of *G. catenatum* blooms in 1998 where a summer bloom early in the year was followed by a smaller autumn bloom (Figure 2.7).

The *Pseudonitzschia* spp. bloom was observed at all three sites in Feb. 1999. It dominated at site F3. *Pseudonitzschia* cells were prolific in the surface water samples where their highest concentrations occurred and they could easily be seen with the naked eye.

Species such as the dinoflagellate *Ceratium* were also observed during 1998 and 1999 and often occurred with diatoms including during the 1999 *Pseudonitzschia* spp. bloom. High proportions of *Ceratium* spp. and other diatoms and unidentified flagellates were present from Oct. to early Dec. 1998. A variety of heterotrophic species were also present throughout the year and were most numerous during algal blooms.

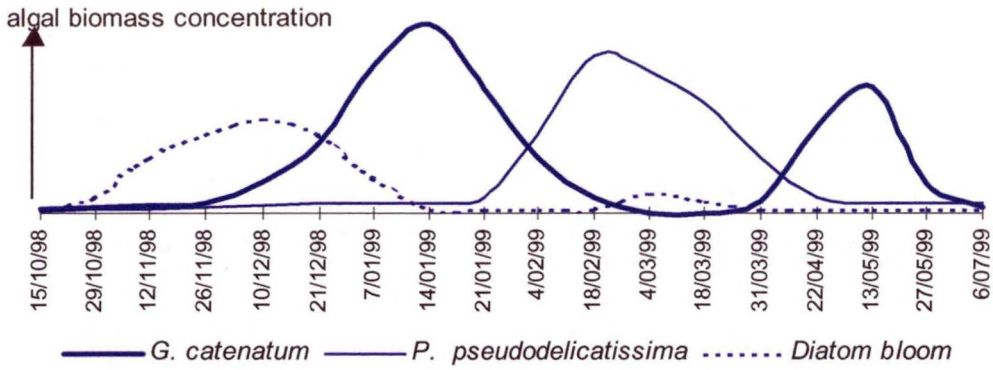

Figure 2.5 Schematic representation of the major algal blooms occurring in the Huon from 1998 to 1999. Scaling whilst not accurate is indicative of cell biomass abundance and period of bloom.

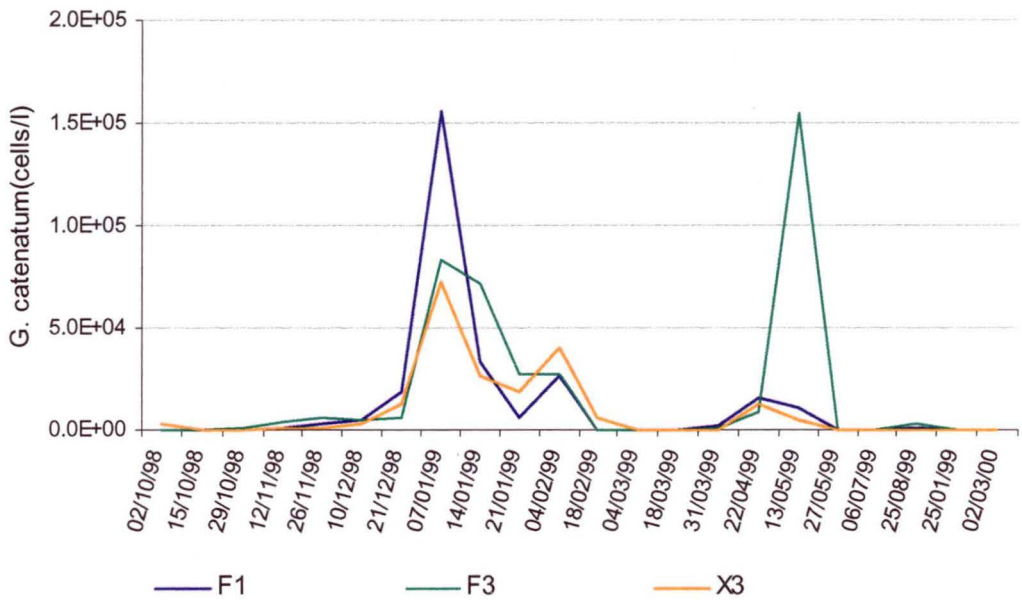

Figure 2.6 *G. catenatum* cell estimates for integrated samples taken from 1998 to 2000 (Parker 2001).

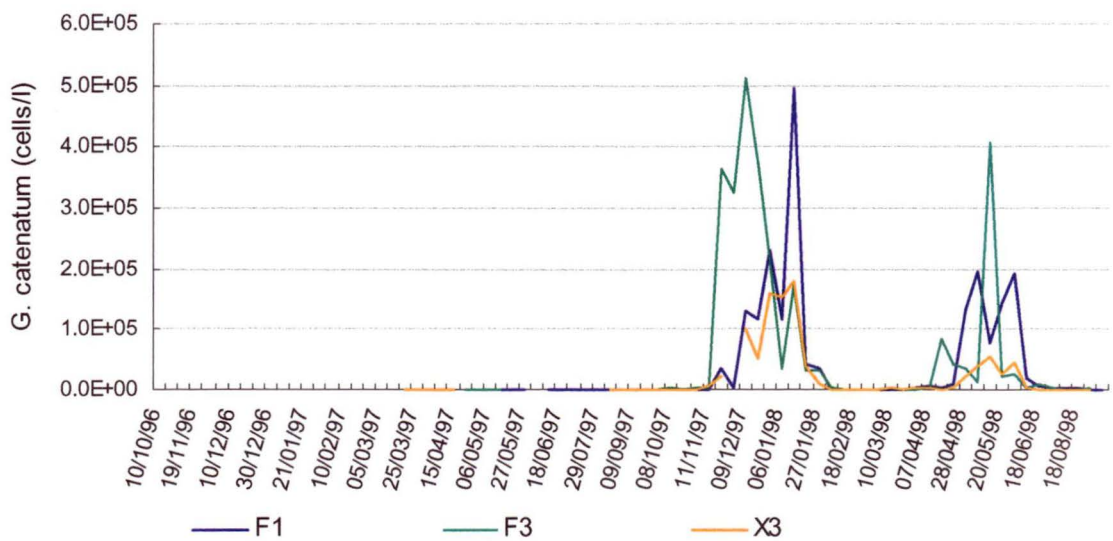

Figure 2.7 *G. catenatum* cell estimates for integrated samples taken from 1996 to 1998 (HST 2000, Parker 2001)

Algal biomass was not indicative of the toxicity of *G. catenatum* blooms (Figure 2.8). In samples taken from Dec. 1998 to Feb. 1999 and May to June 1999, shellfish farms near site X3 were closed because of toxins produced by this alga. Site X3 displayed higher cell numbers during the summer *G. catenatum* bloom than the autumn bloom (Figure 2.8). However, saxitoxin concentrations in the mussel tissue were lower during the summer bloom than the autumn bloom (Figure 2.8).

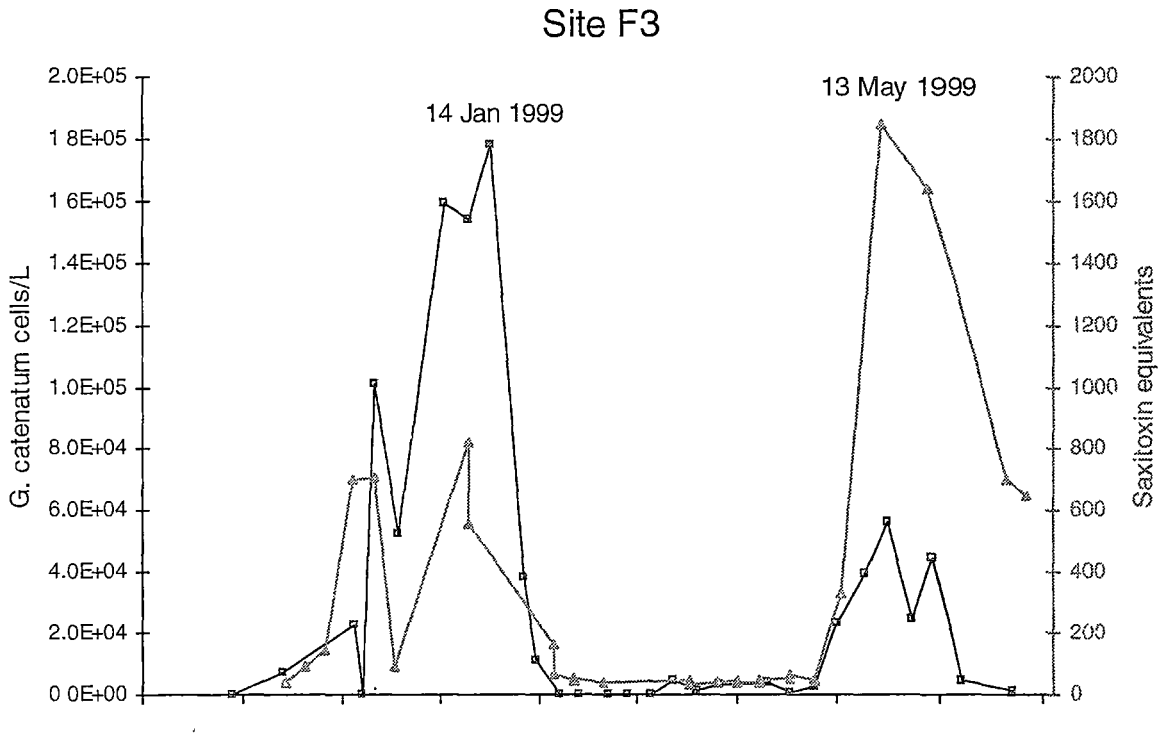

Figure 2.8 Variation of saxitoxins in mussel tissue during 1998 to 2000 compared to *G. catenatum* cell numbers demonstrating bloom size does not necessarily give an indication of mussel toxicity (cell numbers (Parker 2001); saxitoxin data: R. Brown, Manager Tasmanian Shellfish Quality Assurance Program)

2.9 Chlorophyll

Chlorophyll concentrations highlight the two *G. catenatum* blooms and the summer *Pseudonitzschia* spp. bloom (Figure 2.9). Highest chlorophyll levels were observed during the two *G. catenatum* blooms in summer and autumn. Chlorophyll was typically at maximum concentrations in the upper 2 m.

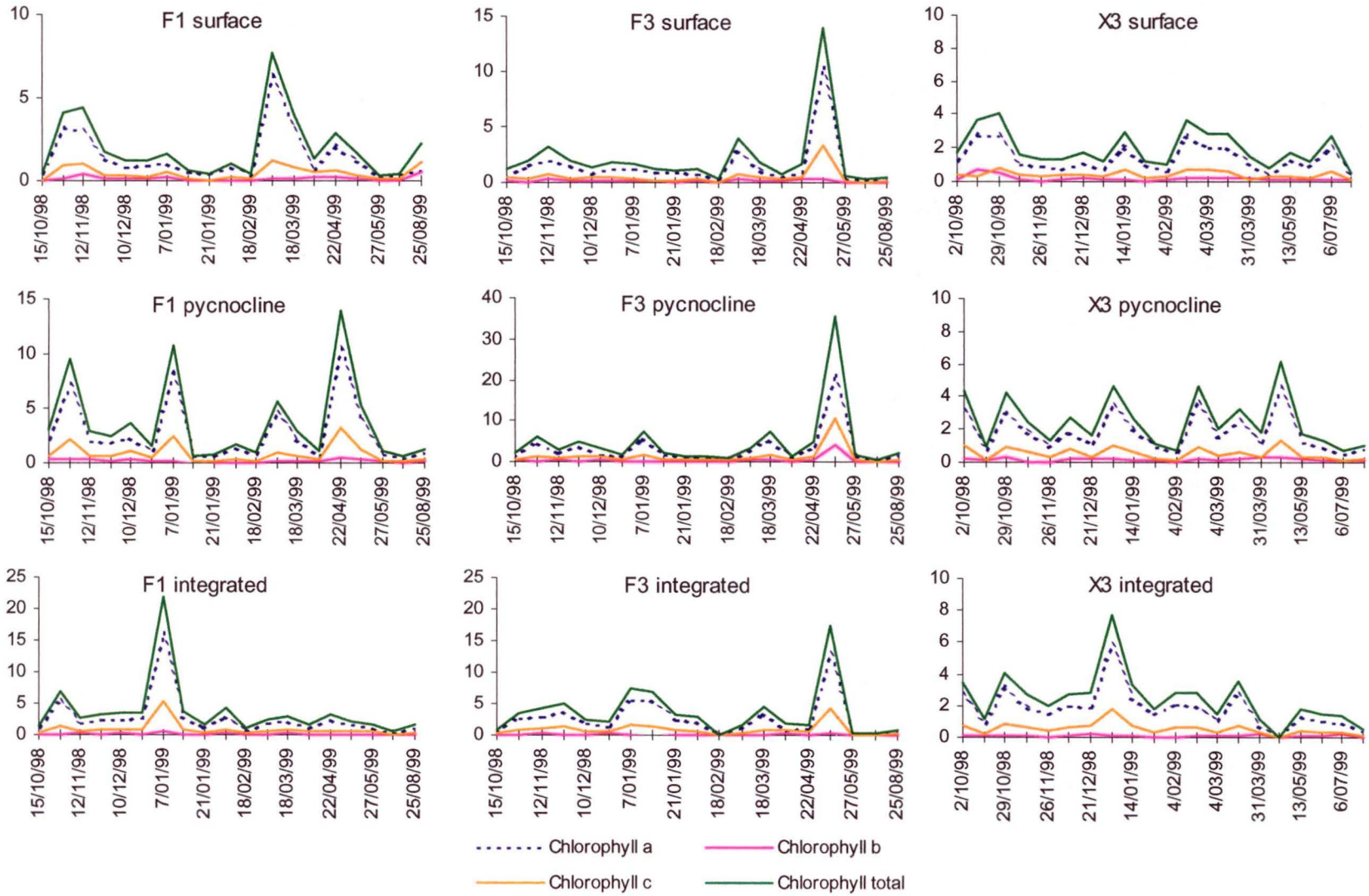

Figure 2.9 Variation in chlorophyll concentrations from 1998 to 1999. Yaxis: mg/l

The highest chlorophyll concentration was 36 mg/l and was observed at the pycnocline for site F3 in May. *G. catenatum* was blooming throughout the water column at site F3 and high chlorophyll concentrations were observed for both the surface and integrated samples (14 and 17 mg/l respectively). Sites F1 and X3 did not demonstrate the same high chlorophyll concentrations as site F3 at this time despite the presence of *G. catenatum* at these sites. The autumn *G. catenatum* bloom occurred earlier at site F1 in late April. Light penetration (3 m, section 2.5) was the same during the observed *G. catenatum* bloom at site F1 in April as at site F3 in the next fortnight in May. The depth of light penetration in May at sites F1 and X3 was double that of site F3 (6 m).

Site X3 contained the lowest chlorophyll concentration throughout 1999 in comparison with the other two sites. The highest chlorophyll levels were observed in the pycnocline and integrated samples at site X3 in mid Jan. 1999. Site F1 had the highest chlorophyll levels for the integrated and pycnocline samples during the Jan. bloom of *G. catenatum.* Surface samples by comparison had low chlorophyll concentrations during the summer *G. catenatum* bloom. Higher temperatures (20 °C) were observed for surface samples during this period when compared with the pycnocline samples (17-18 °C) (Figures 2.3 and 2.4). This observation can be seen in Figure 2.10 where the alga is present in surface waters at 16-18 °C however, once the surface waters reached 20 °C the majority of *G. catenatum* cells have migrated down the water column. Although this temperature difference is small, 20 °C is past the preferred temperature range for *G. catenatum* (Hallegraeff *et al.* 1995b) and thus it may have sought cooler waters.

Chlorophyll levels for the *Pseudonitzschia* spp. bloom were highest in the surface samples and at the pycnocline for all three sites. The small cell size of this diatom results in lower chlorophyll concentrations than those observed for the *G. catenatum* bloom. The *Pseudonitzschia* spp. bloom occurred in the upper 2 m and did not cover the same vertical extent as the *G. Catenatum* bloom.

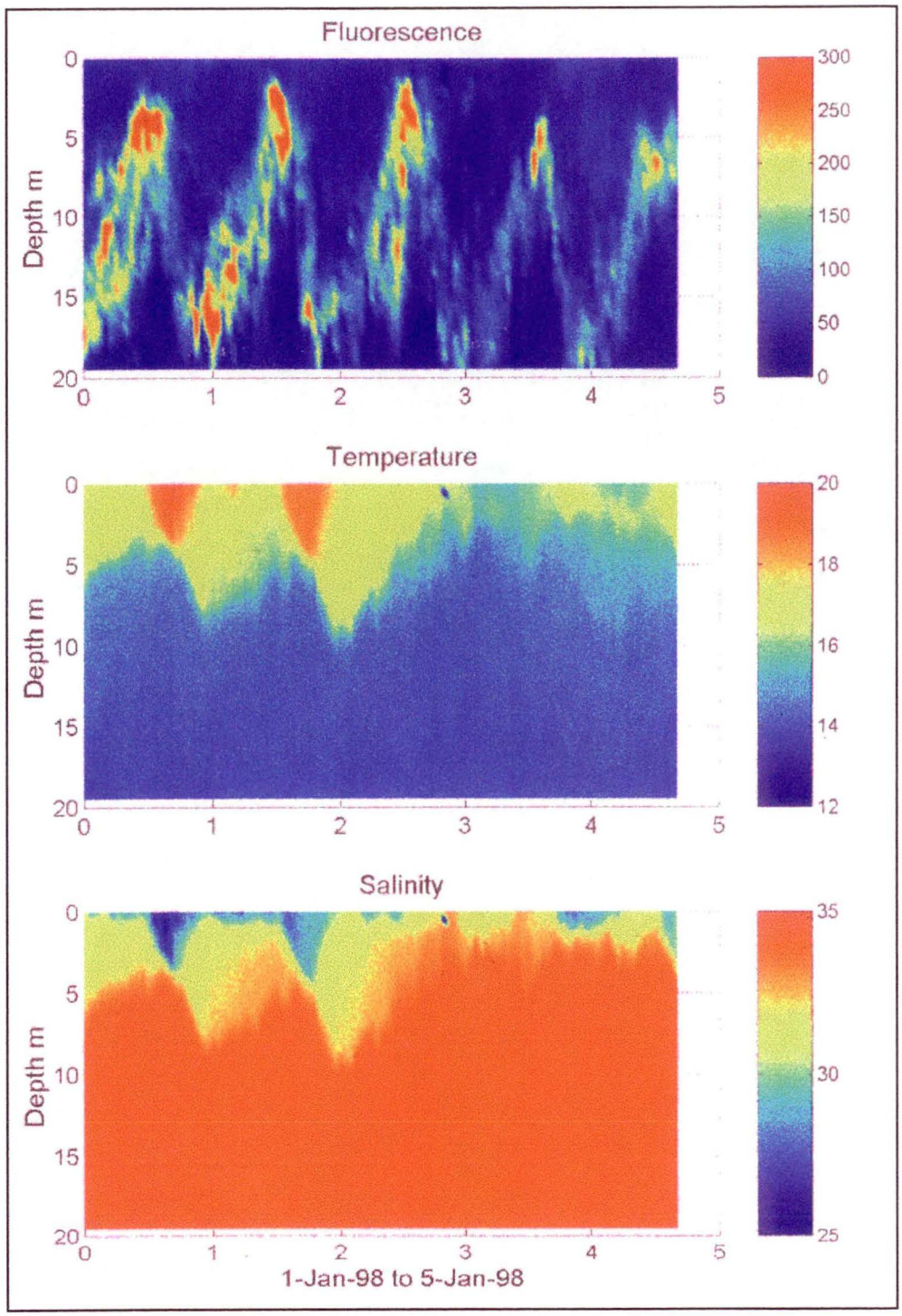

Figure 2.10 Time-depth sections of fluorescence, temperature and salinity at site F1, 1/1-5/1/98 (HST 2000). A strong vertical diurnal migration of *G. catenatum* is shown in the top figure.

Chlorophyll levels indicated the presence of the earlier summer diatom-dominated bloom from Oct. to Dec. 1998 that included the genera *Chaetoceros, Pseudonitzschia, Ceratium* and unidentified flagellates. This algal bloom was observed throughout the water column at Sites X3 and F3 (Figures 2.5 and 2.9). High chlorophyll levels were observed at all three sites during this bloom, particularly in the pycnocline and integrated samples at site F1.

Chlorophyll *a* was highest for all depths and sites. Chlorophyll *c* and *b* were both relatively low in all samples (Figure 2.9). High chlorophyll concentrations, indicative of algal blooms, were always observed at Site F1 before they were observed at the other two sites. Algal blooms at sites X3 and F3 were slightly delayed in comparison to site F1 and occurred concurrently in the following weeks (Figure 2.9). Algal blooms at both sites F3 and X3 although delayed, were prolonged.

2.10 Nutrients

Increased silicate concentrations were observed during the decline of the *Pseudonitzschia* spp. bloom in Feb. 1999 (Figure 2.11). Silicate concentrations were also higher after the first diatom bloom and they increased in the surface samples of sites F1 and F3 at this time. During the summer *G. catenatum* bloom, silicate was also high. Salinity (Figure 2.3) was inversely proportional to silicate (Figure 2.11).

The NO_x totals were predominantly nitrate (Figure 2.11). Nitrate in field samples was often strongly depleted to near the analytical detection limit in summer and early autumn. In early autumn, nitrate concentrations increased to values usually found in marine waters (Figure 2.11). These results concur with the results and conclusions previously established for the estuary (HST 2000). Nitrate levels were low in integrated samples. This was explained by HST (2000) as being due to the low concentrations in the surface waters.

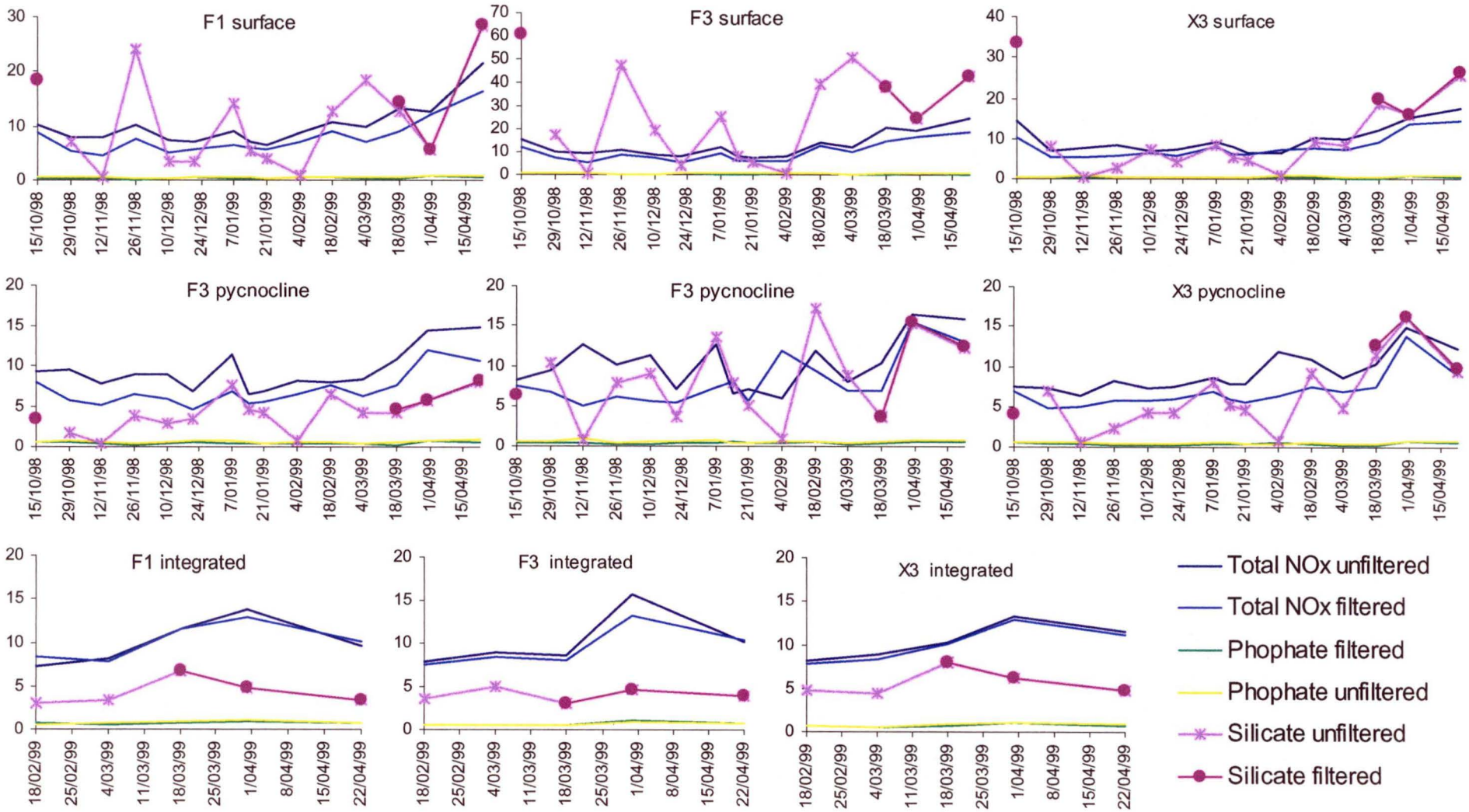

Figure 2.11 Variation in nutrient concentrations from 1998 to 1999. Yaxis: µM

Nitrite demonstrated similar trends at lower concentrations than nitrate. Both nitrate and nitrite levels were higher in the lower marine dominated depths (Figure 2.11). Phosphate levels were also below analytical detection limit on most occasions and remained very low throughout the sampling period. Phosphate levels were highest in the samples taken from the marine influenced deeper waters (Figure 2.11).

2.11 Bacteria

Detailed analysis of bacterial numbers and classifications from 1998-1999 is presented in chapter 7. The following represents preliminary data taken independently during the CSIRO Huon Estuary study 1997 to 1998. Bacterial estimates at site X3 pycnocline and integrated samples from 1997 to 1999 rarely altered from 10^{8-9} cells/l DAPI (CFU, 10^{6-7} cells/l) (Figures 2.12 and 2.13). This agreed with estimates by Lovejoy *et al.* (1998) for the previous year. Bacterial numbers at site X3 from 1997 to 1998 were highest just after all four major algal blooms (Figures 2.13 and 2.7). Bacterial estimates in the water column were made at the periods of maximum and minimum water column temperature during two of the CSIRO surveys (Figure 2.12). Highest numbers were observed during Feb. 1998 for all sites throughout the Huon Estuary. Winter and summer surveys sites in the estuary side arm contained slightly higher bacterial concentrations than for the main estuary (Figure 2.12).

Colony types found in sediment samples were highly varied in comparison to the water column and plankton net particulate samples. The original purpose of sediment collection was to culture algicidal organisms from the sediments rather than provide total biomass estimates. Sediments collected throughout the estuary (Figure 2.1) during winter (July 1998) had low bacterial biomass (CFU 10^4-10^7 cells/g (ww) for all spatial survey sites, DAPI 10^7 cells/g for site A3; 10^9 cells/g (ww) for site S15 (Figure 2.1)).

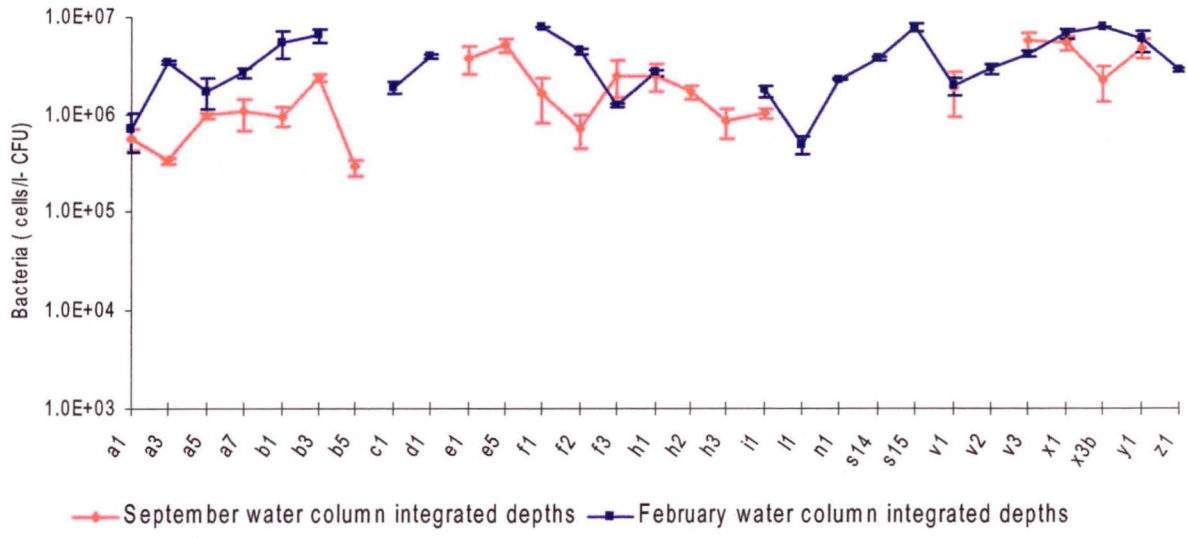

Figure 2.12 Bacterial abundances in the integrated water coloumn samples at the time of minimum and maximum water temperatures (Sept. and Feb. 1998 respectively) at sites thoughout the Huon Estuary. Sites are those marked in Figure 2.1)

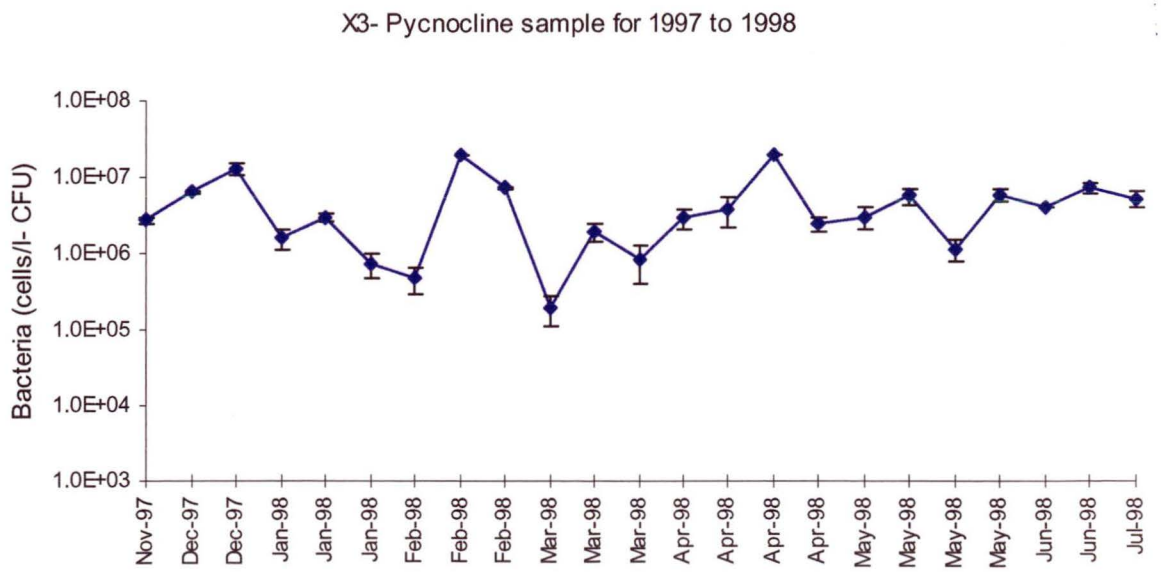

Figure 2.13 Bacterial abundances of the fortnightly pycnocline water samples at site X3 from 1997-98.

The low CFU values result from the poor performance of this technique at representing and enumerating organisms from sedimentary environments. Some of the bacteria would also be anaerobes and samples were cultured under aerobic conditions. A high level of background autofluorescence was observed when using DAPI.

Sediments at the mouth of the estuary near sites A and B (Figure 2.1) were composed of coarse sand while sediments at sites X3, F3 and S15 (which exhibited the highest cell estimates) were fine mud. Bacterial estimates for sediment at sites X3 and F1 were an order of magnitude higher than those in other areas of the estuary. However sediments from site F3 (section 2.1) were similar to those found elsewhere in the estuary despite site F3 being situated 50 m from a fish farm.

Discussion

The physical and chemical properties of the Huon Estuary are more characteristic of northern hemisphere estuaries than other Australian estuaries (HST 2000). The estuary was strongly stratified and nitrogen levels were largely supported by the contribution from coastal seawater and algal blooms. The algal blooms that occurred during 1998 and 1999 were dominated by the alga *G. catenatum*. Diatoms of the genus *Pseudonitzschia* also formed long lasting blooms in late summer and early autumn of 1999.

2.12 Physical variables

The Huon Estuary generally demonstrated strong stratification. Secchi depth was proportional to salinity in the surface layer as episodes of high rainfall coincided with an increase in fresh water and thus an increase in the humic compounds and a resultant decrease in light penetration. Site F1 was most influenced by tidal flow and thus had the highest light penetration depth for the three sites. Sites X3 and F3 were slightly more protected from tidal flows and had lower light penetration. A fresh water influx occurred before the summer and autumn blooms of *G. catenatum* (Jan. 1998 and May 1999). The pycnocline was measured at

approximately 2 m. The pycnocline is significant for the biology of the estuary as most algal blooms occur in and around this region.

The brackish surface water also has a major impact on the biology of the estuary. This surface layer demonstrated the greatest variability of the depths monitored in temperature and salinity. This was due in part to pigmented humic compounds decreasing light penetration and increasing heat absorption during summer. In winter, the surface layer also exhibited the greatest temperature reduction, attributable to the contribution from snowmelt. The marine layer of the estuary demonstrated stable temperatures and salinities throughout the year.

2.13 Chemical variables

The CSIRO study examined the relationship between nutrient levels, suspended particulate matter and algal blooms. Despite the presumption that one of these factors may be the cause of the *G. catenatum* blooms, the study did not find any such correlation (HST 2000).

The biology of the estuary is dominated by seasonal changes rather than nutrient availability. Low concentrations of dissolved inorganic nitrogen (DIN) and phosphorus were observed in 1999 despite the observation of the three large algal blooms. An increase in DIN during winter and a decrease in summer was observed for all years. Nitrate levels in 1997, 1998 (HST 2000) and 1999 changed in a seasonal cycle that was related to the algal blooms, but did not trigger them. The results indicate that all nitrate was used in the estuary and during summer and early autumn the estuary was a nitrogen sink, indicating the influence of primary productivity. During 1999, nitrate increased during autumn after lower levels in summer and was also associated with the late summer *Pseudonitzschia* spp. bloom. During the 1997, 1998 (HST 2000) and 1999 winters, nitrate values were similar at all stations and it was surmised that this reflects strong circulation of marine nitrate though the middle and lower estuary. Diatom blooms in the estuary during this and the CSIRO study were associated with the early spring drawdown of NO_x (HST 2000). NO_x reappeared in the water column in autumn for all years.

Elevated ammonia levels followed all algal blooms from 1997 to 1998, and were associated with drawdown of NH_4^+ (diatoms) and NH_4^+ and NO_x (*G. catenatum*) respectively (HST 2000). Ammonia values were consistently lower at site X3 than at other biological stations. The cause was not clear although mussel farmers speculate that it may be a result of the presence of mussel farms in this arm of the estuary (HST 2000). NH_4 was not measured from Oct. 1999 to Jan. 2000, however NO_x values increased in 1999 after both *G. catenatum* blooms.

The diel vertical migration of *G. catenatum* can be explained in summer by the alga's requirement for nutrients. Nitrate concentrations for the pycnocline and surface samples were higher during the autumn *G. catenatum* bloom in 1999 than the summer bloom. Similar concentrations were observed during the autumn *G. catenatum* bloom in 1998 (HST 2000). Ammonia was low in bottom waters in 1998 so diel migration may indicate a micronutrient limitation of an element more abundant in bottom waters (HST 2000). Potential micronutrients may include bromine, iodine or common seawater compounds not found insufficient concentrations in the brackish surface waters.

Silicate was inversely proportional to salinity from Oct. 1998 to Jan. 2000 (Figure 2.3 and Figure 2.11). This inverse relationship was also observed in the CSIRO study (HST 2000). Silicate levels demonstrated a strong positive correlation with the *Pseudonitzschia* spp. bloom and the earlier summer diatom bloom in 1999.

In the study by HST (2000), variance of chlorophyll was analysed among samples within spatial surveys. For the lower and middle estuaries, variance in surface log(chlorophyll *a*) was relatively uniform across the survey locations. However, pycnocline log(chlorophyll *a*) varied widely across surveys (HST 2000). This demonstrates that although similarities were observed for the biological, chemical and physical data for sites in the middle reaches of the estuary (where the sites of interest in this study are located), there is some variation in the algal blooms and biological dynamics between them. Highest chlorophyll concentrations were noted from 1996 to 2000 in the middle estuary (i.e. sites F3, F1 and X3) when compared to lower and upper estuary sites (HST 2000 and this study). Chlorophyll concentrations were extremely high at site F3 during the 1999 summer *G.*

catenatum bloom. All sites exhibited higher chlorophyll concentrations during the summer bloom than during the smaller autumn bloom. It is possible that chlorophyll levels throughout the water column and cell numbers of *G. catenatum* blooms were not always truly representative of the actual bloom at the site given the capacity of *G. catenatum* for rapid vertical migration.

2.14 Biological variables

Previous research and monitoring demonstrated that dinoflagellate blooms were interspersed with diatom blooms and the two often co-existed (Jameson and Hallegraeff 1994). *G. catenatum* did not bloom for three years between 1994 and late 1997 although large blooms occurred in 1998 (Figure 2.5, HST 2000). The dynamics of the estuary did not appear to differ greatly from previous years when major *G. catenatum* blooms occurred (HST 2000). During 1999 when two *G. catenatum* blooms formed, they were interspersed with a *Pseudonitzschia* spp. diatom bloom and were preceded by a small dominated-bloom containing *Ceratium*, *Chaetoceros* spp. and a number of unidentified flagellate species. Diatoms were generally at highest concentrations in the surface samples. Fatty acid analysis of the 1998 to 1999 samples indicates that the unidentified flagellates may have been prasinophytes (chapter 5). This finding concurs with the 1997-1998 study where similar unidentified flagellates were identified as a mixture of haptophytes and prasinophytes using HPLC pigment markers (HST 2000).

Both autumn and summer *G. catenatum* blooms in 1999 were smaller than those observed in 1998. Correlations between chlorophyll and cell numbers for sites F1, F3 and X3 were high during 1998 (r^2: 0.79 to 0.86) (HST 2000). This correlation was attributable to high biomass blooms by *G. catenatum* in the lower and middle estuaries (HST 2000). This demonstrates the similarity between these sites with respect to algal bloom dynamics. The extension of bloom formation at sites F3 and X3 in comparison to site F1 resulted from the geography and the faster water flow at site F1 compared to sites F3 and X3 which were located in more sheltered areas.

The seasonal phytoplankton successions display a classic temperate seasonal cycle similar to that of a northern hemisphere estuary (Kennedy 1982). The majority of

other estuaries in Australia are 'wet and dry tropical' and subtropical systems (68%, Eyre 1998) and in these estuaries, phytoplanktonic succession is based on episodic freshwater flows.

2.14.1 Bacteria

Other estuarine studies report that there is a shift from a more nitrogen-associated metabolism (proteins) in the main body of an estuary to a carbon utilising community (carbohydrates) at the fresher upper reaches (Cunha *et al.* 2000, Murrell *et al.* 1999). This shift to a carbon-utilising bacterial community is argued to demonstrate the importance of autotrophic processes as a source of substrates during summer and daylight hours. In these upper reaches, bacteria obtain their organic matter mainly from the flux of dissolved organic carbon (DOC) rather than the decomposition of detrital particulate organic matter (POM) (Murrell *et al.* 1999). Bacterial conversion of high (> 3,000 Da) and low (< 3,000 Da) molecular weight dissolved organic nitrogen in another study suggested that the nitrogen-rich compounds were removed from the dissolved organic matter (DOM) and then consumed by bacteria, while the carbon skeleton was unaffected by the degradation processes (Kerner and Spitzy 2001).

In the Huon Estuary, bacterial numbers in the water column were higher overall in the 1998-99 summer than those in winter. Numbers were highest throughout the year at sites in the estuary side arm (including site X3). The study by HST (2000) reported that phosphate, nitrate and nitrite depletion for the estuary sidearm was greater than in main body of the estuary. Algal blooms in the estuary side arm were never sites of peak algal biomass, although the algal species were the same as those in the main estuary (this study, and HST 2000). The removal of algal biomass by shellfish farms may be responsible for this finding (HST 2000.). Alternatively, it is possible that bacteria are removing the nitrogen components from the DOM within the estuary. However, no incorporation studies were completed to examine this hypothesis.

Humic substances and bacteria

The presence of humic compounds (hydrophobic dissolved organic matter) in an estuary may also influence the type and quantity of bacteria present. The Huon Estuary has the highest proportion of chromophoric dissolved organic matter (CDOM) than any other estuary in Australia (HST 2000). In a number of studies, bacterial numbers and bacterial production were significantly higher in estuaries containing humic compounds compared to those without (Esham *et al.* 2000, Bushaw-Newton and Moran 1999, Carlsson 1995). Humic substances irradiated with natural sunlight have also been shown to enhance bacterial growth as a result of increased availability of carbon and nitrogen components (Bushaw-Newton and Moran 1999, Carlsson *et al.* 1999). The study by Bushaw-Newton and Moran (1999) reports that the total biologically available nitrogen formed during a day-long irradiation accounted for about 6 % of the original nitrogen associated with the humic compounds. They reported that that photochemical modification of marine humic compounds might provide a source of labile nitrogen to estuarine and coastal ecosystems (Bushaw-Newton and Moran 1999).

2.14.2 *Pseudonitzschia*

While toxin production by dinoflagellates and their association with toxic algal blooms has been recognized for at least 70 years, toxins produced by algal genera such as *Pseudonitzschia* have only been recognised for the last 14 years (Hallegraeff 1995a). *Pseudonitzschia* species are common inhabitants of many coastal regions and several species and strains are associated with the production of domoic acid. This toxin is responsible for amnesic shellfish poisoning (ASP). One species, *Pseudonitzschia pseudodelicatissima,* is common in marine and brackish waters and has toxic and non-toxic strains.

The dominant bloom-forming *Pseudonitzschia* species in Australian coastal waters is reported to be *P. fraudulenta* (New South Wales), while *P. subpacifica* is also common (Hallegraeff 1994). In Tasmania and Victoria, *P. pungens f. pungens* and *P. pseudodelicatissima* are the dominant species. The species *P. pseudodelicatissima* has been found in high-nutrient open-ocean regions (Buck and

Chavez 1994) and the species has also been described in a number of relatively unpolluted Australian coastal waters (Hallegraeff 1994). *Pseudonitzschia* species that bloom in the Huon Estuary are dominated by *P. pseudodelicatissima* and *P. subpacifica* (HST 2000). Neither of these species are toxic (Hallegraeff 1994). Other *Pseudonitzschia* species found in the Huon Estuary are also non-toxic strains (HST 2000).

In 1999, *Pseudonitzschia* spp. dominated all three sites for up to a month supporting previous identification of this species (HST 2000) as an important contributor to blooms in the Huon Estuary. Larger blooms of *Pseudonitzschia* spp. occurred in 1999 than were observed in the 1997-1998 or 2000. The average cell volume of *Pseudonitzschia* in the Huon Estuary was 200 μm^3 (HST 2000) so although cell numbers of this alga were high the biomass was not as great as blooms formed by *G. catenatum*. High chlorophyll concentrations at all three sites in March were attributable to high cell densities of *Pseudonitzschia* spp..

Diatom species were generally at highest concentrations in the surface samples where there is enough light for photosynthesis. The process of bottom water mixing with surface water would be beneficial for nutrient addition to the *Pseudonitzschia* or diatom blooms. Mixing of these layers also increases the photic depth available because humic compounds are diluted through the water column. Diatoms such as *Pseudonitzschia* cannot move throughout the water column as effectively as *G. catenatum* so they would benefit from the greater depths of increased water clarity and increased nutrients.

2.14.3 *Ceratium*

Blooms of the non-toxic dinoflagellate *Ceratium* are also common in the estuary (HST 2000). The dominant species reported in the HST 2000 were *C. furca, C. tripos* and *C. fucus*. *C. furca* is typically a marine dwelling dinoflagellate. It has been previously reported in estuaries where it was associated with an increase in nitrogen and phosphorus and decreasing fresh water flow (Guerramartinez and Laravilla 1996). The most suitable conditions in estuarine environments in the study by Guerramartinez and Laravilla (1996) were thought to be brackish water

and high concentrations of inorganic phosphorus, or organic material. Although high concentrations of inorganic phosphorus were not contributing to blooms of *Ceratium* spp. in the Huon Estuary, the organic material present in the form of humic compounds makes the estuary a suitable environment for this species. The average cell volume of *Ceratium* in the Huon Estuary was 6×10^4 μm^3 (HST 2000). Large blooms of *Ceratium* species were not observed in the study although *Ceratium* contributed to the algal species in many samples especially during late 1998 during the first diatom-dominated algal bloom.

2.14.4 Gymnodinium catenatum

Gymnodinium catenatum is a major bloom-forming alga in the Huon Estuary and a species that produces paralytic shellfish toxins. In the estuary, the average cell volume of this alga was 1.7×10^4 μm^3 (HST 2000). This large cell size results in a high algal biomass in the water column during blooms.

The vertical extent of bloom formation is characteristic of this alga. It has the ability to survive in waters below the depth of light penetration (3-20 m) Figure 2.10, HST 2000, Doblin *et al.* 2000) because it is highly motile. This motility enables it to move quickly between the photic and nutrient zones. The findings from salinity and light penetration data in this thesis supports previous observations that *G. catenatum* biomass increases with a highly stratified layer (HST 2000, Hallegraeff *et al.* 1995a). Site F3 was the site of greatest stratification and highest cell numbers, so stratification during the two *G. catenatum* blooms may have resulted in the higher cell numbers observed for this site.

Site X3 contained the lowest *G. catenatum* biomass for both summer and autumn blooms in 1999 when compared to other sites, however, toxin levels from the algae were still sufficient to cause shell fish farms to close. Higher toxin levels observed in the smaller autumn bloom are believed to reflect changes in mussel physiology rather than variations in toxicity of *G. catenatum as* during autumn mussels are feeding at high rates (Tas Blue Mussels, pers. comm). It was not possible to gain enough algal biomass to determine whether the vegetative cells themselves may have been more toxic in the second bloom.

G. catenatum has a number of lifestages most simply described as sexual reproduction (cyst formation) and vegetative reproduction (Blackburn *et al.* 1989). Vegetative cell division means that under certain growth conditions there can be an exponential increase in cell abundance. Cyst formation in *G. catenatum* is part of sexual reproduction, and is also a resting or dormant stage allowing the alga to survive conditions unsuitable for vegetative cells. Under suitable conditions, the dormant cyst stage undergoes excystment to produce the vegetative stage.

In the Huon Estuary, all lifestages of *G. catenatum* can co-occur (Parker 2001). Cyst formation in many dinoflagellates occurs in response to a trigger such as low nutrient levels. Throughout the summer *G. catenatum* bloom in 1999 both vegetative and sexual reproduction occurred which was not in response to a nutrient deplete period (Parker 2001). There was constant resting cyst formation throughout both *G. catenatum* bloom development and decline that had not been previously reported for this species (Parker 2001).

The lifecycle of *G. catenatum* in the Huon Estuary is therefore dynamic and varies from year to year. The formation of the resting cyst during all stages of the *G. catenatum* lifecycle of is of particular importance when considering the control of harmful algal species by the addition of algicidal bacteria or their algicidal components. Consequences of this lifestyle characteristic of *G. catenatum* and the relationships with algicidal bacteria will be described in later chapters. If the cyst lifestage is continually produced during bloom development, as well as during bloom decline, then any algicidal component will have to compromise this lifestage to be effective in eliminating the *G. catenatum* bloom.

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3. Taxonomic characterisation of Huon Estuary strains

Summary

Eight of the seventy-five bacteria isolated from the Huon Estuary demonstrated algicidal or polyunsaturated fatty acid (PUFA) producing abilities. These eight isolates were analysed in greater detail for phenotypic and phylogenetic characterisation. Six isolates were algicidal and two were PUFA-producing *Shewanella* strains. Phylogenetic analyses confirmed that four of the eight isolates were novel species. The newly identified species are from the genera *Planococcus* (ACEM 22), *Pseudoalteromonas* (ACEM 4), *Shewanella* (ACEM 6 and ACEM 9) and *Cellulophaga* (ACEM 20). Algicidal strains of *Cellulophaga lytica* (ACEM 21) and *Bacillus mycoides* (ACEM 32) were also isolated. Phenotypic information was determined for the algicidal *Pseudoalteromonas* ACEM 1 (Strain Y) previously isolated from the Huon Estuary (Lovejoy *et al.* 1998). This is the first report of gram-positive bacteria demonstrating algicidal ability (*Bacillus* and *Planococcus*) and one of the first reports of algicidal species from genera other than *Cellulophaga* or *Pseudoalteromonas*. All strains demonstrated the ability to grow in oligotrophic conditions on a variety of media. Fatty acid analysis of the eight isolates indicates that signature lipids may be useful in identifying isolates from the CFB cluster and the *Bacillus* genus in environmental samples because of their distinctive fatty acids.

Introduction

Pseudoalteromonas, and *Cellulophaga* are common genera that include many of the known algicidal bacteria (Doucette *et al.* 1999, Holmström and Kjelleberg 1999, Kondo *et al.* 1999, Doucette *et al.* 1998). They are widespread in many marine environments and are easily cultured. Several of the Huon Estuary isolates belong in these genera. *Shewanella* species are also easily cultured and are a

common species in polar regions in sea-ice and the water column, as well as occurring in more temperate marine environments (Russell and Nichols 1999). The following paragraphs examine the evolutionary history of the genera that are most closely affiliated with the algicidal taxa isolated in this study.

3.1 *Cytophagales: Cytophaga-Flavobacterium-Bacteriodes* cluster

Algicidal bacteria are thought to include bacterial groups whose phenotypic traits are conducive to particle interaction and decomposition (Doucette *et al.* 1998). The order *Cytophagales* includes bacterial species that are able to degrade many different biomacromolecules, are major colonisers of macrophytes (Reichenbach 1992) and make up a major component of bacterioplanktonic biomass (Glöckner *et al.* 1999). The order *Cytophagales* was initially described as encompassing all aerobic, cellulolytic and gliding bacteria (Winogradsky 1929). Recently, this order has undergone dramatic changes and many of the genera have been reclassified, in particular the genera *Cytophaga* and *Flavobacterium* (Nakagawa and Yamasato 1993, Johansen *et al.* 1999). Most marine members of the order *Cytophagales* belong to the family *Flavobacteriaceae*. *Cellulophaga lytica,* a common marine species, was previously named *Cytophaga lytica* (Johansen *et al.* 1999) and is a member of this family. *Cellulophaga lytica* is an aerobic organism requiring elevated salt concentrations for growth and is easily cultivated from marine environments (Reichenbach 1989). *Cellulophaga* species are often found on marine particles after bloom periods (Crump *et al.* 1999) and on the surface of macroalgae (Johansen *et al.* 1999). The ability to degrade many different biomolecules enables *C. lytica* to obtain nutrients from both macro and microalgae (Johansen *et al.* 1999). Their algicidal role is enhanced by their gliding abilities. Other species in this genus are *C. fucicola, C. algicola, C. uliginosa* and *C. baltica* (Bowman 2000).

3.2 *Pseudoalteromonas*

Various species in the genus *Pseudoalteromonas* have undergone reorganisation from the genus *Pseudomonas* (Buck *et al.* 1963) to *Alteromonas* (Baumann *et al.* 1972), to the present classification of *Pseudoalteromonas* (Gauthier *et al.* 1995) The genus *Pseudoalteromonas* encompasses gram-negative, heterotrophic, marine, aerobic, rod shaped bacteria with a single polar flagellum and relatively low G+C (38-48 mol %) (Gauthier *et al.* 1995). *Pseudoalteromonas* species, through a variety of biosynthetic and catabolic reactions, produce bioactive compounds which include antibiotics and secondary metabolites such as tetrodotoxins and anticancer, fish killing and algicidal compounds (see reviews in Mikhailov and Ivanova 1994, Bowman and McMeekin 2001, Lee *et al.* 2000). These compounds may benefit *Pseudoalteromonas* in nutrient and habitat competition in the marine environment. However, production of exopolysaccharides by this genus can also have beneficial effects for other marine organisms in close proximity to the strain through control of bacterial attachment and in the form of nutritional benefits (Holmström and Kjelleberg 1999).

3.3 *Bacillus*

Bacillus mycoides, first described in 1886, is a gram-positive rod-shaped sporulating bacterium. It is in the *Bacillus cereus* group (*Bacillus cereus, Bacillus mycoides, Bacillus thuringiensis* and *Bacillus anthracis*), but is considered a distinct species (Nakamura and Jackson 1995). Recently, a closely related psychrotolerant species, *Bacillus weihenstephanensis,* comprising psychrotolerant '*cereus*' strains, was also proposed (Lechner *et al.* 1998). The *B. mycoides* group is psychrotrophic, has rhizoidal colonial morphology and lacks motility. The group can be distinguished from *B. cereus* by differences in fatty acid profiles (vonWintzingerode 1997) and acetanilide-producing activities (Nakamura and Jackson 1995). A second *B. mycoides* group, *Bacillus pseudomycoides,* exhibits very similar (98 %) 16S RNA sequences to *B. mycoides* and has been recognised as a new species (Nakamura 1998). *B. pseudomycoides* and *B. mycoides* can be

distinguished by significant differences in the proportions of i12:0 and a13:0 in their whole-cell fatty acid profiles (Nakamura and Jackson 1995).

B. mycoides has shown bioactive properties that have been used in the agriculture and biomedical industries (King *et al.* 2000, Pruss *et al.* 1999, Hammad and El-Mohandes 1999, Solujic *et al.* 1999) and can be the causative agent in agricultural infections (Grodnitskaya and Gukasyan 1999). *B. cereus* group produces extracellular compounds that include haemolysin, a soluble toxin lethal for mice, phospholipase C and bacterial-lytic and proteolytic enzymes, (Claus and Berkeley 1989).

3.4 *Planococcus*

Planococcus is closely related to the *Bacillus cereus* group but is essentially a marine genus. At present, this genus contains four species: *P. okeanokoites, P. mcmeekinii, P. citreus* (type strain) and *P. kocurii.* Colony pigmentation is yellow or orange. The original two *Planococcus* species (*P. citreus* and *P. kocurii*) form spherical gram-positive cells (Kocur 1989, Hao and Komagata 1985). *P. okeanokoites* ([*Flavobacterium*] *okeanokoites)* is presently the only rod shaped bacterium described in the genus (Nakagawa *et al.* 1996), however, *P. mcmeekinii* also forms rods during log phase (Junge *et al.* 1998) and does not require Na^+ for growth. *Planococcus* has distinct chemotaxonomic characteristics such as the presence of isoprenoid quinones, menaquinone 7 and menaquinone 8. Fatty acid composition enables differentiation between species. The concentration of Na^+ in the growth medium affects the thickness of the cell membrane in *P. citreus* with Na^+ levels above and below seawater causing a decrease in the cell membrane material (Kocur 1989).

3.5 *Shewanella*

The genus *Shewanella* can be divided into two different clusters corresponding to their ecophysiology and phylogeny. Psychrophilic and/or halophilic species include: *S. gelidimarina, S. benthica, S. hanedai, S. peleana* and *S. woodyi.*

Psychrotrophic and non-halophilic species include, *S putrefaciens, S. frigidimarina* and *S. oneidensis. S algae* and *S amazonensis* by comparison are mesophilic halophiles. Polyunsaturated fatty acid producing members of this genus are predominantly Antarctic species or barophiles from deep-sea vents and include species in the psychrophilic cluster: *S frigidimarina, S. gelidimarina, S. benthica, S. peleana* and *S. hanedai.*

Chapter Objectives:

Objectives of the research reported in this chapter were to:

- identify and characterise the Huon Estuary strains using phylogenetic and phenotypic analysis;
- assess the phylogenetic diversity of the algicidal isolates in comparison with other algicidal strains;
- compare the lipid profiles of the algicidal and *Shewanella* species and identify aspects that may contribute as markers for a genus or group within the marine environment.

Methods

3.6 Isolation

Water and sediment samples were taken from the water column (0-12 m depths) and surface sediment of the Huon Estuary during February 1998. Samples for microbiological analysis were collected in sterile Schott bottles. At the time of collection, salinity was 28-33 psu. Site details and collection methods can be found in chapter 2.

Bacteria from the samples were initially cultured on modified marine agar (800 ml filtered estuary water; 200 ml distilled water; 5 g Bacteriological peptone (Oxoid); 1 g Yeast extract (Oxoid)). Random colonies with differing morphologies were isolated and purified by streak plate technique. These colonies were then purified

and tested for algicidal ability by the methods described in chapters 4 and 5. PUFA producing abilities were ascertained by screening the fatty acid profiles of all purified isolates.

3.7 Phenotypic characterisation

Bacteria were cultured on marine agar for inoculation of biochemical and growth test media. Biochemical tests were performed with API-NE test strips (BioMerieux-Vitek). For carbon and energy source tests, most of the test compounds were used at a concentration of 0.2 % (wt/vol); the exceptions were the carbohydrates, which were tested at a concentration of 0.5 % (wt/vol). The mineral salts medium used contained 2 g NH_4Cl, 2 ml 1 M sodium phosphate buffer (pH 7), 2 ml SL10 trace element solution (10 ml HCl (25%; 7.7 M), 1.5 g $FeCl_2.4H_2O$, 70 mg $ZnCl_2$, 100 mg $MnCl_2.4H_2O$, 6 mg H_3BO_3, 190 mg $CoCl_2.6H_2O$, 2 mg $CuCl_2.2H_2O$, 24 mg $NiCl_2.6H_2O$, 36 mg $Na_2MoO_4.2H_2O$, 990 ml distilled water), 1 l of distilled water and 15 g NaCl. The pH was adjusted to pH 7 with 1 M KOH and the medium was solidified with 1.4 % agar. Comparison of controls without an added carbon source with those growing on a substrate was used to assess carbon substrate use. ACEM 1, ACEM 4 and ACEM 32 were the only isolates that produced a definable pattern in BIOLOG Microplates (BIOLOG Microbial Identification System, Hayward, CA, USA). Additional carbon sources utilized by these three species on the substrates in BIOLOG plates were also assessed.

3.8 Lipid analysis

Isolates were harvested from plates for lipid analysis after 24 hours growth. Lipid analysis was completed using a whole cell methanolysis procedure. Samples were scraped off plates and placed in individual precleaned screw cap test tubes. Methylating reagent (5 ml of 10:1:1, MeOH:$CHCl_3$:HCL) was added and air above the sample evacuated with N_2 gas. The sample was heated at 90 °C for 60 minutes to produce fatty acid methyl esters (FAME). The reaction was cooled, 4 ml H_2O was added followed by 2 ml of 4:1 C_6H_{14}:$CHCl_3$. After mixing, layers were left to

separate. The C_6H_{14}:$CHCL_3$ layer containing FAME was transferred to a vial in preparation for analysis. Fatty acid profiles were determined using a Hewlett Packard 5890 Gas Chromatograph (GC) equipped with a 50 m x 0.32 mm id cross-linked methyl silicone fused- silica capillary column (Hewlett Packard HP5 column) and Fisons GC-mass spectrometer (GC-MS) with conditions as described by Gutierrez *et al.* (1999). Fatty acids were identified prior to GC-MS by comparing retention time data with that obtained for authentic and laboratory standards. The GC data was compiled and analysed with Waters Millennium software. Geometry and position of the double bonds in monounsaturated fatty acid were confirmed using dimethyl-disulfide derivatisation and analysis using GC-MS (Nichols *et al.* 1986).

Fatty acid nomenclature: Fatty acids are designated as total number of carbon atoms: number of double bonds followed by the position of the double bond from the aliphatic end of the molecule. The prefixes i, a br and cy indicate iso, anteiso, branched and cyclopropyl containing fatty acids, respectively.

3.9 Phylogenetic analysis

Genomic DNA was extracted from cells and purified using the procedure of Marmur and Doty (1962). The 16S rRNA genes from these strains were amplified by PCR using the primers 1492r (E. coli numbering system; 5′-GGT TAC CTT GTT ACG ACT -3′) as a reverse primer and 10f (E. coli numbering system; 5′-GTA AGC AGC AGG CCG.GAC AA AG -3′) as a forward primer.

Conditions used for PCR are described in Bowman *et al.* (1996). Briefly, each PCR mixture contained each deoxynucleotide at a concentration of 50 μM, 2.5 mM $MgCl_2$, PCR buffer IV (25 mM NH_4SO_4, 75mM Tris-HCL [pH 9] (Sigma), 0 01 % Tween 20 (SigmaUltra), 50 pmol of each primer, 5 % vol/vol DMSO ($(CH_3)_2SO$), 50 to 100 g genomic DNA and 1 u of thermostable DNA polymerase (Advanced Biotechnologies, Surrey, UK). The PCR reactions were performed in a Corbett Research model FTS-960 thermocycler. The reaction parameters included an initial 5 minutes incubation at 94 °C, 30 cycles consisting of 94 °C for 1 minute, 50 °C for 1 minute and 72 °C for 5 minutes. PCR products were purified with a

Qiaex II gel extraction kit (Qiagen Inc., Chatsworth, California, USA.). Sequences of the 16S rDNA insertion were then generated with an Applied Biosystems model 3728A automated sequencer using a fluorescent dye terminator cycle sequencing kit (Applied Biosystems). The 16S rDNA sequences determined for the strains were compared to the sequences in the GenBank nucleotide database using the BLAST search program of the National Centre for Biotechnology Information (NCBI) website http://www.ncbi.nlm.nih.gov. Analyses of the 16S rDNA sequences datasets utilized PHYLIP version 3.57c (Felsenstein 1993). DNADIST was used to determine sequence similarities using the maximum-likelihood algorithm option. Phylogenetic trees were constructed with the neighbour-joining method by using the program NEIGHBOR. Bootstrap analysis was performed with SEQBOOT and CONSENCE using 250 resamplings of the dataset, using both DNADIST and NEIGHBOUR as well as the program DNAPARS, which constructs trees based on the maximum-parsimony method.

Isolates were catalogued in the Australian Collection of Estuarine Microorganisms, (ACEM). GenBank accession numbers of the four novel isolates investigated in this study are as follows: ACEM 4 (AF295592), ACEM 20 (AY035869), ACEM 22 (AYO35870) and ACEM 9 (AF295593). The accession number for ACEM 1 is AF030381.

Results

ACEM 4 and ACEM 32 were isolated from Huon Estuary sediment while the remaining four algicidal isolates were isolated from the water column (Table 3.1). All six algicidal bacteria occurred in the water column although both ACEM 4 and ACEM 32 were more frequently evident in sediment. ACEM 4 was only isolated six times in water column samples taken from 1998 to 2000. A number of ACEM 4-like strains were isolated during summer but they were not present in samples taken during the cooler months.

Table 3.1 Morphological and phenotypic characteristics of algicidal and PUFA producing bacteria. Dk :dark
ACEM 22, 21, 6 and 9 would not grow successfully on Biolog™ plates.
ACEM 1 and ACEM 4 were also positive for the following Carbon Sources (BIOLOG): α-cyclodextrin, dextrin, glycogen, Tween 40•, Tween 80•.
ACEM 1 was positive for the following additional carbon sources (ACEM 4 was negative), i-erythritol, D-fructose, L-fructose, gentiobiose, m-inositol, β-methyl-D-glucoside, D-raffinose, D-trehalose, turanose, xylitol, mono-methyl succinate, acetic acid•, cis-aconitic acid, formic acid•, D-galacturonic acid, D gluconic acid•, D-glucosaminic acid•, β-hydroxybutyric acid, α-keto butyric acid•, α-keto glutanic acid•, D.L-lactic acid, malonic acid, propionic acid, quinic acid•, D-saccharic acid, succinic acid, succinamic acid•, alaninamide, L-alanine, L alanyl-glycine•, L asparagines, L-aspartic acid, L-glutamic acid, glycyl-L aspartic acid, glycyl-L-glutamic acid, hydroxy L proline, L-leucine, L-pyroglutamic acid•, L-serine•, L-threonine, D.L- carnitine, inosine, uridine, 2,3-butanediol•, D.L-α glycerol phosphate•, glucose-1-phosphate•.
ACEM 32 was positive for the carbon sources above marked with • and also for the following carbon sources (for which ACEM 1 and ACEM 4 were negative): cellobiose, α-hydroxybutyric acid, bromosuccinic acid, D-alanine, D-serine, γ-amino butyric acid, thymidine, phenylethylamine, putrescine, 2-amino ethanol, glucose-6-phophate.

Genera	*Pseudoalteromonas*		*Cellulophaga*		*Plano-coccus*	*Bacill-us*	*Shewanella*	
ACEM Strain	**1**	**4**	**20**	**21**	**22**	**32**	**6**	**9**
Isolation site in Estuary	unknown	mid	entrance	sidearm	mid	mid	entrance	entrance
Map position, see Chap 2	unknown	D1	B1	S15d	F1	76	A3	C2
Isolate Source	unknown	sediment	water	water	water	sediment	water	water
Salinity at site	unknown	33ppt	33ppt	28ppt	29ppt	33ppt	33ppt	33ppt
Colony Characteristics (marine agar 20°C 48 hours growth)								
Surface	smooth	smooth	smooth	smooth	smooth	rough	smooth	smooth
Texture	butter	viscous	butter	butter	butter	dry	butter	butter
Form	circular	circular	circular	circular	circular	rhizoid	regular.	regular.
Elevation	raised	convex	raised	flat	raised	crusty	convex	convex
Margin	entire	lobate	lobate	lobate	entire	erose	undulate	undulate
Opacity	opaque	opaque	translucent	transparent	translucent	opaque	opaque	opaque
Pigment	light or dk yellow	dk green or purple	orange yellow	orange yellow	orange	white	light tan	light tan
Pigment. Organic	yellow	dk green	yellow	orange	orange	-	pink	pink tan
Pigment. Water	-	purple	yellow	yellow	yellow	-	brown	brown
Media pigmentation	-	black green	yellow	yellow	yellow	-	-	-
Shape (log phase/stationary)	rod	rod	rod	rod	rod/cocci	rod	rod	rod
Gliding	no	no	yes	yes	no	no	no	no
Flagella stain	+	+	-	-	-	-	+	+
Gram stain	-	-	-	-	+/variable	+	-	-
Spore forming	-	-			-	elps/mid	-	-
Bacteria Size (um) width, length (log phase culture)	0.1-0 5, 1 4-1 5	0 1-0.5, 1 5-2 5	0.1-0.3; 0.5-1	0 2-0.3, 2-5	1 2	1-1.5; 4-7	0.4-0.5; 0.9-1.2	0.4-0.5; 0.9-1 2
Growth. Temperature							‡	‡
2 °C	+	+	+	+	+	+		
4 °C	+	+	+	+	+	+		
10 °C	+	+	+	+	+	+		
17 °C	+	+	+	+	+	+		
20 °C	+	+	+	+	+	+		
25 °C	+	+	+	+	+	+		
30 °C	+	-	+	-	+	+	-	+
37 °C	+	-	+	+	+	+	-	-
45 °C	-	-	-	-	+	-	-	-
55 °C	-	-	-	-	-	-	-	-
Growth. Salinity							‡	‡
0 psu	-	-	-	-	+	+		
5 psu	+	-	-	-	+	+		
10 psu	+	+	+	+	+	+		
33 psu	+	+	+	+	+	+		
66 psu	+	+	+	+	+	+		
100 psu					+			
DNAse	+	+	-	-	+	-	-	-

Genera	*Pseudoalteromonas*		*Cellulophaga*		*Plano-coccus*	*Bacill-us*	*Shewanella*	
ACEM Strain	**1**	**4**	**20**	**21**	**22**	**32**	**6**	**9**
Utilization of								
adipate	-	-	+	-	-	+	-	-
arabinose	-	-	+	-	-	+	-	-
caprate	-	-	-	-	+	+	-	-
citrate	-	-	-	-	-	+	-	-
citric acid	-	-	-	-	-	-	-	-
DL-hydroxybutyric acid (NaCl salt)	+	+	-	-	-	+	-	+
erythritol	+	+	-	+	-	+	+	+
D-fructose	+	+	+	-	+	+	+	+
fumaric acid	+	+	+	+	+	+	-	+
D-galactose	+	+	+	-	-	-	+	+
gluconate	-	-	+	-	-	+	-	-
gluconic acid	+	+	+	+	+	+	+	+
D-glucose	-	+	+	+	+	+	-	-
D-glutamic acid	+	+	+	+	-	+	+	+
glycerol	+	+	-	-	-	+	+	+
D-lactose	-	+	-	+	-	+	-	+
DL-malate	+	-	+	-	+	+	-	-
malic acid	+	-	+	+	+	+	+	+
Maltose	+	+	+	-	-	+	-	-
D-mannitol	+	-	+	+	+	+	-	-
D mannose	+	+	+	+	-	+	-	-
D-melibiose	+	+	+	-	+	-	+	+
N-acetylglucosamine	+	+	+	-	+	+	-	-
phenylacetate	-	-	-	-	-	+	-	-
L-phenylalanine	+	-	+	+	+	+	+	+
sodium succinate	+	+	+	-	+	+	-	+
D-sorbitol	+	-	+	+	+	+	-	-
starch	+	+	+	+	+	+	+	+
sucrose	+	+	+	+	+	+	+	+
tannic acid	+	+	+	+	+	+	+	+
tyrosine	+	-	+	+	+	+	-	+
Oxidase	-	+	+	-	+	-	+	-
$NO_3 \rightarrow NO_2/NO_2 \rightarrow N_2$	-/-	-/-	+/+	-/-	-/-	-/-	-/-	+/+
Tryptophan deaminase	-	-	-	-	-	-	-	-
Glucose fermentation	-	-	-	-	-	+	-	-
Arginine dihydrolase	+	-	+	-	-	-	-	-
Urease	+	+	+	-	-	-	-	-
Esculin hydrolysis	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+	+	+
β-glactosidase	-	-	+	+	+	+	-	-

‡ see chapter 5

Shewanella isolates were also commonly cultured from samples taken from the water column and were often isolated from below the pycnocline in the water column (see chapter 7).

The algicidal isolates ACEM 20, ACEM 21 and ACEM 22 were closely associated with algal blooms and these and other CFB isolates often dominated the colony forming units (CFU) from net samples (chapter 7). Media dependent pigment variation was observed in ACEM 1 and ACEM 4. All bacteria were tolerant of a range of temperatures and salinities and demonstrated the ability to utilize compounds found in the humic rich estuary water such as tannic acid, fumaric acid, and tyrosine (Table 3.1).

3.10 *Cellulophaga*

ACEM 20 is a novel species in the genus *Cellulophaga.* The nearest relative to ACEM 20 is *C. uliginosa* (Figure 3.1). ACEM 21 is closely related to *C. lytica* based on 16S rDNA sequence analysis (Figure 3.1). Both isolates have gliding motility. During stationary growth phase the gliding, orange-yellow colonies of ACEM 21 exhibit a green iridescence. ACEM 21, ACEM 20 and *C. lytica* require salt for growth (Table 3.1). Other phenotypic characteristics such as utilization of sucrose and optimal growth at ~25°C are also in agreement with characteristics of this genus. Utilization of galactose and glucose is not apparent in ACEM 21 and it is oxidase-negative. *C. lytica* is normally oxidase-positive and utilizes both galactose and glucose. However, *C baltica* is oxidase-negative and *C fucicola* does not utilize galactose and glucose or many other carbon sources (Johansen *et al.* 1999). Interestingly, ACEM 20 is positive for many of these carbohydrates, is also oxidase-positive, and can denitrify. Fatty acid profiles of ACEM 20 and ACEM 21 are dominated by the same fatty acids that are common among other *Cellulophaga* species (Bowman *et al.* 2000). ACEM 20 has a similar fatty acid profile to ACEM 21 and *C. lytica* with high proportions of branched chain fatty acids (BCFA) in particular i15:1(n-10), i15:0 and 15:0 (Table 3.2). In comparison to *C. lytica,* ACEM 21 and ACEM 20 contain lower proportions of β-OH i17:0.

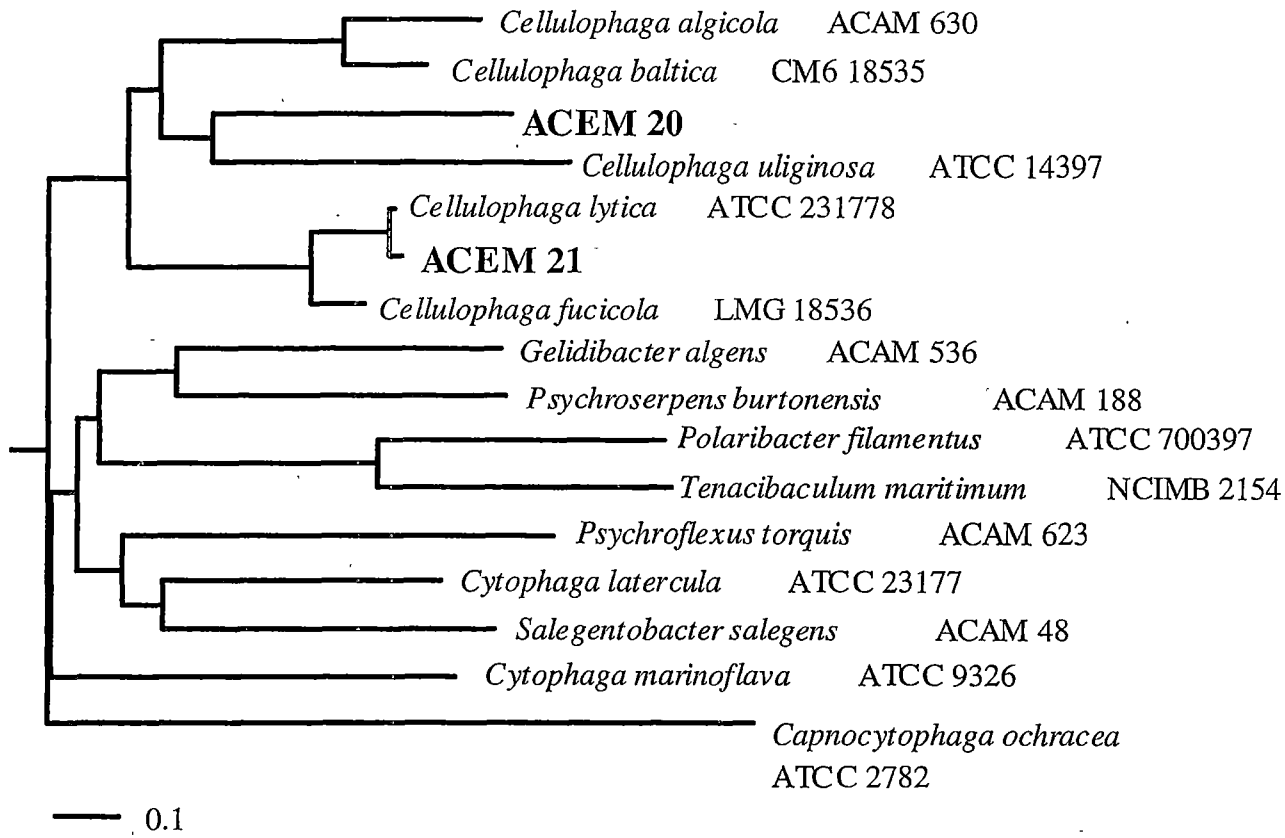


Figure 3.1 Phylogenetic affiliations based on 16S rRNA gene sequence alignment, showing the relationship of ACEM 20 and ACEM 21 to some other members of the genera *Cellulophaga/Cytophaga*. The scale represents sequence dissimilarity value.

Table 3.2 Whole cell fatty acid composition (% of total fatty acid) of algicidal bacteria (Fatty acid profiles of ACEM 6 and ACEM 9 are in chapter 6) tr ≤ 0.5%

ACEM strain no.	1	4	22	21	20	32
Fatty acids percentage						
Saturated						
12:0	0.8	0.8				1.2
14.0	1.4	2.4		2.8	3.3	3.8
15:0	5.6	1.9	4.0	11.6	11.7	1.2
16:0	12.7	14.0	1.3	8.8	4 4	3.1
17:0	8.8	1.9	4.9			tr
18:0	2 4	1.1	0.6			
19·1 or cy 19:0	1.3					
SUM	34	22	11	23	19	10
Branched chain						
i12:0						2.1
a12:0						1.6
ı13.0	1 3			0.8	0.9	11.5
i14:0	tr		7.5	1.2	0.9	6.0
i15:1(n-10)				22.5	13.6	
i15·0	2.7		7.1	25.4	17.3	15.0
a15·0	0.8		38.0	2.0	12.2	5.5
br 16:1						2.2
br 16:1						3.0
br 16:1			8.8			7.6
i16·0	tr	tr	5.0	0.6	0.8	3.0
i17 1		tr	1.0		0.6	8.5
a17·1			1.5			1.1
ı17:0	tr	tr	1.6			3.4
a17.0		6.5	13.2	1.9		1.9
i18:0	tr		1.1			
SUM	5	7	85	54	47	72
β-hydroxy						
10:0	0.7	1.7				
11.0	2.9	1.7				
ı12·0	2.1	2.2				
12·0	2.7	5.0				
13·0	0.6	tr				
ı14:0					3.3	
ı15.0					2.4	
i16:0			tr	3.2	2 3	
16:0		0.4		2.5	0.8	
i17:0		tr		3.2	1.5	
SUM	9	11	tr	9	10	
Monounsaturated						
12:1	0 8	2.8				
15.1		1.7			1.5	
16:1(n-7)	22.1	43.8	3.4	12.6	21.8	17.2
17:1(n-8)	19.9	tr		tr		
17·1(n-6)	1.5					
18:1(n-7)	6.8	10.0	0.9			
SUM	52	60	4	13	23	17
Diunsaturated						
16:2						0 5

ACEM 20 contains high proportions of 16:1(n-7)c in comparison to all *Cellulophaga* species except *C. algicola*, which also contains approximately 20 % of this fatty acid. ACEM 20 contains higher relative levels of a15:0 and 16:1(n-7c) in comparison to ACEM 21. The placement for these two species in the phylogenetic tree based on 16S rRNA, reflects their close similarity to others in the genus and to each other (Figure 3.1). The fatty acids i15:1(n-10)[1], i15:0, a15:0, 16:1(n-7)c, i17:1(n-7) and β-OH i17:0 are the most useful to discriminate *Cellulophaga* species from other members of the *Flavobacteriaceae* (Bowman 2000, Bowman *et al.* 1998b). However, in marine environmental samples the fatty acids i15:1, i15:0 and i17:1 would be the most likely to be detected among the eukaryotic biomass.

3.11 *Pseudoalteromonas*

Both *Pseudoalteromonas* species (ACEM 4 and ACEM 1) were gram-negative, straight rods and required Na^+ for growth. Both showed great phenotypic similarity. ACEM 4 was oxidase-positive, which is characteristic of *Pseudoalteromonas,* however, ACEM 1 was oxidase-negative. Neither species reduced nitrate or produced β-galactosidase. ACEM 1 utilized tyrosine but did not utilize glycerol or lactose. A nearby relative of ACEM 1, *P. piscicida,* also does not utilize glycerol or lactose.

A phylogenetic tree based on 16S r RNA demonstrates the affiliation of ACEM 4 and ACEM 1 to other members of the genus *Pseudoalteromonas* (Figure 3.2). *P. tunicata* is the species most closely related by phylogenetic characterisation to ACEM 4. By contrast, *P. tunicata* did not utilize fructose, sucrose, lactose or glycerol.

[1] In the paper by Bowman (2000) i15:1(n-10)c was incorrectly called a15:1(n-10) c in the text body

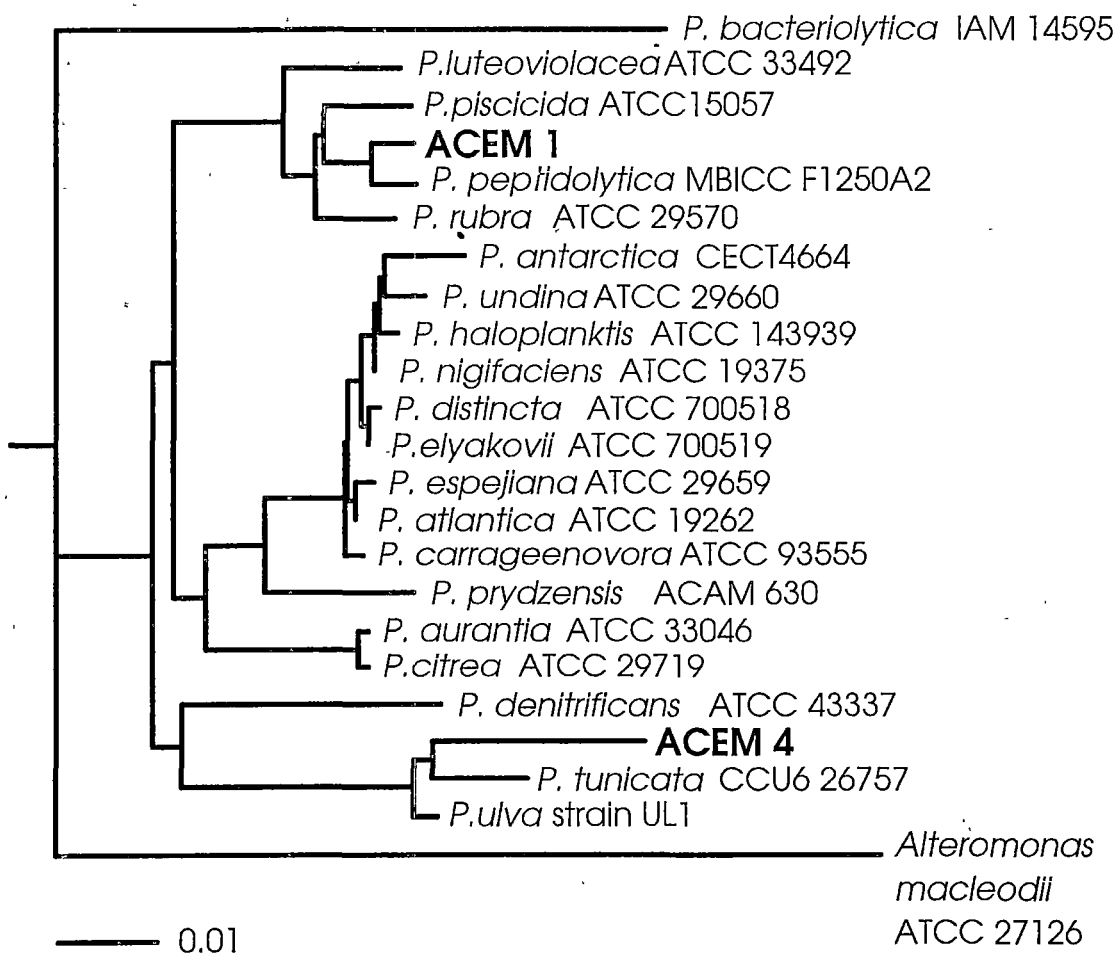


Figure 3.2 Phylogenetic affiliations based on 16S rRNA gene sequence alignment, showing the relationship of ACEM 1 and ACEM 4 to some other members of the genus *Pseudoalteromonas*. The scale represents sequence dissimilarity value.

The fatty acid profiles of both ACEM 1 and ACEM 4 were typical of the genus *Pseudoalteromonas*. Major fatty acids were 16:0, 16:1(n-7)c and 18:1(n-7)c which are similar to other *Pseudoalteromonas* and *Alteromonas* species (Bowman 2001, Svetashev *et al.* 1995). ACEM 1 also contained high proportions of 17:1(n-8)c and 17:0. Unusually for a member of this genus ACEM 4 contained high proportions of a17:0. Both ACEM 1 and ACEM 4 contained up to 11 % β-hydroxy fatty acids. This proportion of hydroxy fatty acids is high for *Pseudoalteromonas* species. *Pseudoalteromonas* normally contain 0-3 % hydroxy fatty acids using either the MIDI (Microbial ID, Inc., Newark, DE, USA) system or the ester-linked method used in this study.

3.12 Bacillus

ACEM 32 has essentially the same characteristics as those of the *B. cereus* group and to *B. mycoides*. It is gram-positive, oxidase-negative and does not reduce nitrate (Table 3.1). A phylogenetic tree of several related *Bacillus* species is shown together with the relationship to the other gram-positive/variable algicidal species examined in this study, *Planococcus* (Figure 3.3).

ACEM 32 produces white rhizoid colonies on agar that are similar to other species of *Bacillus,* although non-motile species have been reported (vonWintzingerode 1997). The phenotypic test which best characterises individuals in this group is fatty acid analysis. Differentiation between the species *B. mycoides, B. pseudomycoides* and *B. cereus* can be observed in the relative levels of 12:0, i12:0, a13:0, i15:0 and 16:0 fatty acids (Nakamura and Jackson 1995). However, fatty acid analyses in the Nakamura and Jackson (1995) study used the MIDI system and therefore the results are not directly comparable. Strains of *B. mycoides, B. pseudomycoides* and *B. cereus* all contain a13:0 however, ACEM 32 does not. ACEM 32 has similar proportions of i13:0, 14:0, i14:0, a15:0, 16:0 and a17:0 to *B. cereus* (*B. cereus* 11.5, 3.1, 5.2, 5.5, 3.9, 1.1 % respectively) but has lower proportions of a15:0 (15 % compared with *B. mycoides* 23 %, *B. pseudomycoides* 19 % and *B. cereus* 31 %) (Table 3.2).

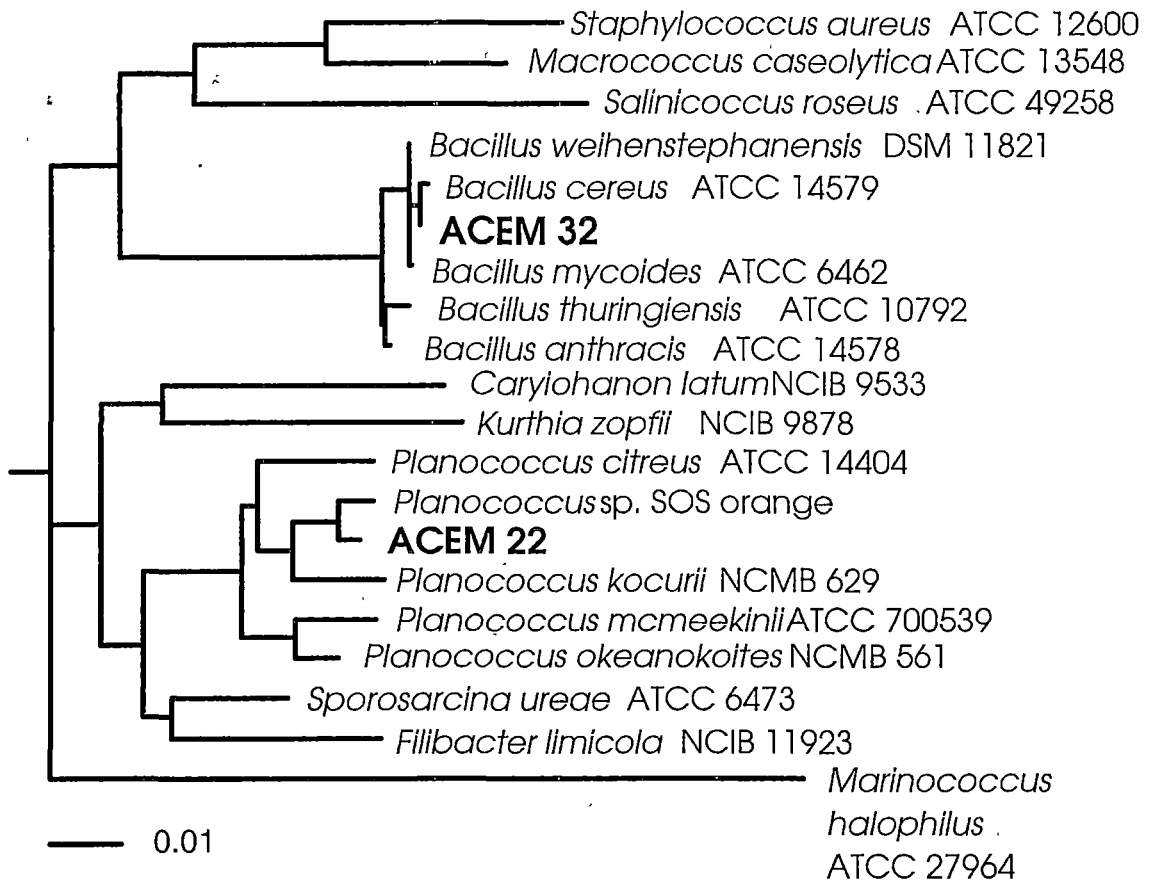


Figure 3.3 Phylogenetic affiliations based on 16S rRNA gene sequence alignment, showing the relationship of ACEM 32 and ACEM 22 to some other Gram-positive genera. The scale represents sequence dissimilarity value.

ACEM 32 contained high proportions of 16:1(n-7)c (17 %), br16:1 (13 %) and br17:1 (9 %) which were not reported for the other species. The fatty acid profile of ACEM 32 distinguishes it from these three valid species. The characteristic fatty acids are however, somewhat unusual (br16:1 and br17:1) and this leads to the possibility that the fatty acids were sufficiently atypical such that the MIDI system would not have identified them. Essentially, the profile indicates that ACEM 32 is easily identifiable as belonging to the *B. cereus* cluster.

3.13 *Planococcus*

Planococcus species have been isolated from marine environments and most species, including ACEM 22, require Na^+ for growth and can grow at very high salt concentrations (Kocur 1989). ACEM 22 has similar morphological lifestage characteristics as *P. mcmeekinii*. It has rod shaped cells during log phase that can stain gram-negative and cocci shaped cells in stationary phase that stain gram-positive. ACEM 22 utilizes glucose, succinate and N-acetyl glucosamine and malate as sole carbon sources. These are also utilised by *P. citreus* and *P. kocurii* (Table 3.1). The most identifiable and unusual characteristic of ACEM 22 is the ability to use a variety of carbohydrates as a sole carbon source unlike other *Planococcus* species. Most other *Planococcus* do not utilise carbohydrates. ACEM 22 is the only species in the genus that does not ferment glucose, but can use fructose, sucrose and other carbohydrates as sole carbon sources. All other species in the genus, apart from ACEM 22 and *P. okeanokoites*, ferment glucose and do not utilize lactose or reduce nitrate. ACEM 22 and *P. okeanokoites* are the only oxidase-positive species in the genus. The fatty acid composition of ACEM 22 is very similar to the other *Planococcus* species and is dominated by BCFA. The principal fatty acids in ACEM 22 are a15:0 (40 %) and a17:0 (13 %) (Table 3.2). *P. mcmeekinii, P. citreus* and *P. kocurii* also contain a15:0 (38, 54 and 56 % respectively) as their major fatty acid. *P. okeanokoites* contains lower levels of a15:0 (14 %, Junge *et al.* 1998). *P. citreus* and *P. kocurii* also have high proportions of a17:0 (13 and 9 % respectively). ACEM 22 demonstrates greatest phylogenetic similarities with *P. kocurii* (Figure 3.3).

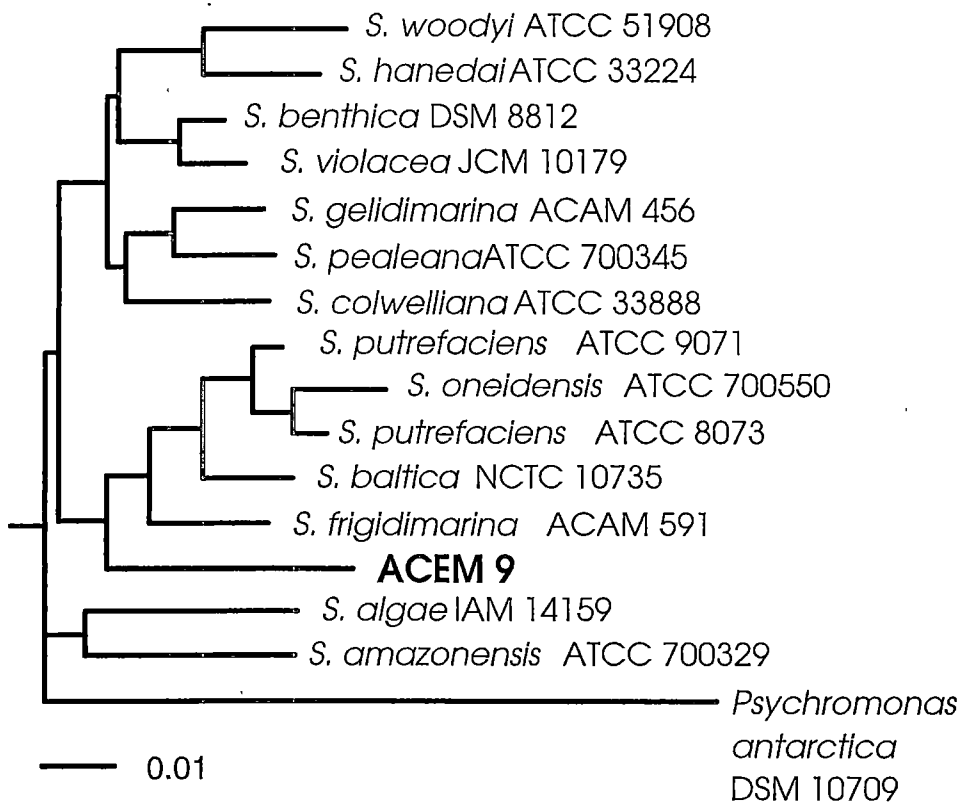


Figure 3.4 Phylogenetic affiliations based on 16S rRNA gene sequence alignment, showing the relationship of ACEM 9 to some other members of the genus *Shewanella*. The scale represents sequence dissimilarity value.

3.14 *Shewanella*

ACEM 6 and 9 are phylogenetically similar based on 16S rDNA sequencing (Figure 3.4) and share similar phenotypic traits (Tables 3.1 and 3.2). Both required salt for growth and were psychrotolerant and oxidase-positive. Optimum temperature for growth was 18-22 °C. All other *Shewanella* species are oxidase positive. Both strains were negative for the production of arginine dihydrolase and fermentation of glucose. ACEM 9 reduced nitrate to nitrite but ACEM 6 did not. All other *Shewanella* species are positive for this ability although S. *hanedai* is variable. One of the closest phylogenetically related species to these two strains, *S. baltica,* also utilized sucrose and grew at 4 °C, but unlike *S. baltica,* ACEM 6 and ACEM 9 did not utilize citrate, DL-malate or maltose and contained 20:5(n-3). Another closely related species, *S. frigidimarina* (Figure 3.4), was positive for utilization of glucose and sucrose and produced lower proportions of 20:5(n-3) than ACEM 6 and 9 (2 - 7 % at 10 °C, Bowman *et al.* 1997). ACEM 6 and 9 produced up to 15 % EPA at 10 °C- chapter 6). Fatty acids and phylogenetic position of this species are described in greater detail in chapter 5.

Discussion

All algicidal isolates were phenotypically similar to other species in their corresponding genera and all demonstrate the capacity to survive in an oligotrophic marine environment. Humic material present in the Huon Estuary includes proteinacious material, phenols, hydroxy acids and glycosides. Most of the algicidal species analysed show an ability to utilize carbon sources that would be found in the humics and tannins of the surface water. All species except ACEM 4 utilized tyrosine and phenylalanine.

The two *Shewanella* strains show a phenotypic profile typical of other species in the genus *Shewanella* and cluster with psychrotolerant species that normally do not produce high proportions of 20:5(n-3). Further analysis and discussion of these two strains is undertaken in chapter 5.

Fatty acid profiles of the *Planococcus*, *Bacillus* and *Cellulophaga* species were distinctive. It would be possible to use these profiles to differentiate their fatty acid contributions from those of other eukaryotes and bacteria in the marine environment. Identification of these bacterial species in the environment would be limited to recognition of classes, but with some potential to refine this to genera. Changes in individual fatty acids or the proportions of fatty acids would enable identification of bacterial community change in the estuary.

All algicidal isolates, except the two *Pseudoalteromonas* species, contained high proportions of BCFA (47 to 85 % of total fatty acids). This indicates that the presence of certain BCFA profiles in environmental samples may be indicative of potentially algicidal genera. In particular, the novel *Planococcus* species (ACEM 22) contained high relative levels of a15:0 and a17:0 which, although not uncommon for this genus, is an atypically high proportion of BCFA with respect to other bacteria. The dominant fatty acids in the *Cellulophaga* species were characteristic of that genus with high proportions of the fatty acids i15:1 and i15:0. Unfortunately, *Pseudoalteromonas* species generally contain fatty acids that are common in other bacteria and eukaryotes so their profiles may not allow them to be distinguished in environmental samples.

The algicidal bacteria described in this study reflect a wider variety of genera than previously reported. This raises the possibility that algicidal bacteria are more common in the marine environment than previously thought. Isolation of new bacterial species that are not from "typical" algicidal genera may simply be as a result of increased research now occurring in this field. Many members of the CFB cluster and *Pseudoalteromonas* are easily cultured. Therefore, it is possible that species less easily cultured, with the same algicidal activities, have not yet been identified.

The increase in research on algicidal genera is due primarily to their potential use for algal bloom control and the bioactive nature of most of the species. Many have shown bioactivity in areas other than algicidal ability. For some genera, such as *Pseudoalteromonas* and *Bacillus,* algicidal activity is just one of the many bioactive abilities that these genera are capable of producing. It appears logical

that if a species is algicidal, it may have other antagonistic or complimentary mechanisms. This has often proven to be the case for genus such as *Pseudoalteromonas* (Holmström and Kjelleberg 1999). All algicidal genera cited produce a range of bioactive compounds derived from different metabolic pathways (Mikhailov and Ivanova 1994, Holmström and Kjelleberg 1999, King *et al.* 2000). This demonstrates the potential of these genera to contribute extracellular exudates in the marine environment that may have a positive or negative effect on the surrounding biota.

The ability to identify potentially algicidal species in the estuary using a combination of fatty acid analyses, fluorescence *in situ* hybridisation (FISH) and traditional morphological techniques will give a better understanding, not only of algicidal species, but of the marine microbial community in which they reside.

4. Algicidal activity of Huon Estuary bacteria

Summary

Seventy-five bacteria isolated from the Huon Estuary were tested for algicidal activity. Five isolates produced algicidal extracellular exudates. Further data is also presented on an algicidal species previously isolated from the Huon Estuary (Lovejoy *et al.* 1998).

All seventy-five Huon Estuary isolates were deposited in the Australian Collection of Estuarine Microorganisms (ACEM). Catalogue names of the algicidal species are ACEM 1 (formally Strain y, Lovejoy *et al.* 1998) (*Pseudoalteromonas sp.*), ACEM 4 (*Pseudoalteromonas sp.*), ACEM 20 (*Cellulophaga sp.*), ACEM 21 (strain of *Cellulophaga lytica*), ACEM 22 (*Planococcus sp.*) and ACEM 32 (strain of *Bacillus mycoides*).

Algicidal supernatant produced by all five bacteria caused cell lysis and death in *G. catenatum* vegetative cells. No change, or reversible ecdysis was noted for two endemic dinoflagellate species. Isolate ACEM 4 also inhibited settlement of barnacle larvae. Resting cysts of *G. catenatum* and *Alexandrium minutum* were not affected by addition of algicidal supernatant. Although cysts remained viable and excysted, vegetative cells lysed after excystment in cultures containing ACEM 1 supernatant. Algicidal components did not appear to control or effect encystment or excystment processes.

Bacterial quorum sensing may play a role in algicidal or inhibitory activity, but this mechanism was not activated via homoserine lactones. Algicidal activity from isolates taken from the field suggested that algicidal activity was influenced by strain or environmental variation. Some species appear capable of losing or switching off the algicidal ability. Therefore, the presence of an algicidal species in the environment may not necessarily signify that they are currently algicidal. Concentrations of algicidal compounds required for algal lysis in laboratory experiments indicate that the five bacterial species can be effective against

vegetative cells when they dominate the bacterial population in the estuary particularly when they are particle attached.

Introduction

The Huon Estuary in Tasmania is an unpolluted waterway with low nutrient levels. Despite this, the estuary experiences periodic outbreaks of the toxic dinoflagellate *G. catenatum* that are detrimental to the local shellfish industry. A two-year scientific study of the biology, chemistry and physics of the estuary found no simple answers as to why the blooms occur or how they can be controlled (HST 2000).

The relationship between algicidal bacteria and toxic algal blooms has been the subject of extensive research (Doucette *et al.* 1999, Lovejoy *et al.* 1998, Doucette *et al.* 1998, Imai *et al.* 1993, Stewart and Brown 1969). Very recently particular attention has been paid to the potential use of algicidal bacteria in bloom control (Nagasaki *et al.* 2000, Yoshinaga *et al.* 1999, Kim *et al.* 1998). However, to date no effective, practical use of these bacteria in the marine environment has been reported.

To gain insight as to the prevalence of other algicidal bacteria in the marine environment Antarctic sea-ice isolates were tested for their algicidal activity. The microbial food web in Antarctic is essential for the higher trophic organisms and an important component of this microbial web occurs in sea-ice. Large algal blooms occur on the ice-water interface and within the sea-ice during summer. Dinoflagellates commonly occur and over-winter within the sea-ice. The possibility of bacterial algicidal activity in this harsh environment was investigated in this study using an Antarctic dinoflagellate and a number of Antarctic diatom and flagellate species. Forty-three bacterial sea-ice isolates were also tested for their algicidal activity on the temperate dinoflagellate species.

Algicidal bacteria include *Pseudoalteromonas* species and various species of the *Cytophaga-Flavobacterium-Bacteroides* cluster (CFB) (Doucette *et al.* 1998, Nagai and Imai 1998). Algicidal species in the CFB cluster are predominantly of

the genus *Cellulophaga*. A *Pseudoalteromonas* species isolated from the Huon Estuary was found to be algicidal against *G. catenatum* vegetative cells as well as other dinoflagellates and flagellates (Lovejoy *et al.* 1998). Another *Pseudoalteromonas* strain isolated in the same study caused a detrimental but reversible effect on *G. catenatum* vegetative cells (Lovejoy *et al.* 1998). Blooms of *G. catenatum* in the Huon Estuary are dominated by the vegetative cell lifestage of this alga although other lifestages regularly co-occur (Parker 2001). Many toxic and non-toxic dinoflagellates form resting cysts which tolerate harsher conditions than vegetative cells (Anderson *et al.* 1983, Persson 2000). Cyst formation has occurred during all phases of the *G. catenatum* bloom in the Huon Estuary (Parker 2001). Research to date on algicidal bacteria has focussed on activity against the vegetative cells of many different toxic dinoflagellates. The capacity of the algicidal components to penetrate, kill or affect the resting cyst lifestage has not been previously examined nor whether algicidal bacteria cause encystment as a protection mechanism.

Dinoflagellate cysts can form seedbeds (Anderson and Wall 1978, Hallegraeff *et al.* 1998) or increase their geographic extent via transportation in ocean currents or ballast water (Hallegraeff 1998). Cyst formation allows the alga to suspend future lifestages until suitable conditions exist for excystment. Macronutrients have not been found to initiate vegetative reproduction or resting cyst formation (Parker 2001, HST 2000).

Due to the bioactive nature of many algicidal bacteria, other biocidal mechanisms have been researched independently. Species of the genus *Pseudoalteromonas* have demonstrated the ability to inhibit biofilm formation in the marine environment (Holmström and Kjelleberg 1999). The importance of bacteria as the initial step in marine biofilm formation has been well established (Cooksey and Wigglesworth-Cooksey 1995, Holmström and Kjelleberg 1994). A problematic aspect of these microbial marine biofilms is the subsequent attachment of biofouling organisms such as barnacle larvae. Some marine bacteria such as *Pseudoalteromonas tunicata* have shown anti-biofouling activity and kill or inhibit the settlement of larvae (Egan *et al.* 2000a, Holmström *et al.* 1998). Such bacteria often occur in a commensal relationship with seaweed and prevent biofouling of

the thallus (Steinberg *et al.* 1997). The antifungal, antialgal and anti-biofouling compounds in *P. tunicata* are all different compounds. The seemingly close association of algicidal activity and inhibition of marine biofilms suggest the use of similar regulatory expression to form these different types of extracellular components.

The mechanisms bacteria activate to either produce the algicidal extracellular components or to commence predatory attack are largely unknown. These mechanisms may include bacteria reaching a certain density before algicidal expression occurs (quorum sensing, see chapter 1). This is typical of many bacteria that form biofilms. Chemicals produced by bacteria such as acetylated homoserinelactones (AHL) can be used to communicate between bacteria in order to regulate expression (Eberl 1999, Bassler *et al.* 1993). AHL are small hormone like molecules. Small AHL are freely diffusible across cell membranes while longer chain AHL must be actively transported across the cell membrane. They are both produced from intracellular intermediates, excreted during growth and accumulate externally (Bassler *et al.* 1993). At certain AHL concentrations during mid to late log phase, the receptor protein produces a phenotypic response and this is termed the threshold concentration. AHL induce their own production and are referred to as auto inducers (AI). The Lux-genes responsible for production of the AHL receptor are LuxI and LuxR. LuxR is the receptor protein, and is membrane associated, and LuxI contains all genes necessary for production of AHL and bioluminescence (Bassler *et al.* 1993). The ability to synthesise AHL molecules is not restricted to enzymes of the LuxI-type AHL synthases. Two AHL synthases, AinS in *Vibrio fischerii* and LuxM in *Vibrio harveyi* have been recognized that direct the synthesis of two different AHL (Eberl 1999, Bassler *et al.* 1993, Gilson *et al.* 1995). However, many different bacterial species make a signal to which *Vibrio harveyi* responds but this signal is not yet fully understood (Bassler 1999). The signal molecule is not an AHL but is termed an AI-2 inducer and has only recently been identified by Bassler and co workers (patent pending).

The objectives of the research reported in this chapter were to:

- identify the algicidal activity and specificity of the 5 new algicidal species isolated from the Huon Estuary (chapter 3) including those that did not belong to "typical" algicidal genera;
- establish whether the vegetative and cyst lifestages of *Alexandrium minutum* and *G. catenatum* were affected by algicidal bacteria or their exudates;
- determine if the algicidal bacteria had other bioactive properties such as inhibition of biofilm formation;
- explore the processes which may be responsible for algicidal activity such as bacterial quorum sensing;
- investigate the prevalence of algicidal activity in marine environments;
- determine if polar marine bacteria were capable of algicidal activity.

Methods

4.1 Algae

Biocidal tests were completed on the algal and microbial species listed in Table 4.1. Algal cultures were maintained at 17 °C under cool white fluorescent light (100 μE/s/m^2, measured with a Biospherical Optics light meter) and alternate 12-hour light/dark cycles. Algal media used either 0 2 μm filtered seawater obtained from off the Tasman Peninsula or autoclaved 0.7 μm filtered Huon Estuary river water. Salinity was adjusted to 28 psu using Milli-Q deionised filtered water or the addition of artificial sea salts (Sigma). Dinoflagellate species were grown in GSe medium (Blackburn *et al.* 1989). Other cultures were grown in standard media (Guillard and Ryther 1962) listed in Table 4.1. All algal cultures tested were non-axenic, uni-algal cultures with moderate to low bacterial loads unless otherwise stated (10^4-10^9 cells/l).

Table 4.1 Taxon, media, strain and source history of species tested in biocidal assays

Taxon	Class	Medium*	Strain	Strain source and history
Gymnodinium catenatum Graham	Dinophyceae	GSe	GCDEO6; GCDE06; GCJP01	S. Blackburn; Derwent, Tasmania; Japan
Gyrodinium sp.	Dinophyceae	GSe	GYPA06	C. Bolch; Port Arthur, Tasmania
Gymnoid strain 1 and 2 and Dinophyta	Dinophyceae	GSe	GX1, GX2, GTR1	M. de Salas; Triabunna, Tasmania
Protoceratium reticulatum (Claparede & Lachmann) Butschli	Dinophyceae	GSe	DPR0	N. Parker; Derwent, Tasmania
Alexandrium minutum Halim	Dinophyceae	GSe	AMAD 06	J. Cannon, S Blackburn; Port River, South Australia
Chattonella marina (Subrahmanyan) Hara & Chihara	Raphidophyceae	GSe	CMPL02	J Marshall; Port Lincoln, South Australia
Skeletonema costatum (Greville) Cleve	Bacillariophyceae	F2	SkeHOA	C. Lovejoy; Huon, Tasmania
Pseudonitzschia pseudodelitcassima	Bacillariophyceae	F2	PPH03	J. Skerratt; Huon, Tasmania
Polarella glaciacola Montresor et al.	Dinophyceae	F2	FL2B	P.Thomson; Eastern Antarctica
Mix of Antarctic diatoms	Bacillariophyceae	F2	-	R. vanDenenden; Eastern Antarctica
Brachionus plicatilis	Aschelminthes	-	-	DPIWE Marine Research Labs
Gymnodinium catenatum Graham	Dinophyceae	GSe	Cysts	N Parker; Huon, Tasmania
Alexandrium minutum Halim	Dinophyceae	GSe	Cysts	N Parker; from strain AMAD 06
Thraustochytridae Cavalier-Smith et al.	Chromista	Unpublished	ACEM A	T. Lewis, South-Eastern, Tasmania

* GSe Media (Blackburn *et al.* 1989) F2 (Guillard and Ruther 1962)

Most Antarctic dinoflagellates required 6 months (2 °C under cool white fluorescent light and alternate 16 hour light 8 hour dark cycles) to attain suitable numbers for algicidal tests. Assays to test algicidal activity of Antarctica bacterial isolates therefore used a multifarious Antarctic diatom culture, *G. catenatum* and *C. marina*. The algicidal Huon Estuary isolates were tested on their ability to lyse the Antarctic dinoflagellate *Polarella glaciacola*..

4.2 Bacterial strains studied

Bacterial isolates were collected and purified as described in chapters 2 and 3. Bacteria were phylogenetically and phenotypically identified (chapter 3). Abundances of algicidal bacteria in the Huon Estuary are described in chapters 5 and 6.

Several other *Pseudoalteromonas* species, related to the two Huon Estuary *Pseudoalteromonas* strains (ACEM 1 and ACEM 4), were also tested for algicidal activity against *G catenatum* and *C. marina*. These bacteria were type strains from the National Collection of Industrial and Marine Bacteria (NCIMB), Aberdeen, Scotland. The strains included *P. espejiana* (NCIMB 2127), *P. rubra* (NCIMB 1890), *P. luteoviolacea* (NCIMB 1893), *P. citrea* (NCIMB 1889) and *P. aurantia* (NCIMB 2033) and 3 strains of *P. piscicida* (NCIMB 1938, 1142 and 645). ACEM 1 is closely related to the species *P. piscicida*. Two bacterial species tested were obtained from the University of NSW. These species were *P. tunicata* (Strain number D2), isolated in Sweden from a tunicate (Holmström *et al.* 1998) and *P. ulvae* isolated from an Australian seaweed (Egan *et al.* 2001a, Egan *et al.* 2000a). ACEM 4 is closely related to the species *P. tunicata*.

For maintenance of bacterial cultures, isolates were grown on full strength marine agar (14 g agar, 5 g bacteriological peptone (Oxoid), 1 g yeast extract (Oxoid), and either 28 g artificial sea salts (Sigma) and 1 l Milli-Q water or 1 l of autoclaved 0.7 µm filtered Huon Estuary river water with salinity adjusted to 28 psu using artificial sea salts). For the biocidal assays, nutrient poor media was $1/10^{th}$ strength of the above medium. Field algicidal experiments used 28 g sea salts and 1 l Milli-

Q water instead of Huon Estuary river water, lest compounds in the river water affected the biocidal ability of the bacteria.

Forty three Antarctic sea ice isolates (Bowman *et al.* 1998b) were cultured in liquid $1/10^{th}$ strength marine agar media (using 28 g sea salts and 1 l distilled water) to 10^{7-8} cells/l which was the equivalent biomass required for algicidal activity to occur for the estuarine species. At 4 °C, the sea ice bacteria generally took between 2 to 3 weeks to attain an equivalent biomass to the temperate isolates. The supernatant used for bioassays was centrifuged at 13,000 rpm and was not filtered for the algicidal tests with *G. catenatum*.

4.3 Characterisation of algicidal compounds

Bacterial pigments were tested for their algicidal activity. Pigments were extracted with acetone, methanol or dichloromethane and the extracts tested on *G. catenatum* only. Extracted pigments also contained unidentified compounds that co-extracted with the above solvents.

The heat stability of algicidal components was tested by incubating algicidal supernatant in Eppendorf tubes suspended in a water bath at 38, 55, 80 or 120 °C (autoclave) for 30, 15, and 10 minutes respectively.

Growth curves of the bacterial isolates were determined to identify differences between algicidal activity in logarithmic and stationary phase cultures. Late log phase cultures (1 ml) were inoculated into 200 ml side arm flasks containing 75 ml of $1/10^{th}$ strength modified marine broth (0.5 g bacteriological peptone, 0.1 g yeast extract, 28 g sea salts, 1 l Milli-Q). The temperature was maintained at 17 or 22 °C. Flasks were placed on a shaker table and agitated at 120 rpm. Optical density measurements and sub-samples were taken every hour during log phase until stationary phase commenced. Sub-samples were then taken at 24 and 48 hours. All sub-samples were filter sterilised though a 0.2 µm filter and frozen at -80 °C.

Size fractionation of algicidal compounds was determined using dialysis tubing (5,000 and 10,000 MU). Further separation of the algicidal compounds was achieved by HPLC using a Waters Alliance 2690 HPLC, coupled with a photo

diode array detector, a reverse phase C_{18} column (Nova-Pak C_{18} 3.9 x 150 mm) and a Finnigan LCQ with APCI source-vaporizer 450, capillary 170, sheath gas 60, aux gas 15, source current 5 μamps, (or Finnigan LCQ with Electrospray source, capillary 200, sheath gas 90, aux gas 15, ESI needle 5KV). The scan range was m/z 100 to m/z 1200 (or m/z 100 to m/z 2000 for the Electrospray source). Data-dependent MS-MS scans were collected from the most intense ions. The elution gradients included a gradient of water-2 % acetic acid-methanol at 0.8 ml/min or a 50/50 methanol: water gradient at 0.8ml/min finishing with 90 % methanol at 25 minutes. Fractions were collected and tested via assays using *G. catenatum.* Controls were run using media blanks.

A normal phase cyano column (Nova-Pak CN HP 3.9 x 150 mm) using a gradient of methanol (A), acetone (B), hexane (C) and water (D) at 0.8 ml/min (95 % A: 5 % C for 3 minutes, then to 100 % A at 30 minutes, then to 50 % A: 50 % D at 40 minutes, which was then held for 20 minutes) was used to separate fractions. Fractions were collected and tested against *G. catenatum.* Unfotunately, all algicidal components remained on the column and would have required derivatisation to ensure removal. Derivatisation would result in loss of algicidal activity and thus HPLC separated fractions could not be tested against any algae. In addition to HPLC, Ion exchange chromatography, Sephadex columns and an XAD column were also used in attempts to isolate the algicidal components but these were unsuccessful. Details of these procedures will therefore not be reported.

4.3.1 AHL assay

Algicidal bacteria were evaluated with an AHL bioreporter, based on the transcriptional activator TraR of *Agrobacterium tumefaciens.* TraR can respond to 3-oxo-AHL, 3-hydroxy-AHL, and alkanoyl-AHL with chain lengths ranging from C_4 to C_{12}. The fusion reporter from the *A. tumefaciens* tumour inducing plasmid has been shown to be the single most sensitive test and versatile detector of all the AHL tests (Cha *et al.* 1998). The method used LB media (Tryptone 10 g (Difco), Yeast extract 5 g (Difco), NaCl (Sigma), 5 g distilled H_2O (1 l) and AB media. AB media consist of media A ($(NH_4)_2SO_4$ 4 g, Na_2HPO_4 4.85g, KH_2PO_4 3

g, NaCl 3 g, distilled water 100 ml) and B (1 M $MgCl_2$ 1 ml, 0.1 M $CaCl_2$, 1 ml, 0.01 M Fe Cl_3 1 ml, 1 mg/ml thiamine distilled water 900 ml). Both A and B are individually autoclaved before combining the two and adding glucose and casaminoacids (DIFCO) to a final concentration of 0.5 %.(Clark and Maaloe 1967). LB plates were supplemented with 4.5 µl/ml tetracycline and 50 µg/ml spectomycin, streaked with *A. tumefaciens* strain A136 and incubated at 30 °C. Plates were then stored at room temperature. After 2 days, 10 ml of LB media supplemented with tetracycline and spectomycin was inoculated with a single colony from the above culture and incubated overnight at 30 °C. 50 ml of AB media was inoculated from 1 ml of the overnight LB *A. tumefaciens* culture. This culture was incubated at 30 °C for 24 hours. Molten AB agar media was cooled to 48 °C and the 24 hour-*A. tumefaciens* culture was then added, mixed and poured immediately into agar plates. Wells were punched in the centre of each agar plate once the agar had set. 50 µl of algicidal supernatant from 24 hour and 48 hour cultures were added to the well. A positive control was included (*V. fischerii*). Petri dishes were incubated at room temperature for 48 hours. The induced blue zone (positive reaction) around the well was measured at 24 hours and 48 hours.

4.3.2 AI-2 assay

Protocols from Joyce *et al.* (2000) were used for the AI-2 assay. Confluent growth from 24-hour algicidal bacteria plate cultures were collected by flooding each plate with 5 ml of $1/10^{th}$ strength Marine Agar broth followed by swabbing the agar surface with a sterile cotton swab. $1/10^{th}$ strength marine broth was inoculated with the cell suspensions to a starting optical density at 600 nm of 0.1, and the culture was incubated at 37 °C, with vigorous shaking (200 rpm). Cell free culture fluids were prepared by centrifugation in an Eppendorf micro-centrifuge (1 min., 13,000 rpm) followed by filtration of the supernatant through Millipore 0.2 µm pore size filters. The supernatants were stored on ice before being assayed for AI-2 activity.

For the AI-2 assay, cell free culture fluids were prepared from the algicidal parental cultures. 10 µl of each preparation were added to wells of a 96-well microtiter dish (IWAKI) and assayed for AI-2 activity with strain *V. harveyi* BB170 as

described in Surette and Bassler (1998) and Surette and Bassler (1999). For each preparation, 10 µl of the corresponding sterile medium was added to the wells as a negative control and 10 µl of *V. harveyi* cell-free culture was used as a positive control for AI-2 activity. The following conditions are known to promote maximal AI-2 production (Surette and Bassler 1998). *V. harveyi* BB170 was grown overnight with aeration at 30 °C in AB medium then diluted 1:5,000 in fresh AB medium, and 90 µl of the diluted culture was added to the wells containing the cell free culture fluids or medium controls. The microtiter dishes were shaken at 200 rpm in a rotary shaker at 27°C. Light production was quantified every hour with a Wallace Model 1450 Microbeta Plus liquid scintillation counter. The data are reported as the fold stimulation of light emission by *V. harveyi* BB170 over that obtained for the corresponding growth medium alone. Assays were performed in triplicate.

4.3.3 Algal assays

The protocol described by Lovejoy *et al.* (1998) was used for algicidal assays. To test bacterial effects on different algal species, triplicate 1 ml samples of algal culture were added to a 24 well micro-plate (Iwaki, Japan). To this, 100 µl of a bacterial culture or filtrate or media control was added. Liquid media used for bacteria were $1/10^{th}$ strength marine broth and full strength marine broth. Duplicate media controls were run in tandem with the experiments. The plates were sealed with parafilm and monitored at time intervals of 0, 5, 10, 15, 30, 60, 90, 120 and 180 minutes and then every hour for up to 6 hours. Cultures were then monitored daily. Most algal cultures lysed within 3 hours. A positive algicidal effect was considered to occur when 80 % or more algal cells were lysed. A dilution series of bacteria or bacterial supernatant was completed to determine bacterial concentrations required to produce algicidal activity.

4.3.4 Larval assay

Larval settlement assays were performed as described in Holmström *et al.* (1992) and de Nys *et al.* (1994). Assays tested the activity of the algicidal bacteria against the settlement of the marine invertebrate larvae *Balanus amphitrite* and a hydroid

species. Test bacteria were inoculated into marine broth in a Petri dish and left overnight. Growth of the bacteria resulted in the formation of a biofilm on the base of the Petri dish. The broth was then poured off leaving the bacterial biofilm; this was washed gently with sterile seawater. 10 ml of seawater containing barnacle or hydroid larvae were then added. The number of settling larvae was determined by microscopic observation after 2 days incubation at 25 °C and compared with controls containing sterile filtered seawater (Egan *et al.* 2000a).

4.3.5 Cyst assays

Three algicidal species ACEM 21, ACEM 22 and ACEM 1 were chosen to test for their effect on the excystment and encystment of *A. minutum* and *G. catenatum* cysts. Supernatants from bacterial cultures and direct addition of bacteria plus supernatants were tested at differing concentrations (10, 100, 500 and 1000 µl, see also Table 4.2).

Table 4.2 Equivalent bacterial concentrations added for encystment and excystment experiments. ACEM 1 caused lysis of the vegetative cells if > 100 µl was added to cyst culture.

Bacterial strain	Effective no. of cells added as supernatant	Volume of supernatant added to 10 ml cyst solution (µl)	*Cells / litre*
ACEM 1	2.6×10^1	100	2.6×10^6
ACEM 21	1.5×10^3	100	1.5×10^8
ACEM 22	6.2×10^4	100	6.2×10^9

The choice of bacterial isolates was made to determine whether algicidal bacteria produce compounds that could effect encystment during periods when they were algicidally impotent and potent. ACEM 1 was chosen because of the highly algicidal nature of this strain and to complement previous research on vegetative cells using this strain (Lovejoy *et al.* 1998). ACEM 21 was chosen due to its rapid swarming capability, its presumed association with the decline of algal blooms, its predatory ability and cytolytic potential. ACEM 22 at the time of testing was not producing algicidal components. It was chosen for this reason as an algicidal

species that is not algicidal may produce other components such as those that could influence encystment (e.g. antibiotic compounds, chapter 8).

All experiments included a set of controls. Blanks included sterile bacterial liquid media ($1/10^{th}$ strength) added at concentrations of 10, 100, 500 and 1000 µl and a blank in which no media was added.

Excystment assays

A. minutum

A. minutum cysts attach firmly to the bottom of the culture vessels because they have a mucoid sheath around the cell (Bolch *et al.* 1991). Containers were emptied of their growth media, gently washed 3 times in sterile seawater (28 psu), and then 10 ml of liquid media was added. Although cysts were washed with sterile seawater the resulting culture was not axenic. 1 ml aliquots of $1/10^{th}$ strength marine broth containing the isolates ACEM 1 ACEM 21 and ACEM 22 were added to the duplicate *A. minutum* cyst preparations. Cyst cultures were tested for the presence or absence of any algicidal isolates after a week, using a dilution series, on triplicate marine agar plates. Plates were incubated for 10 days before identification of bacterial isolates.

G. catenatum

G. catenatum cysts were produced by crossing strains GCDE08 and GCHU11. Resting cysts were isolated individually into a sterile 24 well polystyrene tissue culture plate (Falcon) using a micropipette and then 5 ml of fresh GSe medium was added. Initial enumeration of cyst cells was completed for unviable/viable cells in *G. catenatum* cultures (Parker 2001). The end volume was 5 ml for each culture and a minimum of 11 viable cysts were used per culture. On average, there were 19 viable cysts and 9 non-viable cysts per test solution. The number of viable resting cysts, non-viable resting cysts (cyst wall clearly compromised and contents blackened), and empty resting cysts (i.e. successfully germinated) were enumerated after 8 and 25 days. ACEM 21, ACEM 22 and ACEM 1 were added to the triplicate cyst solutions at differing 0.2 µm filtered supernatant concentrations (0,

100, 200 µl) (Table 4.2). ACEM 1 was also added as an unfiltered bacterial culture (100 µl). Media controls at various dilutions (100 and 200 µl) and triplicate algal controls (0 µl bacterial media) were also tested.

Encystment assays

G. catenatum vegetative cells (strains GCDE08 and GCHU11) were preconditioned in GSe/20 for 7 days to facilitate cyst formation (Parker 2001). 2 ml of each strain was then combined with 5 ml of GSe and the different concentrations of the supernatants, bacterial cells and marine broth added in sterilised 55 mm pre-sterilised petri dishes (approx. 330 cells/ml initial concentration). Strain crosses were placed at 18 °C, and approximately 70 µmol photons PAR /m^2s on a 12:12 light:dark cycle, and monitored for 25 days. The *G. catenatum* resting cysts were counted before addition of the supernatant and then were counted again after 1, 2 and 3 weeks by which time the algal controls had formed cysts. On day 25 a sub-sample was taken and fixed in Lugols iodine solution. The initial *G. catenatum* culture was not axenic (10^9 cells/l). The vegetative cells were tested for encystment with varying concentrations of the bacterial population (10, 100, 500 and 1000 µl) (Table 4.2). The lowest concentration (10 µl) ensured that the dinoflagellates would not lyse. Concentrations of 500 and 1000 µl of algicidal bacteria lysed the cells and 100 µl of ACEM 1 also lysed cells. Duplicate samples of algicidal supernatant were added to the duplicate 10 ml samples of the *G. catenatum* vegetative cells.

4.3.6 Addition of algicidal bacteria to *G. catenatum* cultures

Two trials one day apart were completed to examine the consequence of adding bacterial cells to *G. catenatum* cultures. Log phase *G. catenatum* cultures (5 ml) were placed in 6 well micro-plates (Iwaki, Japan) and each well was tested with increasing bacterial concentrations (Table 1.2). A number of bacterial colonies were harvested from a culture growing on full strength marine agar (incubated 2 days at 22 °C) and suspended in 5 ml of seawater by vortexing. The seawater suspensions were then added to the algal cultures. ACEM 4 and ACEM 1 (10^7 cells/l) were added to the algal culture resulting in the addition of approximately

500 cells/l to the algal culture (both bacterial seawater suspensions were plated onto marine agar media to establish bacterial concentration and type). Blank controls were also counted. Controls consisted of algal culture alone, algal culture with seawater added and algal culture media with bacteria added. The bacterial-algal culture mixes were then plated at 24 hours, 3 days, 1 week and 1 month to see the effect over time of this addition. The effect on *G. catenatum* was also noted.

4.3.7 Algicidal activity of field bacteria

After two years of identifying culturable isolates from the Huon Estuary the distinct morphological characteristics allowed isolation of algicidal bacteria with colonial morphologies similar to algicidal bacterial isolates, before, during and after *G. catenatum* blooms. Seawater samples from the 3 field sites were plated onto full strength marine agar and incubated at 22 °C for a week to attain between 30 and 300 colonies for each sampling date, site and depth (see chapter 7). The appropriate isolates were then selected and purified on full strength marine agar. In effect, colonies that were similar morphologically to ACEM 20, ACEM 21, ACEM 22 and ACEM 1 were treated as bacteria with algicidal potential. Also included in experiments was an orange mucoid colony morphotype that occurred regularly and also showed algicidal activity (tested against *G. catenatum*, data not shown). After 1 week incubation at 22 °C, colonies were transferred to an Eppendorf tube containing $1/10^{th}$ strength marine broth. The cultures were incubated at 22 °C for 2 days. The bacterial suspensions were then centrifuged at 13,000 rpm for 10 minutes. A 100 µl subsample of each of the supernatants was then tested against *G. catenatum* using the previously mentioned algicidal assay.

Algicidal-like bacteria varied in number over the field season. A range of 16 to 89 (average 30) isolates was tested for algicidal activity for each field date. Although bacterial concentrations varied between sample dates similar bacterial types would reappear that were easily recognisable each week or each season. Algicidal activity was registered if cell lysis occurred within 6 hours.

Results

4.4 Pigmentation and morphology

The pigments of algicidal strains were not the algicidal component. Pigmentation of ACEM 1 and ACEM 4 colonies could be dramatically altered using different media. Under nutrient deplete conditions (e.g. $1/10^{th}$ marine agar) the majority of bacteria were still pigmented. ACEM 1 and ACEM 4 produced white colonies under these conditions that co-occurred with their respective yellow or green colonies. The white colonies were not a contaminant as they formed yellow and green colonies, respectively, if replated onto nutrient rich media. If the white colonies were repeatedly replated in nutrient deplete media for a number of generations (n=4-10) algicidal activity in ACEM 1 and ACEM 4 continued to occur. Replating on nutrient rich media led to the return of pigmented colonies. Changes in pigmentation did not appear to affect the algicidal activity (or antibiotic activity for ACEM 1 see chapter 8). Pigment formation in these isolates was therefore not essential to the algicidal activity of the isolates but all isolates nonetheless contained the ability to produce pigment as part of their cell biochemistry.

Media with bacteriological peptone alone or nutrient poor media caused ACEM 1 to produce orange colonies, the normal pigment on solid media was pale to dark yellow with a white halo. Orange pigmentation in ACEM 1 also increased if the culture was grown in large (500 ml) volumes. ACEM 4 had predominantly green colonies but pigmentation varied (purple, black, light or dark green with or without white halo). In nutrient limited media or in stationary growth phase of a nutrient rich liquid media green, surface, lace-like, filiforms were observed for ACEM 4.

The other algicidal species from the Huon Estuary showed little morphological variation with changes in media. In liquid media, ACEM 21 was highly motile and was distributed evenly throughout the broth. If centrifuged at high speed and then left undisturbed the cells would redistribute themselves throughout the media

within 5 minutes. Calculating the time for a majority of cells to reach the top of a 15 cm test tube the approximate speed was 0.6 m/h.

4.5 Growth rates

Growth rates for the algicidal species are shown in Figure 4.1. All algicidal bacteria had rapid growth rates at temperatures and salinity conditions typically found in the estuary. The growth rate of ACEM 1 was particularly fast. This isolate reached log phase in 3 hours and stationary phase in 7 hours at 22 °C (Figure 4.1), and 4 and 11 hours respectively at 17 °C. The growth rate of ACEM 32 in Figure 4.1 is not truly representative of the growth phase of this bacterium. Colloidal balls formed making it difficult to gain an accurate optical density. No bacteria were algicidal in early log phase (Table 4.3). All bacteria were algicidal during mid log phase except ACEM 32. Production of algicidal components in ACEM 32 occurred after 20 hours. Visually it was apparent that ACEM 32 had reached late log phase at 12-14 hours. After 20 hours the culture was obviously in stationary phase, therefore ACEM 32 was only algicidal in stationary phase.

4.6 Loss of algicidal activity

Algicidal bacteria would occasionally lose their algicidal activity. This occurred for all bacteria except ACEM 21. Interestingly, the failure in algicidal activity could often be restored by isolation of a healthy bacterial colony into seawater for 1 or 2 months at 20 °C. ACEM 22 often lost algicidal activity after being cryogenically stored. The algicidal revival method mentioned was used twice successfully for ACEM 22. However, for a period of over a year it would not recover algicidal activity. A method suggested while working at the UNSW[1] re-initiated the algicidal activity in ACEM 22. The procedure involved the addition of the spent,

[1] Ashley Franks Department of Immunology and Bacteriology University of New South Wales

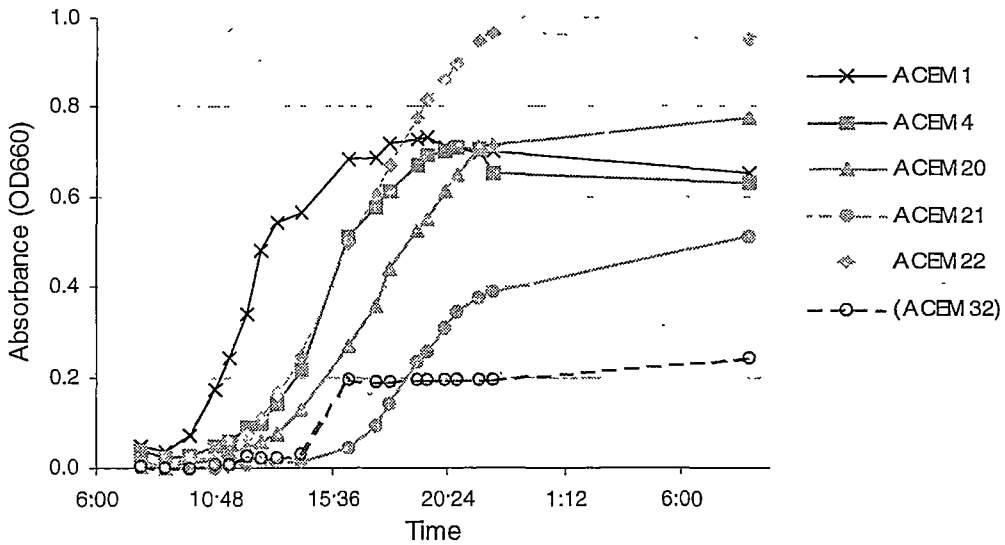


Figure 4.1 Growth curve of algicidal bacteria measured as absorbance (OD 660) at 22 °C. Visually gauging the growth made it feasible to ascertain when log stage had been reached.

Table 4.3 Comparison of bacterial growth stage and the effect of algicidal activity on *G. catenatum* .

Algicidal isolate	ACEM 1	ACEM 4	ACEM 20	ACEM 21	ACEM 22	ACEM 32
Growth phase						
Early Log	-	-	-	-	-	-
Mid Log	50 % cell lysis and cell rounding	10-30 % cell lysis.	< 10 % cell lysis and cell rounding	10-30 % cell lysis.	10-30 % cell lysis.	-
Stationary	≥ 80 % cell lysis	≥ 80 % cell lysis	50 % lysis and cell rounding	≥ 80 % lysis	≥ 80 % lysis	50 % lysis and cell rounding

filtered supernatant of another algicidal bacteria to ACEM 22. This method was also tested for *E. coli* and other environmental strains to identify the possibility of initiating algicidal activity in species that were not algicidal but this did not occur.

Algicidal activity could be lost in ACEM 1 once the culture had been continuously agitated for 3 to 4 days. After this period, the media would appear clear, as though no growth had occurred. Autolysis is a possible cause of this observation. The algicidal activity of this broth was severely effected and became equivalent to the culture in early log phase. The other algicidal isolates did not exhibit autolysis when grown under similar conditions.

4.7 Stability of algicidal components

The bacterial filtrate for all isolates could be left for a week at room temperature (25 °C) or 2-3 months at 2-8 °C with algicidal activity not noticeably affected. Longer periods than this showed a gradual reduction of activity. The supernatant could also be frozen at -20 °C for 6 months without any observable effect, however, after 12 months, algicidal activity had noticeably deteriorated. The ability of the supernatant to retain its algicidal activity after heat treatment was also tested. All supernatants were algicidal after 30 minutes at 38 °C, 15 minutes at 55 °C, 10 minutes at 80 °C and 10 minutes at 120 °C (autoclave). The mode of algal lysis appeared to be the same as before heating (i.e. it did not appear to be a toxic artefact caused by heating). Overall, this indicates that the algicidal mechanism in each of the bacteria was not enzymatic.

4.8 Characterisation of algicidal components

Algicidal components of ACEM 1 and ACEM 4 were found to be less than 5,000 MU. Analysis by HPLC-MS-MS established that the compounds were highly polar and of low molecular weight (214 MW). The algicidal compounds eluted in the first 2 minutes of the elution gradient (water) using the C_{18} reverse phase column.

HPLC analysis by means of the normal phase column resulted in the compounds being retained on the column. A derivatisation process would be required to elute the algicidal compounds. When tested, the derivatisation processes caused algicidal activity to be lost. Use of ion exchange chromatography, Sephadex columns, XAD columns also proved unsuccessful in isolating and or concentrating this fraction.

4.9 Quorum sensing

Chemicals such as acetylated homoserinelactones (AHL) are used by bacteria to monitor their population density and were possible mechanisms in the algicidal activity of the isolates. Algicidal isolates were therefore tested for the two different types of signal molecules that trigger luminescence in *Vibrio harveyi.* One of these signal molecules is turned on by the production of AHL. The tests using *Agrobacterium tumefaciens*, to determine the presence of AHL were negative for all algicidal species. This test is the most sensitive for detection of AHL and therefore suggests that the typical pathway for gene regulation via AHL does not induce the algicidal or other bioactive mechanisms in these species.

The second signal molecule AI-2, controls and induces the Lux gene in a similar way to AHL but is not yet fully understood. This pathway has a role in the mechanism by which these bacteria regulate functions such as quorum sensing. The responder has similar actions to AHL, in that it turns on the same genes, but differs from the gene that causes the formation of AHL. The test is difficult to interpret with strains other than those traditionally used because environmental isolates can be highly variable in behaviour. The algicidal strains all show some activation of the luminescence gene in comparison to the negative control (Figure 4.2). Measurements of bioluminescence during the 2nd and 3rd hour demonstrate that the algicidal bacterial cultures have stabilised (Figure 4.2). In particular, strong signals were observed for the gram-variable and gram-positive species

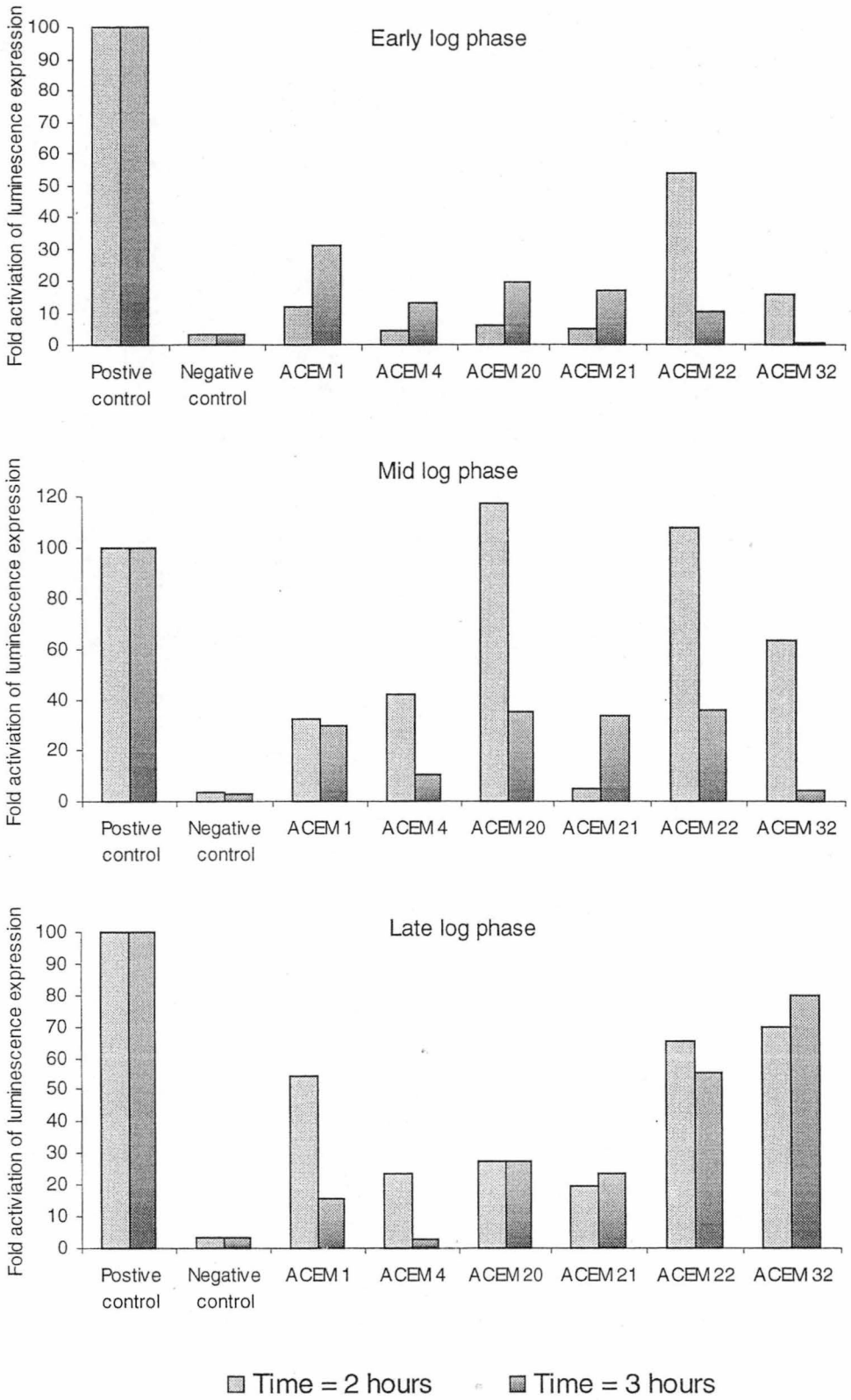


Figure 4.2 Time series of algicidal strains at different log phases, showing induction of the luminescence gene via the autoinducer mechanism AI-2. Y-axis is activity that is reported as fold activation of luminescence of the algicidal strains over the level of luminescence when sterile media were added. Luminescence is reported with respect to the positive control. Positive control: luminous bacterium *Vibrio harveyi*. X-axis shows mechanism induced in the bacterial culture over a period of 6 hours. At and after 4 hours the negative control becomes positive so times after 3 hours are not shown.

ACEM 22 and ACEM 32 respectively; ACEM 22 at late and mid log phase and ACEM 32 at late log phase. ACEM 20 and ACEM 1 demonstrated good activity for all growth phases. The results indicate that for the algicidal species the bacteria appear to be using this mechanism at mid to late stage of log phase.

4.10 Specificity of algicidal isolates

Algicidal supernatants were tested on a range of algal and heterotrophic species previously used in studies of algicidal bacteria (Table 4.4). The main algal species of interest was *G. catenatum* as this is the only toxic alga in the Huon Estuary.

Algicidal components in all 5 bacterial species were extracellular, although both ACEM 21 and ACEM 1 demonstrated the capability to attack directly (Table 4.4). All algicidal bacteria except ACEM 22 were also capable of predatory swarming. The algicidal effect occurred within 15 minutes of adding the filtered supernatant to the dinoflagellate culture and total lysis was usually within 2 hours. Bacterial numbers required for algal lysis were generally 10^{6-8} cells/l (Figure 4.3).

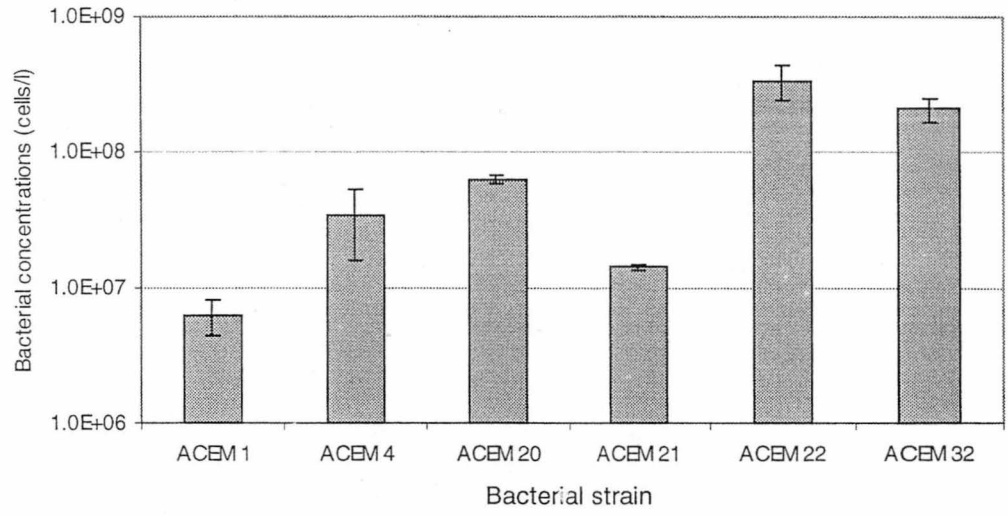


Figure 4.3 Concentration (cells/l) required for bacterial species to cause algicidal effect on *G. catenatum* within 3 hours

Table 4.4 Mode of attack of algicidal species D: direct attack; I: indirect attack, IR indirect attack with recovery, ne: no effect. (Table adapted from Doucette *et al.* 1998). Grey font indicates published results dark font indicates results undertaken in this thesis. * Indicates Tasmanian endemic species.

Algicidal bacterial genera	*Cellulophaga*					*Pseudo-alteromonas*					*Bacillus*	*Flavobacterium*		*Vibrio*	*Planococcus*
Bacterial species/strain†	1	2	3	20	21	5	15	6	1	4	32	9	10	12	22
Host Algae															
Diatoms															
Skeletonoma costatum	D	D		ne	ne		I	ne	ne	ne	ne	ne	ne	ne	ne
Chaetoceros didymum	D			ne	ne	I			no	ne	ne				ne
Ditylum brightwellii	no	D				no							no	no	
Eucampia zodiacus	D						I								
Mixed Antarctic diatoms				ne	ne				ne	ne	ne			no	ne
Thalassiosira sp	D	D					I						no	no	
Raphidophytes															
Chattonella antiqua	ne	D	D			I		ne					ne		
Chattonella marina		D	ne	I	D/I	I	I	ne	D/I	I	I	ne			I
Heterosigma akashiwo		D	ne			ne		ne	D/I			ne	ne	I	
Fibrocapsa japonica		D	ne												
Dinoflagellates															
Gymnodinium catenatum	ne	D	ne	I	D/I	I		I	D/I	I	I	I	I	I	I
G. catenatum cysts					ne				ne						ne
Scrippsiella		ne													
Protoceratium reticulatum *				ne	ne				ne	ne	ne				ne
Polarglaciola (polar dinoflagellate)				I	D/I				I	I	I				I
Alexandrium catenalla													I	I	
Gymnodinium sanguineum									D/I						
*Gyrodinium sp.**				ne	IR				IR	IR	ne				ne
*Dinophyta**				ne	ne				I	I	ne				
*Gymnoid**				I	I				I	I	I				I
Alexandrium minutum				ne	I				IR/I	I	I				ne
A. minutum cysts					ne				ne						ne
Prasinophyte (Mantoniella)									ne						
Crptophyte (Chroomonas)									no						
Cyanobacteria (Oscillatoria)									no						
Thraustochytrida				ne	ne					ne	ne				ne
Rotifer				ne	ne					ne	ne				ne

† 1 Mitsutani *et al.* (1992) 2 Imai *et al.* (1993) 3 Furuki *et al.* (1991) 20 (ACEM 20), 21 (ACEM21), 4 (ACEM 4), 32 (ACEM 32), 22 (ACEM 22) Skerratt 5 Imai *et al.* (1995) 6, 10, 12 Yoshinga *et al.* (1995) 7 Lovejoy *et al.* (1998) 9 Fukami *et al.* (1991) 14 Lee *et al.* 2000

All bacterial supernatants apart from ACEM 22 lysed the vegetative forms of the toxic flagellates and dinoflagellates *G. catenatum, Chattonella marina* and *Alexandrium minutum.* This is in contrast to the study by Lovejoy *et al.* (1998) in which *A. minutum* cells rounded but recovered after 24 hours. The variation between this study and the study of Lovejoy *et al.* (1998) may be due to the maintenance of bacterial cultures in this study on full strength marine agar or liquid media before completion of the biocidal assays at $1/10^{th}$ strength. In the experiment by Lovejoy *et al.* (1998), ACEM 1 was maintained in $1/10^{th}$ strength MA. ACEM 22 lysed *G. catenatum* and *C. marina,* but not *A. minutum.* No effect was observed on any of the diatoms tested, the rotifer or thraustokytrid species. No effect was observed on any of the cyst stages of the life cycle of *A. minutum* or *G. catenatum* (see section 4.3.5 and Table 4.4).

Algicidal activity against four endemic Tasmanian dinoflagellates was tested (Table 4.4). The algicidal components were deleterious for two dinoflagellate species, however, two of these species recovered and survived after treatment. One endemic dinoflagellate species, *Protoceratium reticulatum* was almost unaffected and no cells lysed.

ACEM 20 and ACEM 21 are both members of the CFB cluster and had unusual algicidal activity. Gliding bacteria typically use a direct rather than indirect mode of attack. ACEM 21, a strain of *Cellulophaga lytica* utilised gliding and swarming (ie direct attacks) and also produced exudates that were capable of lysing the algae. ACEM 20, the other *Cellulophaga* species isolated, was capable of swarming. It did not use obvious, direct methods of predatory attack, inducing algicidal lysis via extracellular exudates. ACEM 1 also used predatory methods although this was most apparent once the algal cells had started leaking and bacterial cells started to swarm.

The mode of lysis using extracellular compounds was similar for all algicidal bacteria and to published data for strain ACEM 1 (Lovejoy *et al.* 1998). After 15 minutes, chain-forming species such as *G. catenatum* would separate into single cells. Single cells would then start to show cell rounding and thinning of the cell wall (30-45 minutes) followed by lysis of the cell wall at 1.5 to 4 hours. During

the period of cell rounding, some cells would not become rounded for up to an hour whereas others would have started to lyse. Occasionally large temporary cysts would form in cultures that contained algicidal bacteria and these would also eventually lyse. In another study, temporary resting cysts formed on the addition of algicidal bacteria to vegetative dinoflagellate cells (Nagasaki *et al.* 2000).

Figure 4.4 shows algal lysis over time. Figure 4.4a was taken before the addition of the supernatant. Cells appeared to remain unaffected for 2 to 10 minutes after supernatant addition. Figure 4.4b shows the *G. catenatum* cells separating. Figure 4.4c shows a single unrounded cell of *G. catenatum* still unaffected by exposure to the algicidal component surrounded by other rounded cells. Figure 4.4d demonstrates the effect of adding the algicidal bacteria to the algal culture after a minimum of 1.5 hours.

When bacterial cells of ACEM 21 and ACEM 1 are added to *G. catenatum* cells, swarming is apparent to the degree that the algal cells are physically moved around the field of view. The swarming effect was not apparent if the supernatant was added alone, but cell lysis occurred within a similar time frame. If bacteria were added with the liquid media, greater disintegration of cells (not lysis) was observed in all cultures apart from those with ACEM 22 added. The physical breakdown of the algal cells appeared to enable the bacterial swarm to become more effective once the integrity of the algal cell wall was broken.

Of the additional *Pseudoalteromonas* species tested for algicidal activity against *C. marina* and *G. catenatum*, only 3 species lysed the two toxic algal species. These were *P. rubra*, *P. tunicata* and *P. ulvae*.

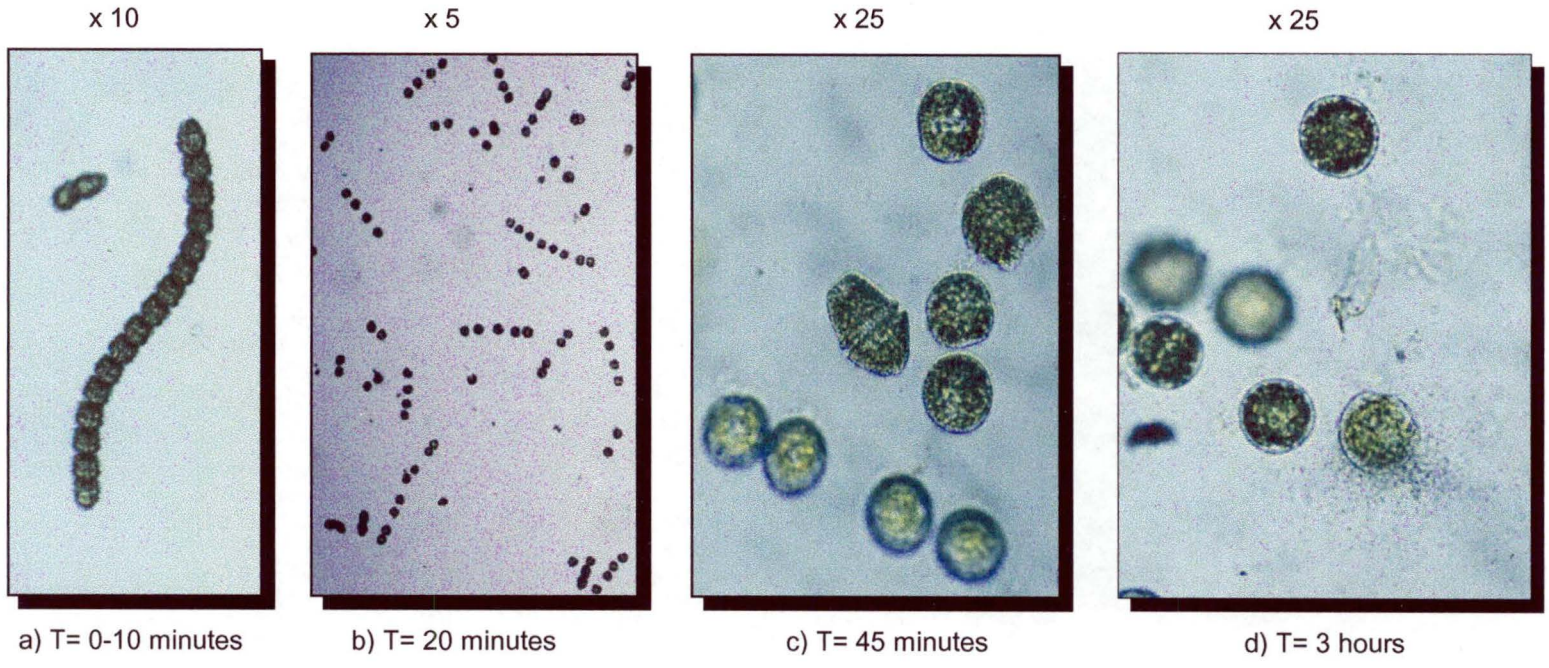


Figure 4.4 Images showing time series of *G. catenatum* vegetative cells after addition of algicidal bacteria and supernatant.

To determine whether the occurrence of algicidal bacteria was a common occurrence in other marine environments, exudates of the Antarctic sea-ice bacteria were tested on a mixed culture of Antarctic diatoms as well as *G. catenatum* and *C. marina*. No Antarctic bacterial isolates showed any algicidal effect, although 3 showed a detrimental but reversible effect. All algicidal bacteria from the Huon Estuary lysed the polar dinoflagellate *Polarella glacialis* no effect was observed in the mixed Antarctic diatom culture (Table 4.4).

4.10.1 Excystment

A. minutum

A. minutum cysts were not affected by addition of the bacteria with supernatant. The cysts proved viable and excysted. In the culture containing ACEM 1, once the vegetative cells of *A. minutum* excysted they lysed and this is thought to be caused by the continuing presence of the algicidal exudates added from ACEM 1.

Only white colonies ($4.6 \times 10^{12} \pm 1.9 \times 10^{7}$/l) were present a week after the addition of ACEM 22 exudate plus cells which infers that the original bacterial population from the cyst culture dominated. ACEM 1 was dominant a week after addition to the *A. minutum* cysts although white colonies were also present in high proportions (~50 %). Bacterial concentrations in the cyst culture were less after a week in the ACEM 1 treatment than those in ACEM 22 treatment ($3.2 \times 10^{7} \pm 1.1 \times 10^{7}$/l).

It was difficult to estimate the presence of ACEM 21 due to the gliding nature of this isolate on plates. It was present a week after addition to the cyst culture although white colonies were present at higher proportions. Unlike ACEM 1 vegetative cells of *A. minutum* did not lyse after excystment in the cultures with ACEM 21 or ACEM 22 added.

G. catenatum

No major effect was noted for any of the bacterial cultures on the viability of cysts or a decrease in excystment of *G. catenatum* (Figure 4.5). The supernatant demonstrated no treatment effect on the viability of cysts.

Similar to the result of the excystment experiment with *A. minutum*, once the vegetative cells of *G. catenatum* excysted they lysed in the culture containing ACEM 1 cells or 200 µl supernatant.

4.10.2 Encystment

Promotion of encystment in *G. catenatum* was not observed when using supernatant or bacterial cultures from the algicidal strains ACEM 1, ACEM 21 and ACEM 22 (Figure 4.6).

Overall, there was no positive effect on encystment of *G. catenatum* by the addition of the algicidal bacteria or by adding low levels of algicidal supernatant that did not kill the vegetative cells (< 200 µl). Both ACEM 21 and ACEM 1 killed the vegetative cells at concentrations of 100 µl. Vegetative cells were also negatively affected by the addition of higher concentrations of $1/10^{th}$ strength marine broth (500 µl and 1000 µl) with both sets of duplicate cultures declining relatively early.

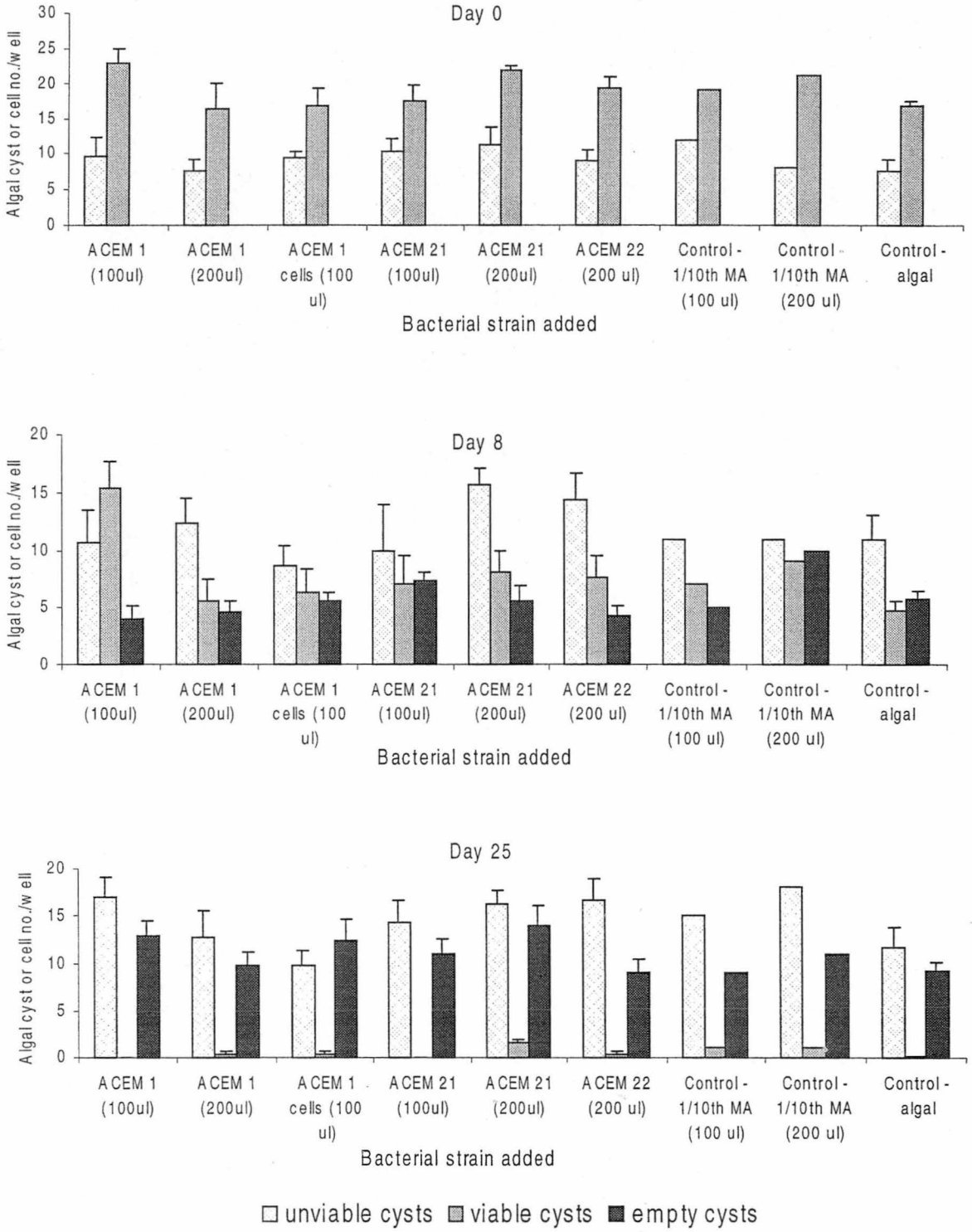


Figure 4.5 Variation in the excystment of *G. catenatum* cysts after addition of various concentrations of the bacterial cultures: ACEM 22, ACEM 21 and ACEM 1 and bacterial media and algal controls

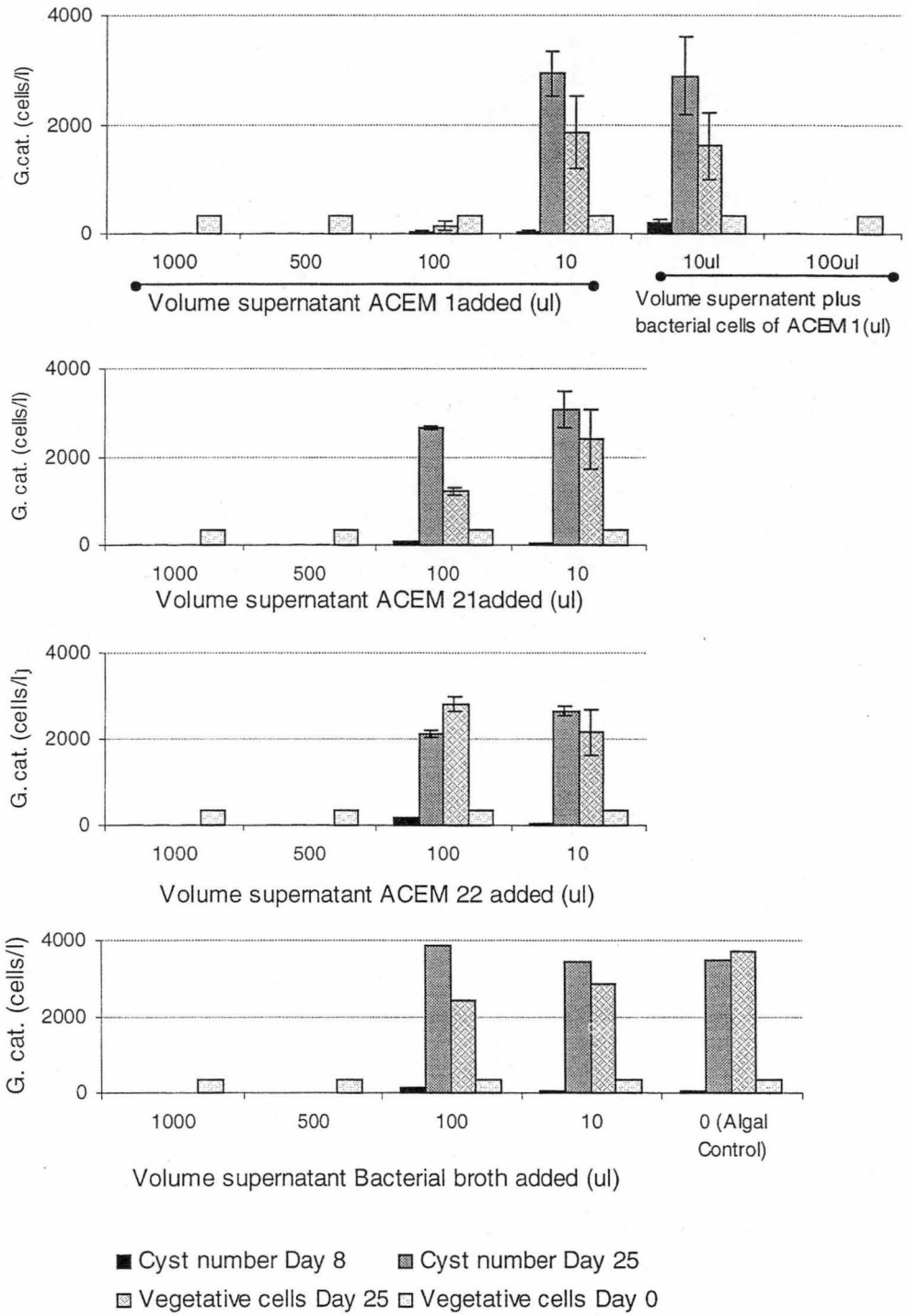


Figure 4.6 Variation in the encystment of *G. catenatum* vegetative cells after addition of ACEM 22 ACEM 21 and ACEM 1 and media supernatants, or supernatants and cells, at differing concentrations. SN: supernatant.

4.11 Larval attachment

The algicidal species were tested for the ability to kill or inhibit barnacle and hydroid larval settlement (Table 4.5). Only one strain, ACEM 4, inhibited barnacle settlement but it did not kill the barnacle or hydroid larvae and it did not inhibit hydroid settlement.

Table 4.5: Percentage settlement or germination of barnacle and hydroid larvae against a biofilm consisting of the algicidal bacteria

Bacterial isolate used for biofilm	Percentage settlement or germination (n=3)	
	Barnacle larvae	Hydroids larvae
ACEM 21	86.1±3.7	79.8±5.4
ACEM 20	82.6±9.3	70.0±10.8
ACEM 32	72.6±1.8	57.5±28.6
ACEM 22	80.6±4.5	81.8±1.9
ACEM 1	71.3±7.4	66.3±9.4
ACEM 4	**3.1±4.4**	80.5±7.1
No biofilm	81.6±7.8	8.8±6.3

4.12 Addition of bacterial cells to *G. catenatum* cultures

ACEM 1 and ACEM 4 cells were added without their growth media and at low cell numbers to logarithmic phase *G. catenatum* vegetative cells. Both ACEM 1 and ACEM 4 were algicidal but bacterial cells were added at concentrations that would not immediately kill the alga (10^5 cells/l). The initial *G. catenatum* culture was not axenic. Before the addition of the algicidal bacteria, the bacterial population of the culture was 10^7 cells/l (Figure 4.7). All the culturable colonies from this algal culture were tan with a clear halo. The presence or absence of the added algicidal bacteria were then observed and enumerated over the following days through observation of morphological variation. (Figure 4.7, Table 4.6).

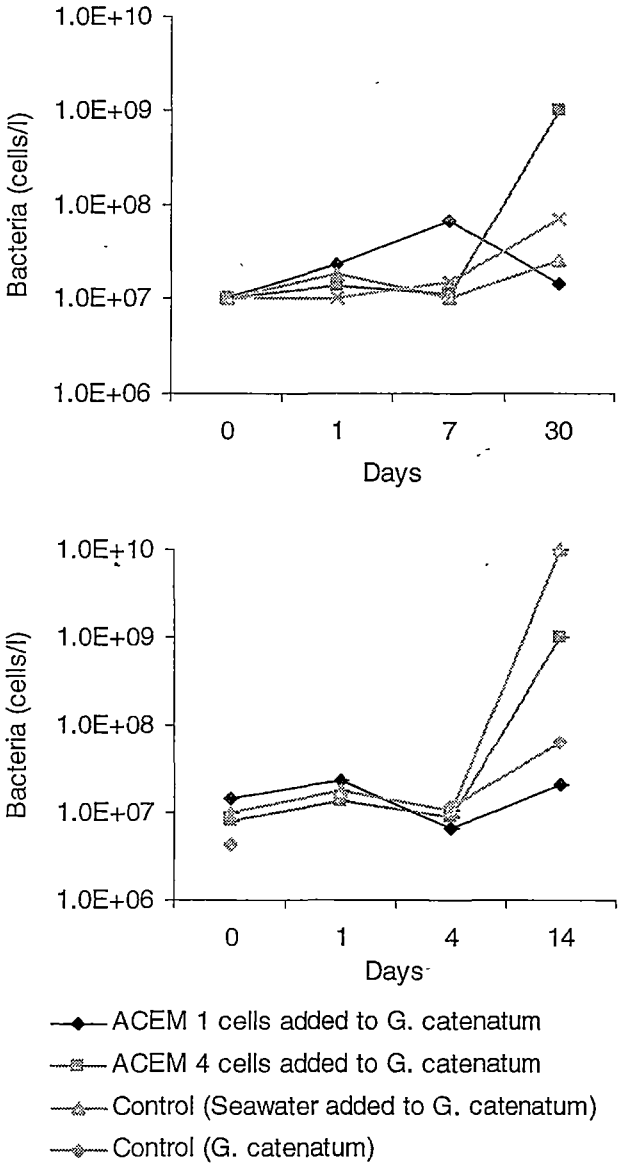


Figure 4.7 Replicated experiments showing variation of bacterial concentration in *G. catenatum* cultures over time to which ACEM 1 and ACEM 4 log phase cells were added.

Table 4.6 Colony pigmentation variations observed for the bacteria in *G. catenatum* cultures over time after the addition of ACEM 1 and ACEM 4 log phase cells.

Colony pigmentation	Bacteria present in *G. catenatum* culture before bacteria added	After 24 hours	After 1 week	After 1 month
G. catenatum with ACEM 1 added	100 % tan	ACEM 1	90 % ACEM 1 10 % cream	50 % ACEM 1 50 % white or tan
G. catenatum with ACEM 4 added	100 % tan	10 % tan 50 % white 40 % ACEM 4	30 % ACEM 4 70 % white	100 % white
G. catenatum with seawater added	100 % tan	100 % tan	30 % white 70 % tan	50 % white 50 % tan
G. catenatum alone	100 % tan	100 % tan	100 % tan	100 % tan

A day after the addition of ACEM 1 cells to the *G. catenatum* culture, all colonies were typical of ACEM 1 and at cell concentrations high enough to be algicidal (Figures 4.1 and 4.7). However, *G. catenatum* cultures were unaffected.

Bacterial numbers in the *G. catenatum* culture containing ACEM 4 after one day were mostly white with only 10 % being the original tan pigmented colonies (Table 4.6). The seawater control, and the *G. catenatum* control contained the original tan colonies. ACEM 1 and ACEM 4 were present throughout the study in the control of algal media and bacteria although many ACEM 4 colonies were white or light green. This infers that the *G. catenatum* culture with ACEM 4 added also contained ACEM 4 but it was not expressing the green pigment. The white colonies were not tested for algicidal activity or the ability to return back to their original green pigment.

A week later the *G. catenatum* culture with ACEM 1 cells added contained ACEM 1-like colonies which were dark yellow. The control containing ACEM 1 in algal media exhibited these same dark yellow colonies. This pigmentation was a common feature for ACEM 1 under nutrient stress. The *G. catenatum* culture containing ACEM 4 and algal media control contained darker green as well as light

green and white colonies. The *G. catenatum* controls had the original tan pigmented morphotype. *G. catenatum* in all cultures remained healthy.

A month after ACEM 1 was added to *G. catenatum* culture approximately 50 % of CFU were ACEM 1 (Table 4.6). The ACEM 4 treated culture contained higher concentrations of bacteria than either ACEM 1 or the algal controls at this time. A week later all *G. catenatum* cultures except the culture that contained ACEM 1 had lost viability. The antibiotic properties of ACEM 1 (chapter 8) were not affected by the lack of algicidal activity and may have assisted the *G. catenatum* culture to survive longer than the controls. The experiment was completed 3 times increasing the bacterial cell numbers each time. The second experiment is shown in Figure 4.7 with initial addition of bacterial cells at 10^7 cells/l. The *G. catenatum* culture with ACEM 1 added was always the last to lose viability unless supernatant was added at concentrations that could kill the alga.

4.13 Variation in algicidal activity of bacteria

Algicidal activity varied dramatically for the algicidal-like colonies isolated from the estuary at different sampling dates. Up to 90 % of the algicidal-like isolates were algicidal for one date yet similar isolates in the following weeks showed no such activity (Figure 4.8). Interestingly, many algicidal-like isolates were not "algicidal" at the peak of a *G. catenatum* bloom even though they were present before and after the bloom declined.

Algicidal-like isolates were present on each of the sampling dates, and although the proportions of the isolates changed, similar species were observed in consecutive weeks. These species also varied in their algicidal activity. For example, the spreading iridescent orange ACEM 21-like isolate was algicidal whenever present. In comparison, gram-positive ACEM 22-like orange isolates that occurred at the peak of the bloom were not algicidal at this time although similar isolates were algicidal earlier and later. It can be surmised that particular strains or environmental conditions are required for algicidal activity to occur.

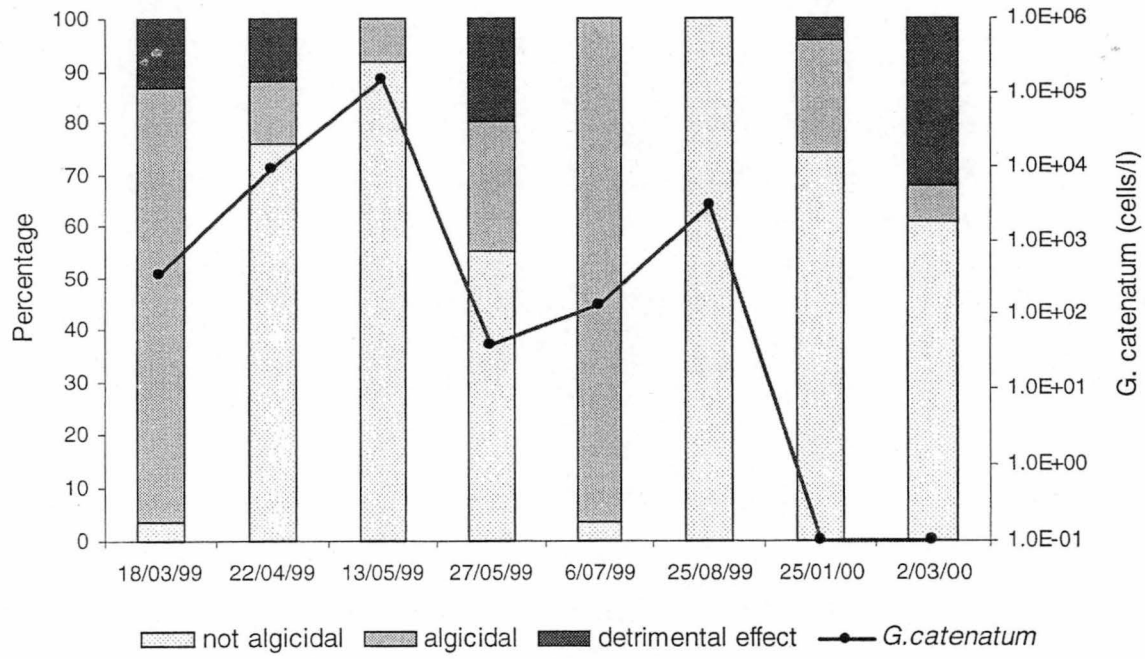


Figure 4.8 Algicidal-like bacteria isolated from the field over the season showing algicidal activity for particular dates and comparison with *G catenatum* vegetative cell numbers (*G. catenatum* counts Parker 2001).

Discussion

Many bacterial secondary metabolites, bioactive and algicidal compounds, are only produced during stationary or late log phase (Fukami *et al.* 1992, Lovejoy *et al.* 1998, Hukami *et al.* 1992). All algicidal species apart from ACEM 32, produced algicidal compounds in both late log phase and stationary phase. Previously, ACEM 1 was reported to produce algicidal components during log phase (17 °C, sample taken at 16 hours) (Lovejoy *et al.* 1998). However because of the rapid growth of this strain, it is probable that the majority of cells would have already been in stationary phase. The rapid growth rates of all the algicidal bacteria in this study indicate that all strains have the ability to rapidly take advantage of suitable conditions in the natural environment should they occur.

This study is the first time gram-positive or gram-variable species have been reported as algicidal. ACEM 22 was the only species that did not show swarming activity. ACEM 32 only produced algicidal exudates in stationary phase. These two species are also unusual because most algicidal bacteria are from the CFB cluster or the genus *Pseudoalteromonas*. It is possible that descriptions of algicidal species to date have been influenced by bacterial culturability. Some CFB and many *Pseudoalteromonas* are easy to culture so these species are the first to be identified as algicidal. As research on algicidal bacteria continues, it is possible algicidal species will be found to be widespread and common in other bacterial genera.

Morphological characteristics such as formation of biofilms or the ability to rapidly move throughout liquid media are effective methods of survival for algicidal bacteria. The swarming capacity of ACEM 1 was first observed by Lovejoy *et al.* (1998), and although not an unusual bacterial ability, the swarming attacks of ACEM 21 and ACEM 1 were physically aggressive in comparison with ACEM 20, ACEM 4 and ACEM 32. Rapid movement may enable the bacteria to more effectively access nutrients in the marine environment which can be a place of patchy nutrient availability.

In other studies of algicidal *Cellulophaga*-like species, all but one have shown predatory mechanisms only. Interestingly, both *Cellulophaga* species in this study produced an algicidal exudate. In the case of ACEM 21, this was in addition to a predatory mechanism. Recently, another *Cellulophaga* species unrelated to the two described in this study (89 % 16S rDNA sequence similarity to ACEM 20) was also reported to use extracellular mechanisms (G. Doucette, personal communication).

The results of the heating experiment do contradict previously published data on ACEM 1 that state after microwaving, algicidal activity disappeared from ACEM 1 (Lovejoy *et al.* 1998). The differences between the two results can potentially be explained by long storage time in culture (4 years) affecting aspects of algicidal activity. It was suggested that with 15 minutes of autoclaving the chemicals would react and form a more potent compound that would kill the alga rather than using exudate-mediated lysis. Close tandem experiments with autoclaved and unautoclaved samples indicated no observable differences in the way the cells reacted. The tests at 55 °C and 80 °C support this finding and suggest that the lytic reaction is unchanged as a result of heating.

Pigments were not the algicidal component as non-pigmented colonies exhibited algicidal activity and were shown to be capable of reverting back to their original pigmented state. Loss of bioactive components in relation to complete pigment loss has been previously observed in other bacterial studies where non-pigmented mutants of algicidal bacteria were studied (Egan *et al.* 2000b). They found that unpigmented cells occasionally formed that were not algicidal and that remained unpigmented. The results of Egan et al. (2000b) and this study infers that pigment formation may be associated with algicidal activity, but does not have to be expressed for the algicidal activity to exist.

No algicidal bacteria showed the ability to produce AHL. Many bioactive bacteria do not have AHL mechanisms, instead they appear to use an alternative induction pathway which can activate at lower cell concentrations than required for AHL (Bassler 1999). This second group of transponders in gram-negative bacteria were recently identified as a group of furanones by researchers (B. Bassler pers. comm.)

but owing to patent submission, their structures have not yet been published. This alternative AI-2 mechanism may play a role in switching on algicidal activity for the gram-negative bacteria once the bacteria reaches a specific cell density. The results indicate moderate to good induction of this second mechanism when compared to the negative control. ACEM 1 demonstrated a very good response for early log phase and ACEM 21 for mid log phase.

Gram-positive bacteria do not generally employ AHL as signals. They secrete processed peptide signalling molecules via a dedicated exporter protein (Bassler 1999). For example in *B. subtilis*, two peptide signals allow the bacteria to choose between competence for DNA uptake and sporulation. The secretion mechanics of the export of the peptides has not been identified (Bassler 1999). The positive results in the autoinduction experiment for ACEM 32 and ACEM 22 is likely to be as a result of this gram-positive quorum signalling mechanism.

Biofilms in the marine environment generally commence with bacterial attachment. Diatoms, oyster or barnacle larvae and other microorganisms attach to the bacterial biofilm using them as a nutrient source and thus producing more complex biofilms. Biofilm formation can be unwelcome, an example being the fouling of boat hulls. Some *Pseudoalteromonas* species can inhibit barnacle attachment as well as kill barnacle and hydroid larvae (*P. tunicata*, Holmström and Kjelleberg 1992 and *P. ulvae* Egan *et al.* 2001a and b). None of the algicidal species in this study demonstrated any ability to kill barnacle or hydroid larvae and only ACEM 4 showed the ability to inhibit settlement. ACEM 4 is the closest relative of *P. tunicata,* a species that shows strong antifouling activity, inhibits the barnacle and hydroid larvae and is also toxic and kills both (Holmström *et al.* 1992). ACEM 4 inhibits the barnacle larvae by the use of a non-toxic method and therefore the mode of action would appear to differ from that used by *P. tunicata*. *P. ulvae* and *P. tunicata* also possessed algicidal activity. Inhibition of larval settlement is therefore the result of a different mechanism than algicidal activity.

Interestingly, both *P. ulvae* and *P. tunicata* possess the same algicidal activity against *G. catenatum* and *C. marina* as ACEM 1 and ACEM 4. *P. rubra* was the only other *Pseudoalteromonas* species to show algicidal activity and cause algal

lysis. The genus *Pseudoalteromonas* is known for its many bioactive compounds (Mikhailov and Ivanova *et al.* 1994, Gauthier 1976,) so this result is not surprising. Other *Pseudoalteromonas* species tested such as *P. luteoviolacea* (McCarthy *et al.* 1994), *P. aurantia* (Gauthier and Breittmayer 1979) and *P. piscicida* (Buck *et al.* 1963) have all produced antibiotic or bioactive compounds. In this study they did not demonstrate any algicidal activity. Given the nature of these algicidal components these results may be caused by bacterial strains in culture collections losing algicidal viability over time.

Elucidation of algal lytic compounds has been elusive for 10 years. Researchers in the area have put effort into attempts at their isolation, purification and characterisation (Doucette *et al.* 1998, Dakhama *et al.* 1993, Baker and Herson 1978). Part of the difficulty in isolation is the highly polar nature of the compounds and the loss or degradation of the bioactive components when trying to concentrate them or form derivatives. Algicidal compounds and their characteristics vary markedly across the many species of algicidal bacteria (Doucette *et al.* 1998). The one similarity that appears to hold true is that all are highly polar which would make them readily diffusible in the marine environment. The compounds can be heat labile (Baker and Herson 1978) or heat tolerant (Dakhama *et al.* 1993), of a large (Lee *et al.* 2000) or small MW (Fukami *et al.* 1992, Dakhama *et al.* 1993), made up of a number of compounds or only one, and can be species specific or non-specific in their mode of action (Doucette *et al.* 1998). The algicidal compounds in ACEM 1 were a small molecular weight (<300 MW), highly polar and heat stable. Algicidal compounds in the other 5 species were also heat tolerant, although all lost activity if left for an extended period of time.

Understanding the association with and potential for control of toxic algal blooms has directed most algicidal bacteria research (Nagasaki *et al.* 2000, Doucette *et al.* 1999, Yoshinaga *et al.* 1999, Holmström *et al.* 1999, Kim *et al.* 1998, Lovejoy *et al.* 1998, Doucette *et al.* 1998, Imai *et al.* 1993, Stewart and Brown 1969). Very little is known about algicidal bacteria that affect non-toxic algal blooms. There is also the question of how common algicidal bacteria are and whether the algicidal

activity of these species occurs continuously or sporadically in the marine environment.

All algicidal species investigated in this study employed indirect methods of lysing algal cells and two isolates also used direct attack. Achieving a bacterial density high enough to induce lysis of dinoflagellate vegetative cells is possible when algicidal bacteria dominate the bacterial population in the Huon Estuary. Attachment of bacteria to marine snow or algal cells also creates a zone of artificially high bacterial numbers and thus higher potential for more effective algicidal activity.

Although research on algicidal bacteria and their effect on the vegetative life-stages of *G. catenatum* and other cyst forming dinoflagellates has been completed (Lovejoy *et al.* 1998), no research has been published on the effects on the viability of the cyst life-stage. If algicidal bacteria are to be effective against toxic dinoflagellate blooms, it is this life-stage that would be the most valuable to eliminate.

The algicidal bacteria examined in this study do not affect the encystment or excystment of the cyst life stage of either *G. catenatum* or *A. minutum.* The cyst lifestage offers the alga a high degree of protection from environmental extremes and lack of nutrients as well as algicidal compounds (Anderson *et al.* 1984). For practical purposes, treatment with ethanol or H_2O_2 can destroy the cyst (Parker, Hallegraeff pers. coms.). Due to this excellent method of protection and the algicidal mechanisms described so far, it is doubtful that algicidal bacteria in the water column would be effective against dinoflagellate cysts. However, in the sediment, where cyst seedbeds form, different chemical conditions occur than those in the water column. Processes such as microbial fermentation and thus the production of ethanol or other metabolites may enable some bacterial species to be effective against the cyst lifestage.

The algicidal compounds examined in this study do not appear to initiate cyst formation in *G. catenatum.* Encystment would seem a natural defence reaction of the alga when these compounds were present, however this was not shown to

occur in this study. Adachi et al. (1999) isolated a number of unidentified non-algicidal bacteria (analysed by means of the most probable number (MPN)) during a bloom of *Alexandrium tamarense*. They reported that when these bacteria were added to a culture of *Alexandrium catenella* there was a positive correlation with encystment. The comparison of the *A. tamarense* bloom and encystment of a different species (*A. catenalla*), while of interest does not fully discount other possible causes of encystment in the natural environment, particularly as encystment was not monitored in the original bloom of *A. tamarense*.

The inability of algicidal bacteria to kill or effect the cyst life stage of *G catenatum* or *A. minutum* is of major significance in the potential use of algicidal bacteria in the marine environment as a method of controlling harmful algal blooms. The bacterial extracellular components or the active compound could be added to kill vegetative cells. However, the addition of algicidal bacteria to seed the local bacterial community, is less likely to be successful. For experiments with ACEM 1 and ACEM 4 in this study, the results indicate that the bacterial strain, or the environment may control the algicidal mechanism. Bacteria added at low concentrations without their supernatant did not affect the algal culture, even though ACEM 1 appeared to dominate and be at concentrations high enough to create an algicidal effect after 24 hours. Similar results were observed with the algicidal isolates tested in the field. Isolates demonstrated algicidal activity on one sampling date yet no activity on the next indicating that there may be an environmental or strain preference trigger that occurs in the natural environment.

Algicidal bacteria appear to be common in the marine environment. Whether this algicidal activity is continuous remains in question. There has been a general assumption that these bacteria are releasing their lysing components in the natural ecosystem either constantly or once a certain cell density has been reached. The research in this thesis suggests that some algicidal bacteria may not always exude algicidal components, and may have periods in the natural environment in which they are non-algicidal. The reason for this variation may be environmental or be caused by strain variation. Detection of these bacteria in the marine environment using molecular mechanisms is therefore useful to assess a "potential" level of algicidal ability. Some algicidal bacteria such as those belonging to the genus

Pseudoalteromonas have been shown to produce many bioactive compounds that are not algicidal. In the marine environment, there is likely to be a greater metabolic benefit for these different bioactive mechanisms to be activated individually or as the bacteria requires, rather than simultaneously and continually.

Acknowledgements

The cyst experiments were completed in collaboration with N. Parker a PhD student at the University of Tasmania.

5. Identification of algal and bacterial genera in the Huon Estuary using lipid biomarkers

Summary

An examination of the lipid and fatty acid profiles of the Huon Estuary microbial community was undertaken for samples collected from October 1998 to June 1999. Specifically, fatty acid analysis was used to identify changes in bacterial and algal communities located at different depths in the marine environment. Particular attention was paid to variations in microbiota and their association with blooms of the harmful alga *Gymnodinium catenatum*.

Analysis of branched chain fatty acids (BCFA) indicated that the *Cytophaga-Flavobacterium-Bacteriodes* (CFB) cluster was significant in the microbial degradation of algal blooms in the estuary. Results from fatty acid analyses suggested that the CFB cluster was more common in the photic zone and during the chlorophyll maximum. Higher bacterial numbers with lower BCFA concentrations indicated that γ proteobacteria or other bacterial species without BCFA may be more abundant in the deeper, marine influenced waters. The fatty acid profiles, particularly the occurrence and proportions of BCFA, also suggest periods of rapid species change in the microbial community between the major algal blooms. A recurring seasonal BCFA profile was observed for all sites that were dominated by *iso*-BCFA.

Results from fatty acid analyses effectively pinpointed algal blooms at specific depths and dates. During the two *G. catenatum* blooms in 1999, estimation of algal abundance using total fatty acid concentrations closely agreed with microscopic enumeration of *G. catenatum* cell numbers. Lipid class analysis also indicated the physiological status of the three algal species during their bloom periods. An increase in degradation and storage products was apparent once the bloom had declined while higher levels of membrane components occurred during the growth stage.

Fatty acid analysis differentiated between different bacterial isolates in culture and in the marine environment. Combined with other techniques, use of these signature compounds gives insight into the marine microbial community as a whole and the microbial relationships involved in algal blooms.

Introduction

Signature lipids and fatty acid profiles can act as bacterial or algal chemical markers, and have been used to investigate many marine environments such as deep-sea hydrothermal vents, sewage outfalls, sea-ice, water column, wetlands and sediments (e.g. Mergaert *et al.* 2001, Marteinsson *et al.* 1999, Boon *et al.* 1996, Skerratt *et al.* 1995, Green *et al.* 1992, Nichols *et al.* 1990, White 1983). Specific fatty acid profiles are often used in bacterial taxonomy to identify and differentiate between bacterial genera and species. Although other biochemical and molecular methods can quantify the total bacterial biomass or bacterial community composition, they provide limited information about species interaction and the microbial population as a whole.

In the marine environment lipids are derived from many sources. Large organisms contain higher lipid concentrations per individual than smaller organisms, however, small organisms may dominate the biomass in field samples because of higher numbers. Branched chain fatty acids (BCFA) are only produced by bacteria, so in marine waters they can be used to gain insight into the microbial community structure (Edwards *et al.* 2001, Harvey and Macko 1997, Wakeham 1995, Gillan *et al.* 1983). Algal species also contain signature lipids that can differentiate between groups such as diatoms and dinoflagellates.

Measurement of the total lipid content and lipid class composition allows an estimate of the biomass and physiological status of algal blooms over a sampling period. High levels of polar lipid indicate a growth stage in the community, whether it is diatom or flagellate. High proportions of triacylglycerol indicate a community past its growing peak, where the algae are storing lipid, but the community is not undergoing degradation.

Fatty acid profiles, including the use of BCFA analysis, can identify changes in the bacterial community between discrete samples. Variation in 20:5(n-3) and 22:6(n-3) may indicate a change in the algal community from diatoms to dinoflagellates. C_{18} PUFA can be used in conjunction with other fatty acid markers to identity heterotrophs or diatom species. Variation in ratios of the various fatty acids can also give an understanding of changes in community structure.

Although variation in lipid profiles may occur because of significant changes in culture conditions, there is as yet little published evidence for such large shifts in nature. The use of lipid signatures has also been validated in many environmental studies (Edwards *et al.* 2001, Meziane and Tsuchiya 2000, Boon *et al.* 1996, Skerratt *et al.* 1995, Wakeham 1995, Mancuso *et al.* 1990). Culture conditions can vary dramatically, although the natural environment would be expected to restrict the survival of specific microbial strains to much narrower conditions of growth (White and Findlay 1988).

Signature lipids therefore can identify and quantify species present within a marine or estuarine community. Analysis of a single water sample can provide a lipid "snapshot" of the community structure and can show the relationship between diatoms, dinoflagellates and bacteria. Over a time series, these signature lipid snapshots can be useful for understanding the influence of seasonal and environmental factors.

Objectives of the research reported in this chapter were to:

- identify the characteristic fatty acids of the dominant culturable bacterial population and group them into bacterial genera or clusters;
- group algicidal bacteria within these culturable populations;
- distinguish algal blooms and bacterial communities in the marine environment using fatty acid analysis;
- determine seasonal changes in the physiological status of the marine community by examining membrane and storage lipid components;

- ❖ analyse the marine community by studying different fatty acid groups: polyunsaturated fatty acids (PUFA), branched chain fatty acids (BCFA), monounsaturated fatty acids (MUFA) and principal components analysis (PCA).

Methods

Methods details and complementary results for the same sampling sites, dates, and depths as described in this chapter are discussed in chapter 2 (physical, chemical and algal abundance), chapter 3 (morphology) and chapter 7 (field identification of bacterial species and fluorescence *in situ* hybridisation (FISH)).

5.1 Collection and culture of individual bacterial isolates

Water samples for bacterial isolation were collected in autoclaved 2 l Schott bottles. Samples were collected from sites throughout the Huon Estuary and placed in the dark at ambient river water temperature for transfer to the laboratory. Water samples were plated onto solid marine agar media (see Methods chapter 2) and incubated at 20 °C for 1 to 2 weeks. Individual bacterial colonies were isolated and streaked onto marine agar to obtain a bacterial monoculture. The purified isolates were incubated at 20 °C for one week before lipid analyses. Colonies were placed in test tubes for transmethylation (section 5.4). Replicate isolates from two Petri dishes were used for lipid analyses. Replicate fatty acid analyses of a field water sample plated from two duplicate field seawater samples was also included (Mixed plate number 1 and 2 in Figure 5.1).

5.2 Field samples: Water column sample collection

Water samples for fatty acid analysis of field samples were collected from the three sites using a 5 l Niskin bottle for the surface and pycnocline samples and a Lund tube for the integrated water samples. Water depths were selected for the pycnocline samples were selected on the basis of salinity change. Pycnocline depth was generally 2 m. Samples for lipid analysis were collected in pre-cleaned glass 2

l containers, placed in ice and kept in the dark, and were filtered onto premuffled GFF glass fibre filters. Samples were filtered, frozen and stored at -20 °C within 3 hours of water collection. Lipid analyses were completed within a month for sample dates Nov 12 1998 to Feb 18 1999 and within three months for sample dates Mar 4 1999 to May 4 1999. Labelling of sampling dates in the figures use the abbreviations: a:12/11/98, b:26/11/98, c:10/12/98, d:21/12/98, e:7/01/99, f:14/01/99, g:21/01/99, h:4/02/99, i:18/02/99, j:4/03/99, k:18/03/99, l:31/03/99, m:22/04/99, n:13/05/99.

5.3 Total lipid analyses

Field net samples were extracted by the modified one-phase $CHCl_3$-MeOH-H_2O Bligh and Dyer (1959) method (White *et al.* 1979). After phase separation, lipids were recovered in the lower $CHCl_3$ layer. Solvents were removed under vacuum and concentrated under N_2. Total lipid samples were stored at -20°C in 1.5 ml vials until analysis the following day.

A portion of the total lipid extract of the net samples was analysed for total lipid content and lipid class composition with an Iatroscan MK V Th10 TLC-FID analyser (Iatron Laboratories, Japan) (Volkman and Nichols 1991). Samples were applied to silica gel SII Chromarods using Drummond micropipettes (1 µl). Chromarods were developed in a glass tank lined with pre-extracted filter paper. The solvent system used for lipid separation was $C_6H_{14}/(C_4H_{10})O/CH_3COOH$ (60/17/0.2; v/v/v). After development, the Chromarods were oven dried for 10 minutes at 80 °C and analysed immediately to minimise absorption of atmospheric contaminants.

5.4 Fatty acid analyses

Preparation of fatty acid methyl esters (FAME) involved the addition of a 10:1:1 MeOH:CH_2Cl_2:HCl solution. Samples were heated at 100 °C for 1 to 2 hours before extraction with 4:1 C_6H_{14}:CH_2Cl_2. CH_2Cl_2 containing C_{23} FAME was added as an internal injection standard. Samples were treated with BSTFA (N,O-

bis(trimethylsilyl)trifluoroactemide) to convert hydroxy fatty acids to their O-trimethylsilyl derivatives. Fatty acid identification was completed using GC and GC-MS conditions described in Bowman *et al.* (1998a). The location and configuration of the double bonds in the MUFA of the bacterial isolates was determined for the major peaks by GC-MS analysis of the dimethyldisulfide adducts as described by Nichols *et al.* (1986). Some fatty acids present in the field samples are not represented in figures displaying fatty acid data or mentioned in the text, as they only appeared once in the whole season (e.g. cy19:0 or 19:1, pycnocline sample at site F3 1/12/98, 2%). Other components were at low concentrations during the season (e.g. 20:1, other C_{20} PUFA, 20:0, 21:0, i15:1, a15:1, i13:0, a13:0, i14:0, other C_{18} PUFA). If the unrepresented components were BCFA, the total proportion of all BCFA for the site or depth was < 0.2% of the total fatty acids. If the components were PUFA, MUFA or SFA, then the total of all minor components for any particular sample was < 2 %.

The cultured isolates were separated using hierarchical cluster analysis based on similarities in their fatty acid profiles (section 5.5). 16S rDNA sequence analysis was completed on algicidal bacteria, *Shewanella* species, ACEM 24 and ACEM 25 similar to the Method section in Chapter 3. The genus *Bacteriodes* was characterised based on the high proportion of i14:0 and a17:1 which are indicative of this genus (Turova and Osipov 1996, Olsen 1994, Wilkinson 1988), morphotype characteristics and FISH (chapter 7). Other isolates had molecular confirmation of their fatty acid classification using FISH with class and genus specific probes (chapter 7).

Fatty acid nomenclature: Fatty acids are designated as number of carbon atoms: number of double bonds followed by the position of the double bond from the aliphatic end of the molecular. The prefixes: i, a and cy indicate iso, anteiso and cyclopropyl containing fatty acids, respectively.

5.5 Cluster analyses

Fatty acid biomarkers can be interpreted three ways: by multivariate analyses, by the presence/absence of individual fatty acids (Boon *et al.* 1996), or by the analyses

of the chemical composition of the total microbial community as individuals that are then treated as a combination of chemical profiles (Osipov and Turova 1996). The first method classifies samples into groups based on overall similarity and the second allows an understanding of changes in the abundance of individual microbial taxa (Boon *et al.* 1996). The third method involves a higher level of analysis of a bacterial community. The marine environment is complicated by the presence of a wide range of algal and heterotrophic species and the method of Osipov and Turova (1996) requires less biological complexity. The method is only successful where high concentrations of bacterial species are present such as in activated sludge and soils. Only the first two methods were considered appropriate for this research.

Hierarchical average cluster analysis was used to group the isolated bacteria into different phenotypes based on their fatty acid profiles utilising JMP (Version 4; SAS Institute Inc.) software. The results were expressed as a dendogram. Variations in fatty acid and BCFA profiles for the 273 water samples collected over the season were also examined with this method of analysis, as well as principal components analysis (PCA). The results of hierarchical cluster analysis were expressed as a dendogram and the different clusters were attributed a colour. Each colour represents a change in the fatty acid profile.

The software used for PCA analysis was PCA CANOCO Version 3.12; ter Braak 1998 (1990). PCA is an ordination technique used to reduce the dimensionality of multivariate data sets and enable graphical presentation of the relationships between features (ter Braak and Šmilauer 1998). PCA assumes that species are linearly related to each other and to environmental gradients, and has been used in aquatic systems to simplify ecological data (Grimalt and Olive 1993, ter Braak 1987). PCA was chosen over canonical correspondence analysis (CCA) and detrended correspondence analysis (DCA) after data was tested and found not to be unimodal.

Thirty-three variables (fatty acids) were included in the analysis of 273 different sites and depths. Statistical analysis was also completed with ANOVA to test the statistical significance of the differences among the obtained means of samples

from the given dates and fatty acids. Two assumptions important to the use of ANOVA are that the data-sets are normally distributed and that there is no direct relationship between variances and the means (Underwood 1981). Data transformation methods were tested including concentration data, logarithmically transformed data, and ratios. The clearest results were observed after the entire dataset was autoscaled using the normalizing and averaging function. This method normalizes the data by subtracting each observation from the mean and dividing by the standard deviation. Normalisation was required because the concentration of individual compounds varied considerably between sample sites and in many cases the standard deviation was close to the mean for minor components. PCA was carried out on the normalized correlation matrices. It was necessary to discard (Grimalt and Olive 1993) one sample set (site F3 pycnocline, May 13) from the PCA as this sampling date was a major outlier. Two minor fatty acids were also removed (20:1 and 20:3). When tested their presence in the dataset had no effect as their proportions were between 0-1.5% of the total fatty acid composition.

Two methods were used to reduce the data set. First the database was split by dividing the variables into their compound subgroups (e.g. integrated surface and pycnocline depths). PCA was then carried out separately on each group. Integrated samples were analysed together and independently from the surface and pycnocline for the PCA, as the integrated samples contained a small component of the surface and pycnocline samples.

Results

5.6 Fatty acid composition of cultured Huon Estuary isolates

The fatty acid compositions of 30 Huon Estuary bacterial isolates were analysed to identify the typical profiles for culturable bacteria from the estuary (Table 5.1). The profiles showed distinct variation between isolates. The major fatty acids were similar for all species and included: 15:0, i15:0, a15:0 and br15:1. Hydroxy fatty

Table 5.1 Total fatty acid composition (%) of culturable bacterial isolates from the Huon Estuary.

ACEM number	30	26	12	29	24†	13†	4†	17	33	16	11	23†	19†	34	15	14†	18†	28†	1†	5†	31†	3†	8	22	7	9	6†	27	21†	25†	20†	32†	Mix1†	Mix2†
12:1							2.8									5.4	3.2	1	0.8	1.7	1.3	5.5						0.2						0.4
12:0							0.8								7.8	1.6		2.6	0.8	0.8	3.1	2.6	0.5		3.4	3.9	3.6	0.1				1.2	2.8	3.3
βOH 12:0							2.2												2.7			0.4											0.4
i13:0																	0.6	0.7	1.3			2.4				9.6	9.1	0.6	0.8		0.9	11.5		
i14:0	1.1	2.5	4.2				2.4	18.2	22.3	4.3	2.4	0.8	8.0	1.0	7.5				0.1		2.6	2.4	4.8	7.5	3.9		1.3	0.8	1.2	1.0	0.9	6.0		1.9
14:0	0.7					6.2				0.8			1 0				1.1	1.3	1.4	2.1	1.2	0.9	0.9		0.6	5.5	5.2	0.2	2.8	0.5	3.3	3.8	0.7	0.7
i15:1	5.4		2.4		13.0					2.4		5.3		5.6											0.7			32.0					2.6	1.0
a15:1																												38.9	22.5	7.1	13.6			
i15:0	14.0				14.3			46.5	37.8	75.3	78.3	27.6	27.9	59.4	43.5	59.3		4.8	2.7	0.7			25.3	7.1	26.4	17.2	14.1	4.2	25.4	16.7	17.3	15.0	3.7	2.4
a15:0																			0.8	2.6			37.4	38.0	27.4		1.8		2.0		12.2	5.5		
15:1	46.0	72.3	76.3		5.8		1.7										2.8				5.0	4.8				1.6				3.6	1.5			
15:0	1.0		1.2		13.2	2.0	1.9		1.8	1.5		1.6	1.1	1.4	1.9		1.9	4.3	5.6	3.6	4.3	5.4	2.5	4.0	2.5	2.7	4.5	0.1	11.6	15.4	11.7	1.2	1.6	1.3
br 16:1									16.9															8.8						1.7		7.6		
i16:0	10.1				1.6		0.1	19.2			2.3	6.9	9.8		13 5	1.2		0.8	0.1				9.7	5.0	8.3		0.4	1.2	0.6		0.8	3.0		
16:1		4.1	8.3		1.8			8.6	15.0	8.2	3.0	7.6	16.5	10.8	9.7	2.1				0.9		1.0	2.3					6.4		6.5				
16:1n-7		2.3		1.6	8.1	20.0	43.8	7.5	1.6		1.5	35.5	9.2		2.7		56.7	44.8	22.1	41.9	42.5	31.9	2.4	3.4	6.6	23.3	15.2	1.4	12.6	6.1	21.8	17.2	25.9	25.0
16:0	2.2	2.8	0.8	1.4	4.0	23.1	14.0			0.8	0.9	2.0	9.4	2.3			6.0	10.6	12.7	16.2	10.1	9.3	0.4	1.3	0.6	14.3	12.1	4.8	8.8	6.9	4.4	3.1	17.4	17.0
βOH i15:0					17.0							4.9	2.0																1.9	11.5	2.4			
βOH 15:0			1.3				6.5											1.0		4.6		0.1	2.0	1.0										
i17:1							0.2			0.9	2.2		3.4		2.3	6.3							7.7	1.0	4.0			0.3		5.0	0.6	8.5	2.9	0.4
a17:1											1.5				6.2									1.5	11.6							1.1		2.4
i17:0	3.2	7.7	5.5		2.3	2.7	0.1		4.7		3.1		0.8	3.0		1.1					12.9		0.5	1.6	4.1		0.8	1.5		1.5		3.4	0.5	4.2
a17:0		8.4									4.8		4.6	16.6		21.8							3.6	13.2						5.0		1.9		
17:1	16.4				4.4	1.4	0.4			5.7		1.6	3.9		4.7		11.2	13.7	19.9	7.5	1.6	16.9				3.4	4.8		0.4				5.5	5.1
17:0					2.0	1.1	1.9										0.9	3.0	8.8	4.4	3.1	4.0		4.9		1.9	2.8	0.9				0.1	1.5	1.8
βOH i17:0					8.3																								3.2	9.9	1.5		2.8	2.2
βOH 17:0				4.2	0.5	24.6																							0.4				2.2	2.5
18:1				91.3		17.1	10.0										8.6	6.1	6.8	7.0	6.2	4.0		0.9		5.7	4.6	0.3					22.9	23.4
18:0				1.6			1.1											0.5	2.4			0.3		0.6			0.6	4.9				1.4	1.5
20:5n-3																										10.9	14.6							
Sum	100	100	100	100	96	98	90	100	100	100	100	94	98	100	100	99	93	95	89	94	94	92	100	100	100	100	95	99	94	98	93	90	94	97
Total BCFA	47	3	7	0	33	1	3	84	77	89	87	42	53	66	78	67	12	20	25	11	4	22	85	69	82	30	32	78	53	32	46	58	15	13

† **Also includes the following fatty acids (if less than 1%, percentage is not shown):** ACEM 24: 16·1, βOH a17 0 ACEM 13: 18·1 ACEM 4: βOH i12 0 (5 0%), βOH 10 0, 13·1, 13 0, βOH 11 0, βOH 13 0, 18 1, βOH 16 0 ACEM 23: 16:1 (6 2%) ACEM 19: 16·1, (1 3%), βOH 16·0 (1 1%) ACEM 14: βOH 10 0 (1 2%) ACEM 18: 11 0 (2 1%), βOH 10 0 (2 1%), βOH 11.0 (1 7%), 14·1 (1.1%). ACEM 1: 11 0, βOH 10 0, 13 0, βOH 11 0 (2 9%), βOH i12 0 2 1%, βOH 13 0, 17 1 (1 5%), i18 0, 18 1, 19 1 or cy19 0 ACEM 28: βOH 10 1 (1 1%), 13 0, βOH 11 0 (1 8%) ACEM 31· i12:0 (2%), br13 1 (1 8%), 13 0 (1 1%), 14:1 (1.1%). ACEM 3: i12 1, i12 0,12 1 x3, βOH 10 0 (2 8%), 13.0 (1 3%), 14 1, βOH 17 0 ACEM 5: 11 0 (1 5%), βOH 10 0 (1 9%), βOH 11 0, 14 1, βOH 13 0 ACEM 21: 13 0, βOH i16 0(3 2%), βOH 16 0 (2 5%) ACEM 27: br11 1, br12.1, 18·1 ACEM 22: i18·0 (1 1%) ACEM 20: βOH i14 0 (3 3%), βOH i16 0 (2 3%) βOH 16 0, ACEM 25: i12 0, ACEM 32: i12 0 (2 1%), a12·0 (1 6%), 13·0, 16.2, br16 1x2 (2 2 and 3 0%) ACEM 6: i12 0, i13 0, C_{18} PUFA, C_{20} PUFA, 22·6n-3. Mix1: br14·1 (1 2%), 17·1 (2.6%) Mix 2: 16:2, 14·1 (1 6%)

acids were also observed in many isolates. BCFA accounted for an average of 42 % (std. dev. 5) of the total fatty acids. The highest proportion of BCFA for an isolate was 89% (Table 5.1).

Combined phylogenetic and phenotypic analyses of the six algicidal and four other bacterial isolates as well as the use of FISH oligonucleotides for bacterial classes and genera (chapter 7) demonstrated that the 30 isolates chosen for fatty acid analyses were from a broad and representative range of commonly found marine and estuarine bacteria. They included gram-positive and gram-negative cocci and rods, and members of the CFB and proteobacteria clusters. Six of the cultured isolates were identified using 16S rDNA sequence analysis as new species (chapter 3 and unpublished data for ACEM 24 and ACEM 25).

Hierarchical cluster analysis was used to group the 30 bacterial isolates based on similarities of their total fatty acid profiles (Figure 5.1). The bacterial isolates form groups similarly to phylogenetic clusters based on 16S rRNA sequences. Based on fatty acid profiles, the two algicidal *Pseudoalteromonas*, the two *Shewanella* and the two *Cellulophaga* isolates clustered closely to each other (Figure 5.1). The *Planococcus* species clustered with other gram-positive bacteria. The majority of bacteria isolated from the estuary were from the CFB and proteobacteria clusters. The CFB cluster accounted for 50 % of the total isolates analysed. Two main groups of this cluster were observed with separation occurring because of the very different fatty acid profiles (Figure 5.1).

The fatty acid profiles of ACEM 16 and ACEM 34 are similar to those of the CFB cluster. This result is consistent with the FISH results. High proportions of i15:0 are often associated with gliding species within the CFB and high proportions of *anteiso*-C_{17} fatty acids are common in genera related to *Flavobacterium*. Unusually, both isolates contain over 75% i15:0, a very high proportion for any genus in the CFB or gram-positive cluster. Lower proportions of this fatty acid are generally observed for these clusters (8-35% O'Leary and Wilkinson 1988, Bowman *et al.* 1998b, 20-55% Wilkinson 1988)

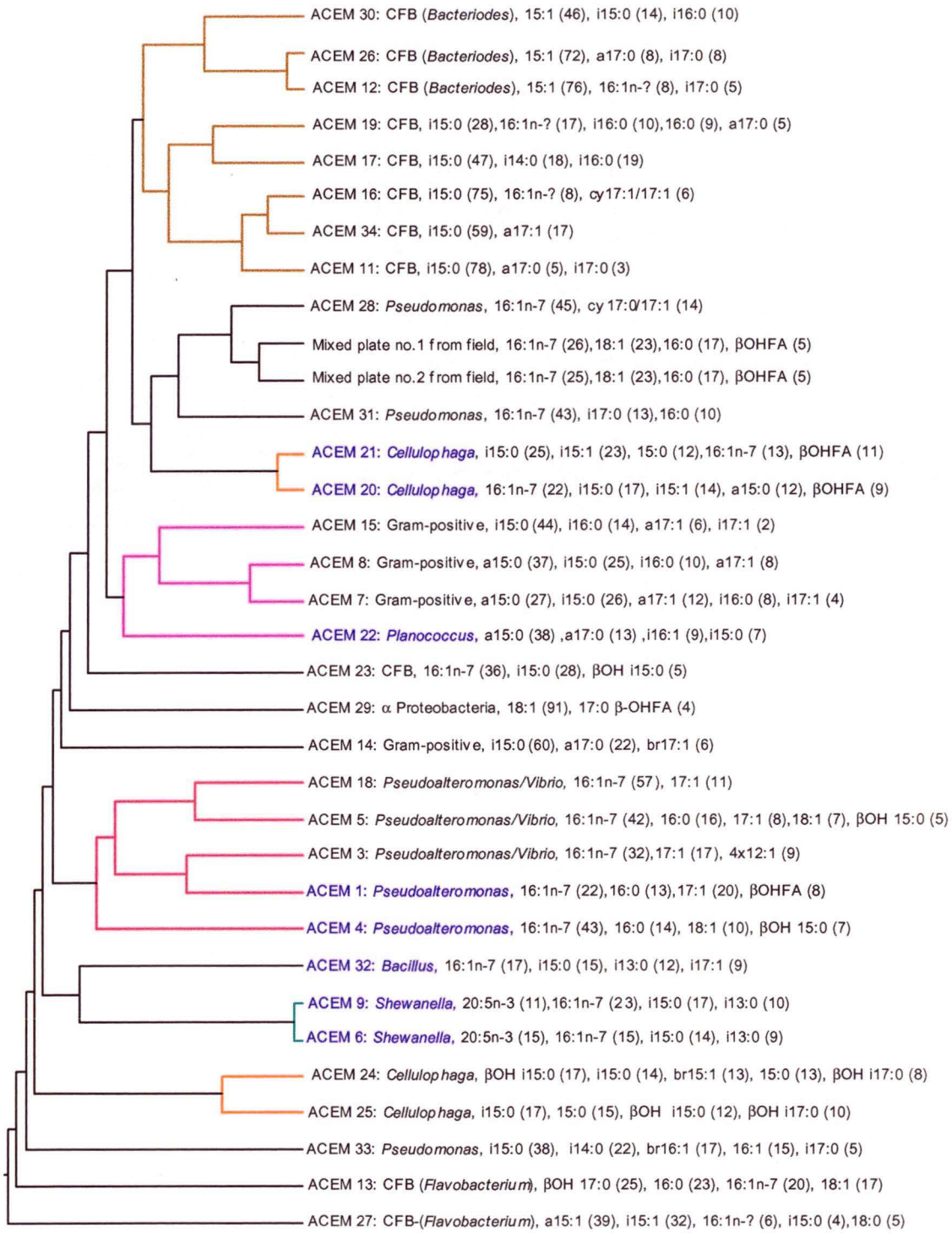


Figure 5.1 Bacteria isolated from Huon Estuary clustered according to fatty acid profiles. The major fatty acids and relative level (% of total) are shown for each species. Bacterial genera were identified either phylogenetically or with the use of fluorescent in situ hybridization oligonucleotides and phenotypically. Algicidal and *Shewanella* isolates are in blue. If a number of βOH make up a significant proportion of the total fatty acids they are referred to as βOHFA. 16:1n-? indicates a significant 16:1 fatty acid that is not 16:1n-7 or 16:1n-5.

The pink ACEM 29 isolate contained a very high proportion of 18:1 (91%) and was identified (phenotypically and through the use of FISH) as an α proteobacteria species. It was the only α proteobacteria isolated.

Fatty acid analyses of the combined colonies from the two duplicate field seawater samples (Mixed plate number 1 and 2 in Figure 5.1) showed lower levels of BCFA and beta hydroxy fatty acids (β-OHFA). The profiles were more closely related to genera such as *Pseudomonas* and *Pseudoalteromonas* which are often reported as being over-represented in plate counts. Few other species possessed the same low relative levels of BCFA or hydroxy fatty acids as observed for the *Pseudoalteromonas* species in this study. The dominance of the genera *Pseudoalteromonas* and *Pseudomonas* in the mixed plate fatty acid profile was because they readily grow on solid media and form larger colonies than many other species. Their large colony size overwhelmed other bacterial colonies. However, other genera were present in large numbers in field samples and were easily cultured. Based on fatty acid analysis, only 7 *Pseudoalteromonas* or *Pseudomonas* species were identified from the thirty species isolated. These findings were supported by sequence analysis and FISH using class specific probes (chapters 3 and 7).

5.7 Lipid class composition of field samples:

Lipid class analysis of plankton net samples (0-12 m depth) over the season examines the composition of all organisms greater than 20 μm. These samples would include a small proportion of microorganisms, such as bacteria, attached to larger particles. Variation in lipid class composition was similar for site F1 and site X3 (Figures 5.2).

Throughout the season the major lipids were polar lipids. These are cell membrane components and included glycolipid, phospholipid and chlorophyll. Polar lipid

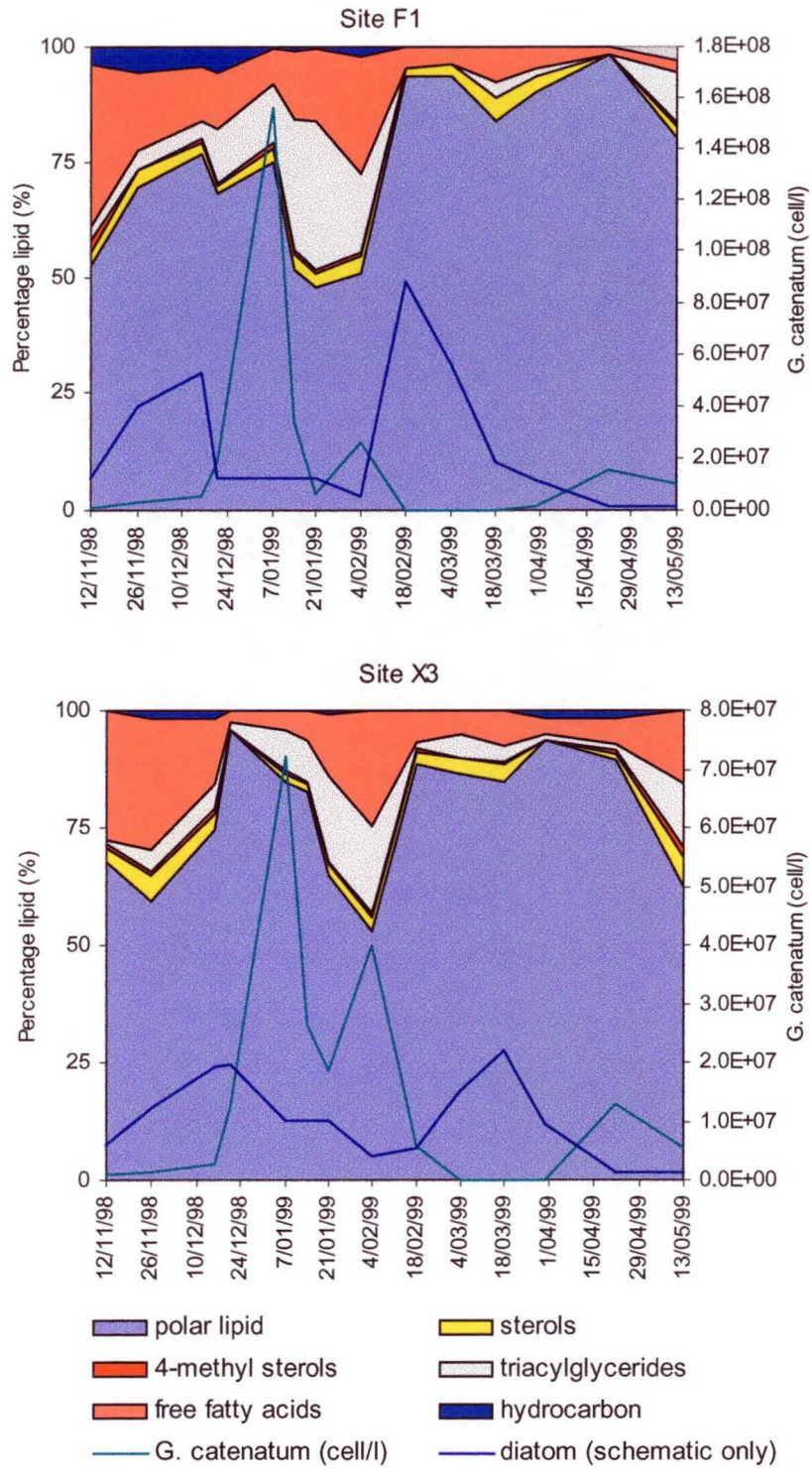


Figure 5.2 Variation in lipid class composition for particulates >20 μm (0-12 m depth) collected from the Huon River water column at sites F1 and X3 during 1999-2000. 4–methyl sterols are derived predominantly from dinoflagellates

content of samples remained high during the initial stages of algal blooms as algal cell abundance increased. Energy demands of phytoplankton cells in growth phase are high so during this period triacylglycerol (TG) levels were low as storage lipids were rapidly utilised.

Before the summer *G. catenatum* bloom, there were very high proportions (30-35%) of FFA (Figure 5.2). During the initial stages of this bloom, both the storage lipid (TG) and free fatty acids (FFA) decreased while membrane-derived components increased. An increase in TG was first observed at the peak of the summer *G. catenatum* bloom. The increase in TG then intensified at both sites until the end of the bloom. During the autumn *G. catenatum* bloom, the proportion of TG was lower than for the summer *G. catenatum* bloom. FFA were at relatively high levels throughout early summer and early autumn.

Sterols were present at higher proportions during periods of diatom and dinoflagellate blooms. The lipid class labelled 4-methyl-sterols (Figure 5.2) has been identified as indicators for many dinoflagellate species (Volkman *et al.* 1998). They were present at higher proportions during both blooms of *G. catenatum.*

Highest proportions of hydrocarbon were present during late spring and early summer (Figure 5.2). Their occurrence corresponded to the presence of the first small summer diatom/flagellate bloom. Hydrocarbons detected in samples from this study could be derived from phytoplankton or may be caused by oil contamination, (e.g. from the boats used for sampling). No unresolved complex mixture (UCM) hump (typically found in crude oil and diesel) was present in the GC traces hence the hydrocarbons present in the sample were not of petroleum origin. In addition, when the highest proportions of hydrocarbons occurred, algal species such as *Ceratium* and *Chaetoceros* were present (chapter 2, section 5.10).

5.8 Fatty acid composition of field samples

5.8.1 Total fatty acids

The three sites sampled over the field season showed variations in fatty acid content and composition in response to changes in the abundance and proportions of diatom, dinoflagellate or bacterial species present (Figure 5.3). Throughout the season, integrated water samples always contained the lowest TFA concentration (Figure 5.3) and the highest proportions of SFA (Figures 5.4 and 5.5) for all sites when compared with either the surface or pycnocline samples. SFA levels in integrated water samples were 5 to 20 % higher than other depths for all three sites (Figure 5.5).

During the summer *G. catenatum* bloom, PUFA proportions were elevated at all sites and were similar to values previously observed for blooms of this species (this study, 33 to 37 %, Figure 5.5: Hallegraeff *et al.* (1991) 27-41 %). The highest PUFA proportions occurred on Dec 21 for sites F1 and X3 surface samples and Jan 7 and Jan 14 for the pycnocline samples (Figure 5.5). These coincided with the summer bloom of *G. catenatum* (chapter 2).

Blooms of *G. catenatum* have a very high biomass, as their cell size is an order of magnitude larger than other microalgae (chapter 2). Thus, they contribute a large proportion of lipid to the water column. During bloom periods, fatty acids from *G. catenatum* would therefore exceed those of other microbial species present in the water column. It is possible under these conditions to estimate cell numbers of *G. catenatum* using fatty acid concentrations. Algal cell numbers for the integrated sample at site X3 during the *G. catenatum* bloom were highest on Jan 8 at 83 cells/ml (integrated sample, Figure 2.7, chapter 2). *G. catenatum* was essentially a mono-species bloom for this sample at this site. The TFA concentration was 89 µg/l which results in a concentration of 1070 pg/cell TFA. This assumes that all TFA originated from *G. catenatum* cells.

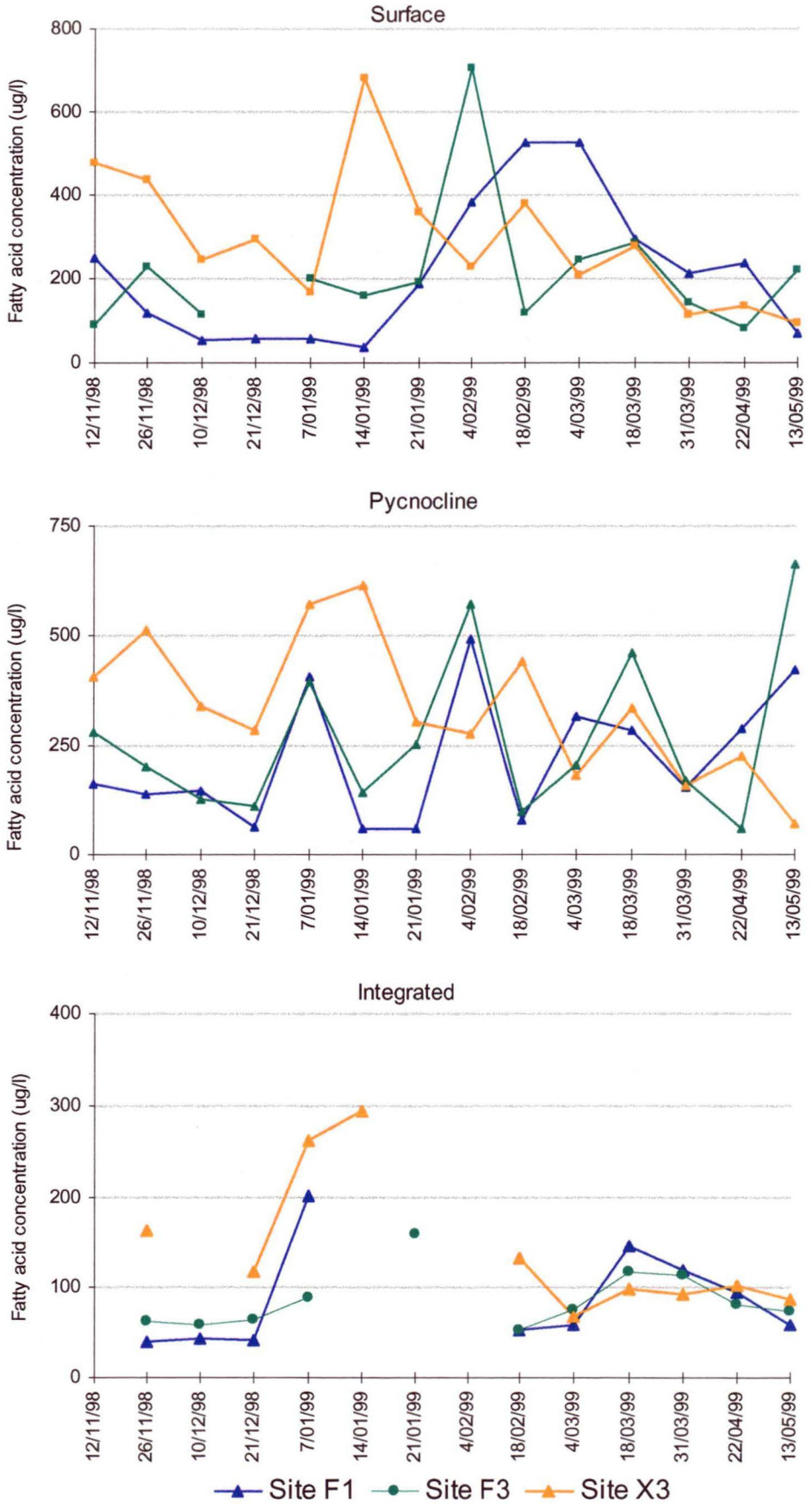


Figure 5.3 Variation in total fatty acid concentration of particulate matter (> 0.7 µm) in the Huon Estuary water column at 3 sites and 3 depths in 1998-99.

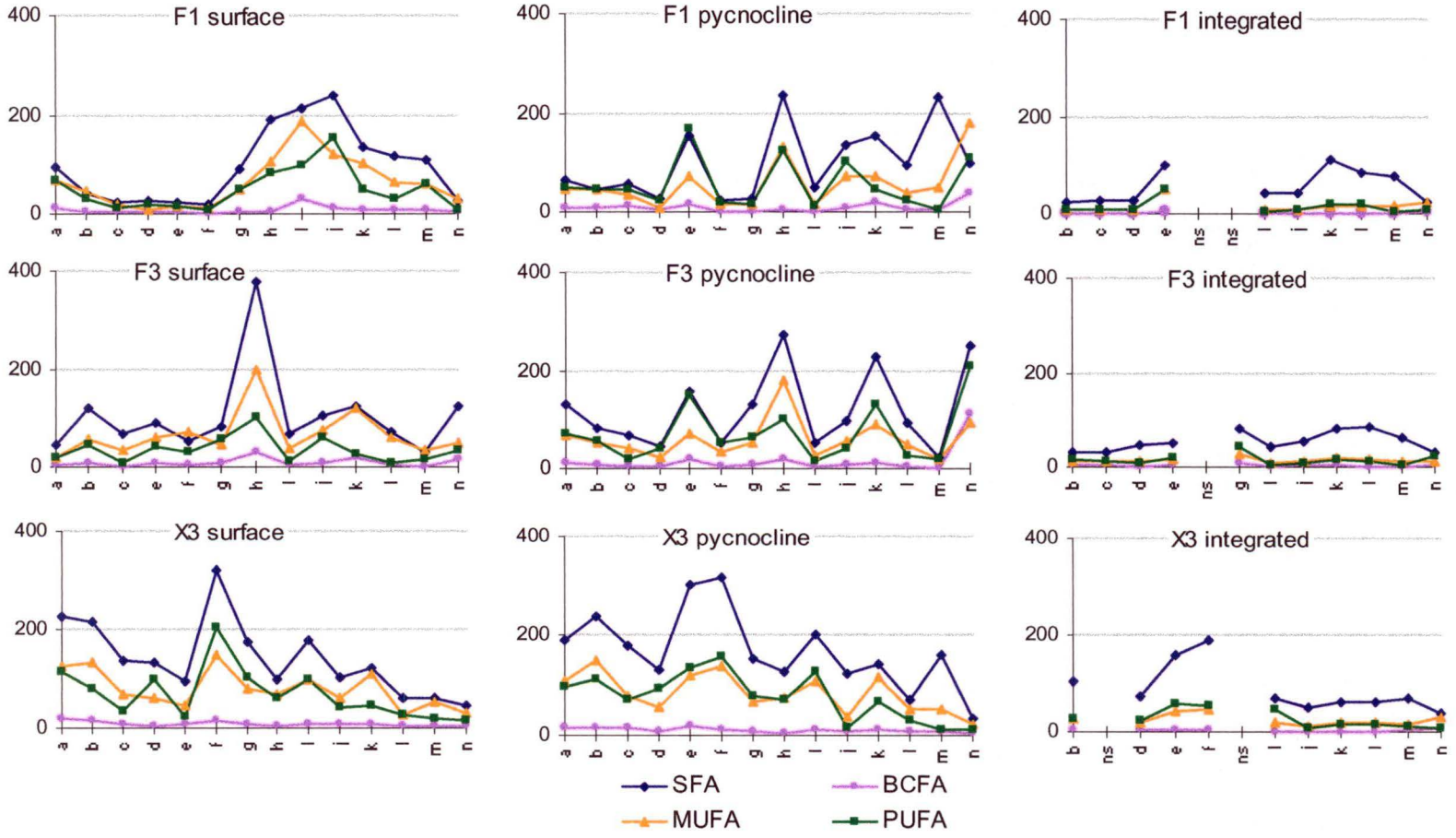


Figure 5.4 Variation of concentrations of SFA, MUFA, BCFA and PUFA in the Huon River at 3 sampling sites and 3 depths during 1998-99. Y axis: µg/l, X axis: Sampling dates (a:12/11/98, b:26/11/98, c:10/12/98, d:21/12/98, e:7/01/99, f:14/01/99, g:21/01/99, h:4/02/99, i:18/02/99, j:4/03/99, k:18/03/99, l:31/03/99, m:22/04/99, n:13/05/99, ns: not sampled).

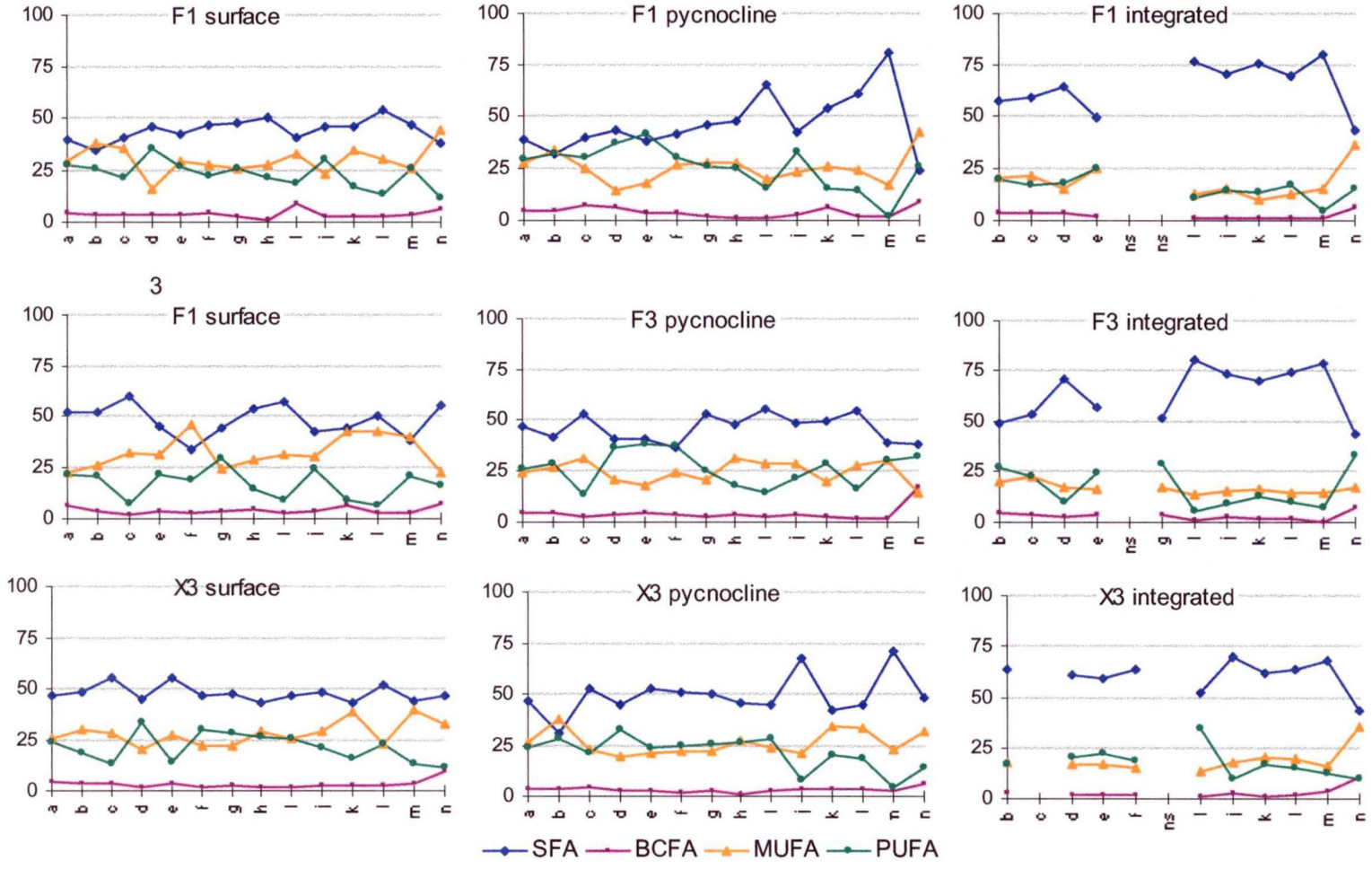


Figure 5.5 Variation in percentage of SFA, BCFA, MUFA and PUFA in the Huon River at 3 sampling sites and 3 depths during 1998-99. Y axis %, X axis: Sampling dates (a:12/11/98, b:26/11/98, c:10/12/98, d:21/12/98, e:7/01/99, f:14/01/99, g:21/01/99, h:4/02/99, i:18/02/99, j:4/03/99, k:18/03/99, l:31/03/99, m:22/04/99, n:13/05/99, ns: not sampled)

The autumn *G. catenatum* bloom contained 158 cells/ml (site F3 integrated sample, Figure 2.7, chapter 2) and 80 µg/l TFA. Assuming the same relationship, this was consistent with 506 pg/cell TFA. These estimated cellular TFA concentrations are in agreement with literature values for pure cultures of *G. catenatum* (536 cells/ml (log phase) contained 431 µg/l TFA giving 804 pg/cell TFA, Hallegraeff *et al.* 1991). The pure cultures demonstrated less variability than observed for this field study (cultures: 426 pg/cell (log), 734 and 602 pg/cell (both early stationary)). However, the estimated cell numbers for field samples containing *G. catenatum* numbers as determined in this study were within these ranges.

After the summer *G. catenatum* bloom, the water column was dominated by the diatom *Pseudonitzschia* spp. (predominantly *P. pseudodelicatissima*) (Jan 21 to Mar 18). During the *Pseudonitzschia* spp. bloom, the concentration and proportions of PUFA again increased (Figures 5.4 and 5.5). Surface samples at sites F3 and F1 and pycnocline samples at site F1 contained the highest proportions and concentrations of PUFA on Mar 4. On the next sampling date (Mar 18), the pycnocline samples for sites F3 and X3 demonstrated a similar increase in fatty acids. This may have been caused by the movement or bloom of this species to lower in the water column. High proportions and concentrations of PUFA were observed at site X3 on Feb 18 indicating that the *Pseudonitzschia* spp. bloom occurred earlier at this site. The bloom on Feb 18 occurred throughout the water column at site X3. The bloom decreased by Mar 4, and lower PUFA proportions were evident.

At the beginning of the autumn *G. catenatum* bloom (Apr 22), PUFA concentrations and proportions were very low for sites X3 and F1 and high proportions and concentrations of SFA were observed (Figures 5.4 and 5.5). Conversely, high PUFA proportions (30%) were observed at site F3 where the *G. catenatum* bloom dominated. Despite low PUFA proportions on Apr 22, the pycnocline sample at site F1 contained the highest TFA concentration of the season (Figure 5.3) as it was dominated by high concentrations of SFA (Figure 5.4). Site X3 samples contained lower SFA concentrations and on May 13, PUFA increased corresponding with the occurrence of the autumn *G. catenatum* bloom. Overall,

the pycnocline samples contained the highest proportions and concentrations of PUFA for all sites and depths.

Branched chain fatty acid (BCFA) concentrations were highest during or after diatom or dinoflagellate blooms and varied with depth (Figure 5.4 and 5.5 and section 5.8.4). The highest proportions and concentrations of BCFA were at the pycnocline and to a lesser extent at the surface. The greatest concentrations of BCFA therefore corresponded to the areas of highest chlorophyll *a* (chapter 2). The proportions and concentrations of BCFA for the integrated samples were generally lower than at the other two depths for all three sites for most of the year (Figures 5.4 and 5.5). During the autumn *G. catenatum* bloom (May 13), BCFA concentrations were particularly high for the pycnocline sample at sites F3 and F1. However, all sites and depths contained high proportions of BCFA. Site F3 on May 13 contained the highest proportion of BCFA (17%- section 5.8.4) and the autumn *G. catenatum* was observed to be in decline. Overall, site X3 had the lowest concentration and relative levels of BCFA throughout the season and for all depths. This site also contained the highest concentrations of PUFA for all sites and depths.

The total fatty acid profiles indicate that the estuary was relatively homogeneous across the three sites. Differences related to variations in depth and salinity rather than between sites. The fatty acid groups are presented in the following sections separated into their individual components: PUFA (section 5.8.2), MUFA (section 5.8.3) and BCFA (section 5.8.4).

5.8.2 Variation of PUFA in relation to algal bloom

High proportions of 20:5(n-3), 14:0 and a high 16:1 to 16:0 ratio (>1) indicate the presence of diatom species while a low 16:1/16:0 ratio and high 22:6(n-3) indicates the presence of dinoflagellates and other flagellates (Figure 5.6 and 5.8). An elevated 18:5(n-3)/18:3(n-3) ratio has also been used as an indicator for dinoflagellates (Viso and Marty 1993, Nichols *et al.* 1984) (Figure 5.7). Site X3 consistently had a higher 16:1/16:0 ratio indicating the dominance of diatom

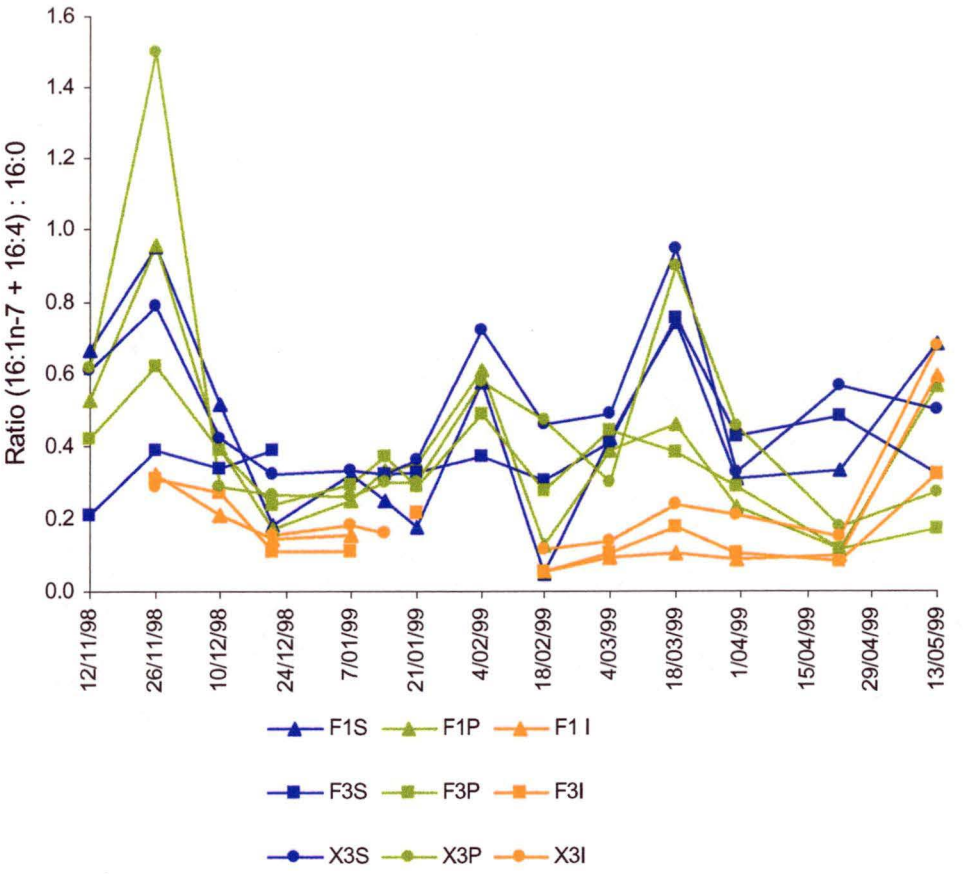


Figure 5.6 Variation of the 16:1 to 16:0 ratio over the sampling season indicating the change from diatom influence (high ratio) to dinoflagellate influence (low ratio)

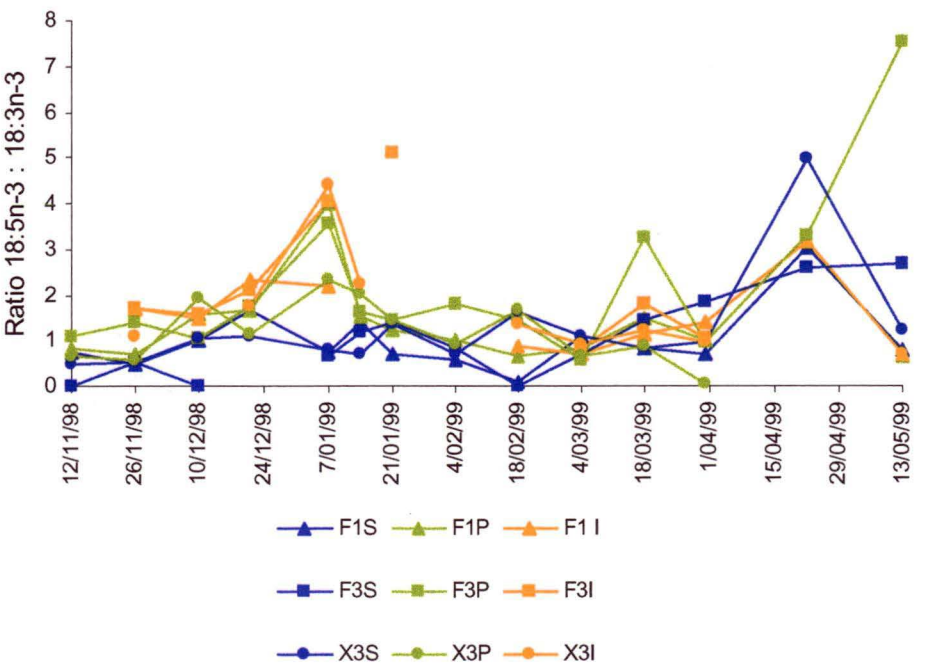


Figure 5.7 Variation of the 18:5n-3:18:3n-3 ratio over the sampling season indicating the change from dinoflagellate influence (high ratio) to diatom influence (low ratio).

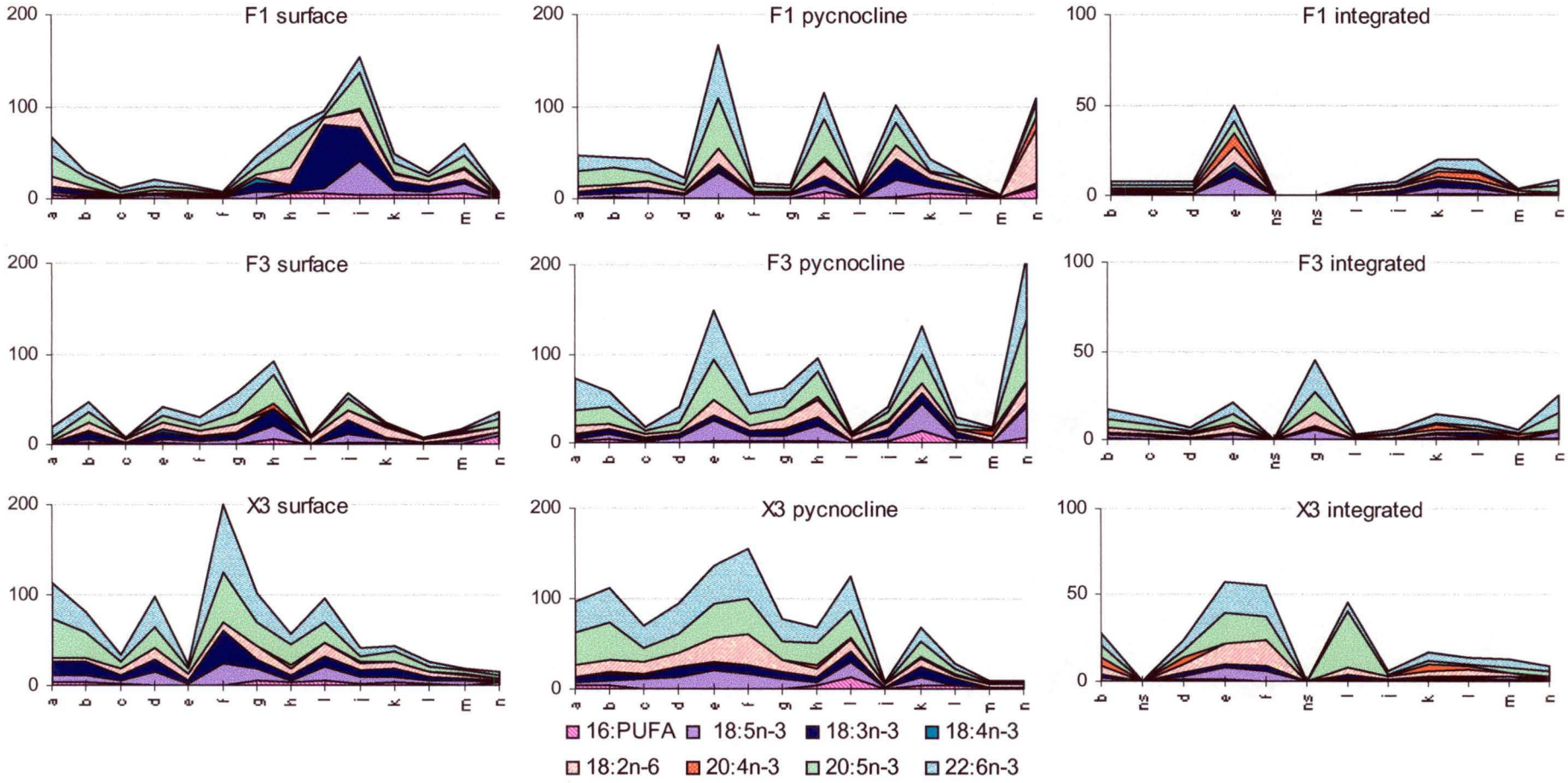


Figure 5.8 Variation of PUFA concentration in the Huon River at 3 sampling sites and 3 depths during 1998-99. Y axis: µg/l, X axis: Sampling dates (a:12/11/98, b:26/11/98, c:10/12/98, d:21/12/98, e:7/01/99, f:14/01/99, g:21/01/99, h:4/02/99, i:18/02/99, j:4/03/99, k:18/03/99, l:31/03/99, m:22/04/99, n:13/05/99, ns: not sampled).

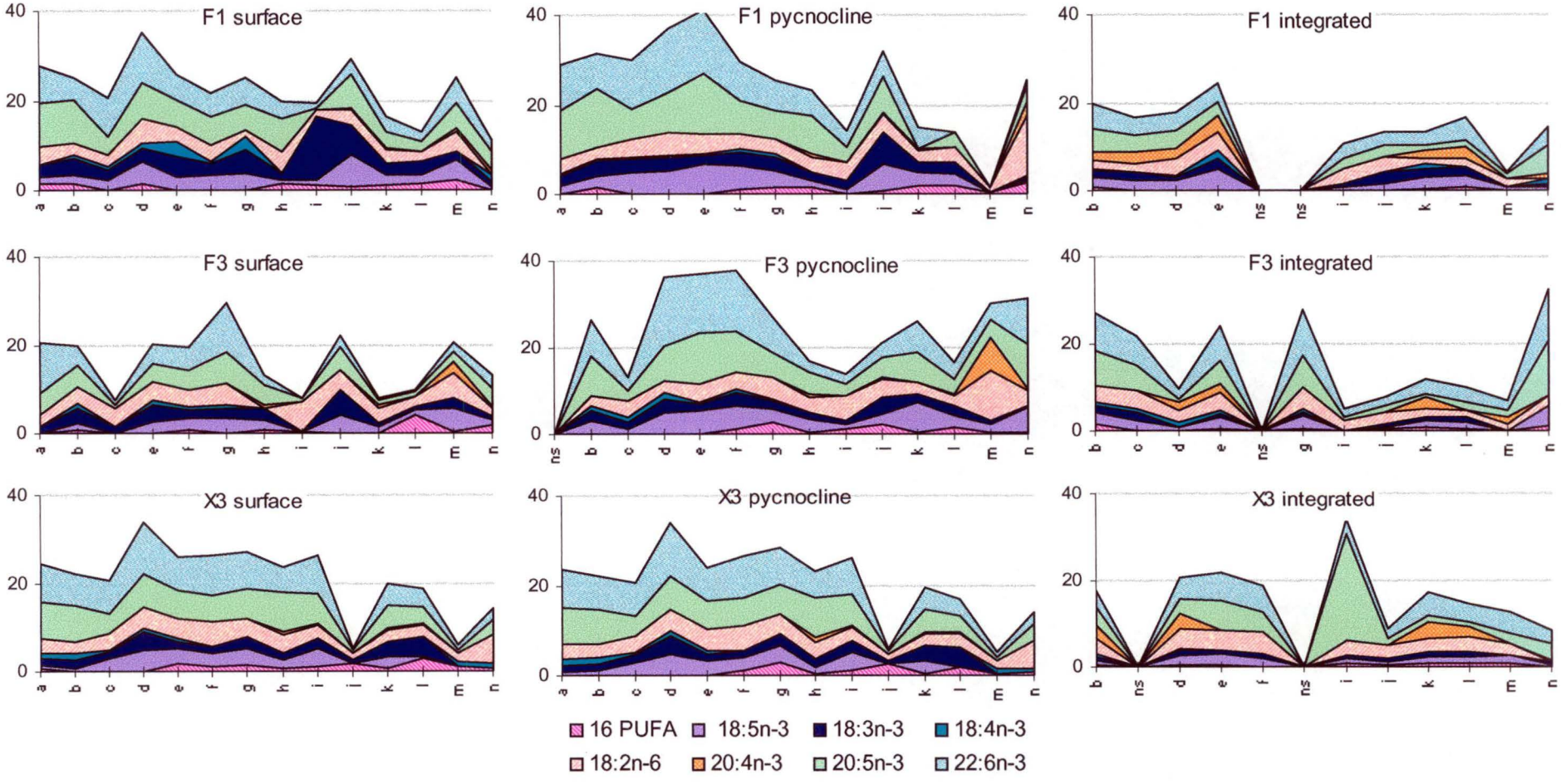


Figure 5.9 Variation in PUFA percentage in the Huon River at 3 sampling sites and 3 depths during 1998-99. Y axis: %, X axis: Sampling dates (a:12/11/98, b:26/11/98, c:10/12/98, d:21/12/98, e:7/01/99, f:14/01/99, g:21/01/99, h:4/02/99, i:18/02/99, j:4/03/99, k:18/03/99, l:31/03/99, m:22/04/99, n:13/05/99, ns: not sampled)

species (Figure 5.6). Sites F1 and F3 had higher levels of 18:5(n-3)/18:3(n-3) indicating a greater proportion of dinoflagellate species (Figure 5.7).

G. catenatum occurred at highest concentrations in the pycnocline sample for all sites and during all algal blooms (chapter 2). The summer *G. catenatum* bloom occurred at the pycnocline at all sites and showed high proportions of 22:6(n-3) (Figure 5.8). Relatively high levels of 20:5(n-3) (Figure 5.8) were also present at proportions similar to those shown for pure cultures of *G. catenatum* by Hallegraeff *et al.* (1991). The ratio of 22:6(n-3) to 20:5(n-3) for cultured *G. catenatum* is generally above 1.5. This ratio occurred during the peak of both *G. catenatum* blooms. Higher proportions of another PUFA produced by *G. catenatum,* 18:2(n-6) (3-5 % Hallegraeff *et al.* 1991), were also observed during both *G. catenatum* blooms (Figure 5.8). This marker occurred to a lesser extent throughout the year and during the *Pseudonitzschia* spp. bloom. During the autumn *G. catenatum* bloom the proportions and concentrations of 22:6(n-3) and 20:5(n-3) were lower than throughout the summer bloom at most depths except the pycnocline and integrated samples at site F3 (Figures 5.8 and 5.9). However, 18:2(n-6) was higher in the pycnocline samples than during the summer *G. catenatum* bloom.

The integrated samples for all sites had lower proportions and concentrations of PUFA when compared to the other depths. However, the integrated samples contained higher proportions and concentrations of 20:4 for all sites than either the surface or pycnocline samples. The integrated sample at site X3 contained a high level of 20:5(n-3) during the *Pseudonitzschia* spp. bloom on Feb 18. All other sites also demonstrated an increase in PUFA around this period. Although the bloom of *Pseudonitzschia* spp. occurred on Feb 18, the surface samples at sites F1 and F3 contained only C_{18} PUFA. These fatty acids are not found in *Pseudonitzschia* spp.. This phenomenon re-occurred at the next sampling date (Mar 4) for the pycnocline and surface samples at site X3. However, Mar 4 samples demonstrated an increase in C_{16} and C_{20} PUFA indicating the presence of *Pseudonitzschia* spp.. Concentrations of C_{18} PUFA for Feb 18 were also high. Large numbers of *Pseudonitzschia* spp. (predominantly *P. pseudodelicatissima*)

were present on Feb 18 (3 x 10^5 cells/l; Parker 2001), while unidentified flagellates and heterotrophic species such as tintinnids, rotifers, copepods and ciliates were noted in the sample.

5.8.3 Variation of MUFA

Over the season, the dominant MUFA was 16:1(n-7) (Figure 5.10), which is a common fatty acid present in many marine microorganisms. However, during the *Pseudonitzschia* spp. bloom, surface samples at sites F1 and F3 contained 18:1(n-7) at high concentrations. High proportions of 18:1(n-9) were observed during the autumn *G. catenatum* bloom. This fatty acid can be used as a marker indicative of bacteria or the presence of heterotrophic species (Wakeham 1995).

Species such as *Pseudoalteromonas* and *Vibrio* do not contain BCFA, but do contain fatty acids such as 18:1(n-9). High concentrations of 18:1(n-9) were observed at site X3 throughout the year. Vaccenic acid (18:1(n-7)) has also been used as a bacterial signature (Derieux *et al.* 1998). This fatty acid is common for γ proteobacteria, and dominated at sites F1 and F3 in the surface and pycnocline samples during the transition between the summer *G. catenatum* bloom and the *Pseudonitzschia* spp. bloom.

The less common C_{16} MUFA 16:1(n-5)c, has been used as a marker for some marine gliding species in the CFB cluster although the genus *Cellulophaga* only has low proportions of this fatty acid. Concentrations of 16:1(n-5)c were highest on Feb 18 and Mar 18 in the site F1 surface sample where concentrations of *Pseudonitzschia* spp. were highest (Figure 5.11). Although levels of other BCFA typical of the CFB cluster were high, the fatty acid 16:1(n-5)c was not high during either *G. catenatum* blooms or the first diatom bloom. These results suggest a different genus from the CFB cluster such as *Cellulophaga* were present in the microbial community during these blooms.

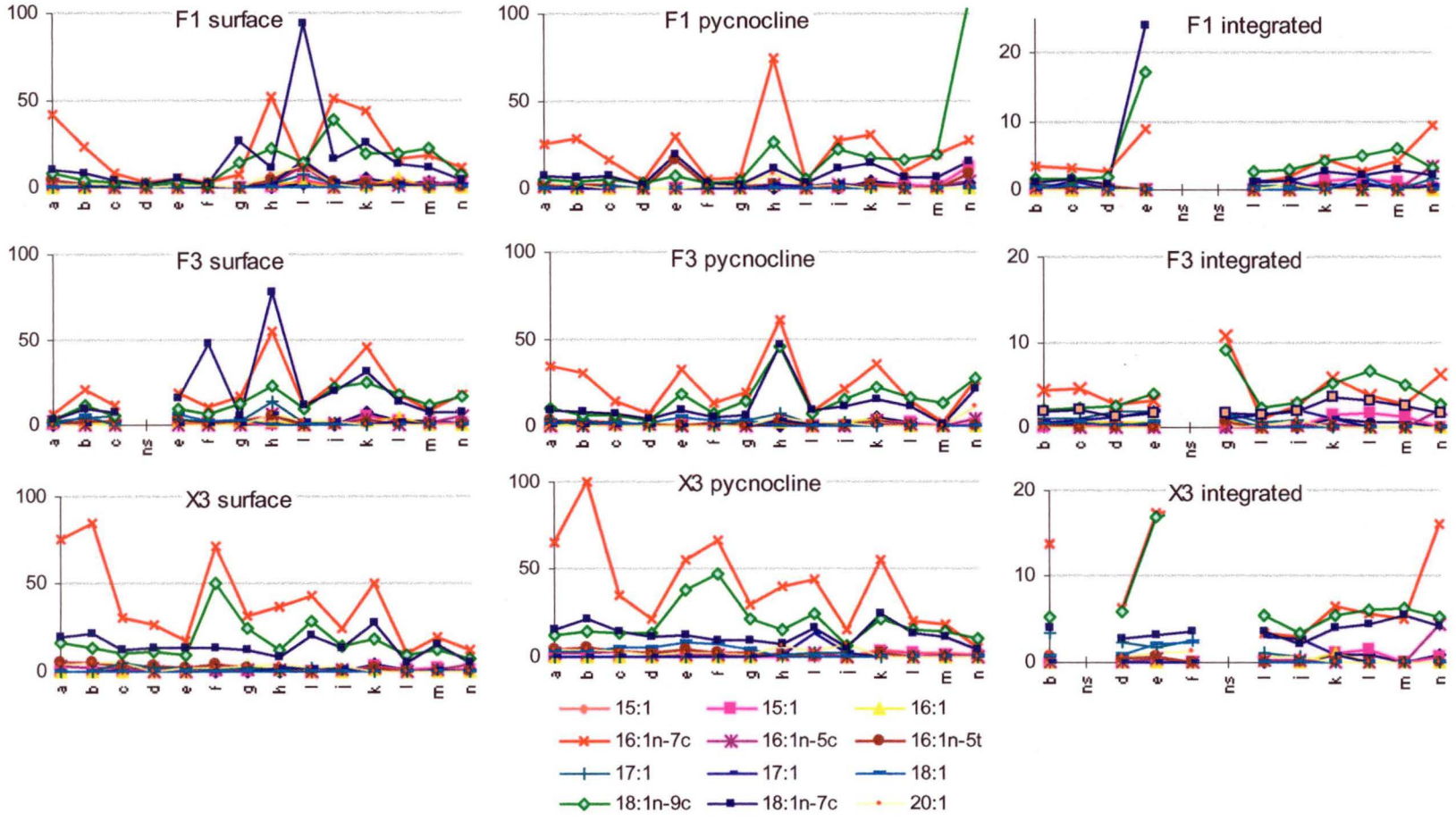


Figure 5.10 Variation in monounsaturated fatty acid (MUFA) concentrations in the Huon River at 3 sampling sites and 3 depths during 1998-99. Y axis: µg/l, X axis: Sampling dates (a:12/11/98, b:26/11/98, c:10/12/98, d:21/12/98, e:7/01/99, f:14/01/99, g:21/01/99, h:4/02/99, i:18/02/99, j:4/03/99, k:18/03/99, l:31/03/99, m:22/04/99, n:13/05/99, ns: not sampled).

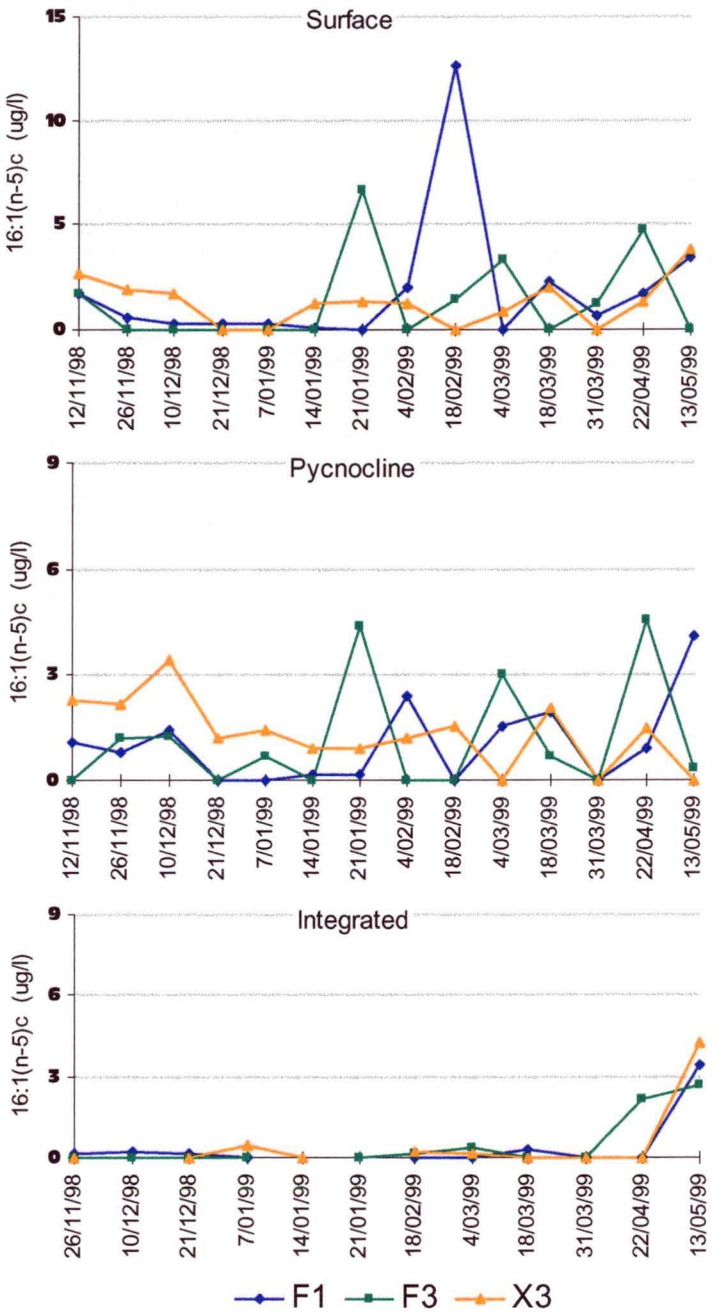


Figure 5.11 Variation in concentrations of 16:1(n-5)c in the Huon River at 3 sampling sites and 3 depths during 1998-99. Y axis: µg/l, X axis: Sampling dates

5.8.4 Variation of BCFA

Changes in BCFA over the season and between depths reflect the variation in the absolute and relative abundance of different bacterial species (Figure 5.12 and Figure 5.13). The highest proportion of BCFA was observed for the autumn *G. catenatum* bloom on May 13. BCFA were 17% of the TFA (111 µg/l, Figure 5.12) for the pycnocline sample at site F3 where the *G. catenatum* bloom was at highest abundance. The major fatty acids during the bloom were predominantly br17:1 fatty acids for all sites and depths. The concentration and proportion of BCFA was very high, particularly allowing for the high concentration of lipids contributed from *G. catenatum* in this sample. The *G. catenatum* cells at site F3 were healthy with high numbers of vegetative cells (154 cells/ml, Figure 2.7 chapter 2). The highest cell numbers of *G. catenatum* occurred on May 13 for site F3 and Apr 22 for sites X3 and F1. Although algal cells were healthy on May 13 fatty acid and lipid composition analysis (section 5.7 and Figure 5.13) demonstrated that the preceding date, (Apr 22) contained healthier cells with lower levels of FFA, TG and BCFA. High proportions of fatty acids from the family *Flavobacteriaceae* were observed on May 13 (BCFA particularly br17:1). The fatty acid profile corresponded with the presence of high proportions of bacteria from the CFB cluster (chapter 7).

Bacterial numbers on May 13 were estimated at 2.08 x 10^8 cells/l for the integrated sample at site F3. This value was calculated from the field BCFA concentration of 5 µg/l and an approximate measure of fatty acid per bacterial cell (*E. coli*; 0.024 pg/cell, Atlas and Bartha 1993). The calculation assumed that BCFA described 100% of the bacterial population which was dominated at the time by CFB (83%, site F3, chapter 7). By comparison, microscopic examination using DAPI stained cells for this sample was 4.1 x 10^8 cells/l (chapter 7). BCFA should underestimate bacterial concentrations as not all bacteria contain BCFA. Bacterial species also exist at different growth stages and are more diverse in their size and cell volume than *E. coli*. The use of the BCFA concentration to estimate cell numbers accounted for 50% of the DAPI stained cells. This is consistent with results

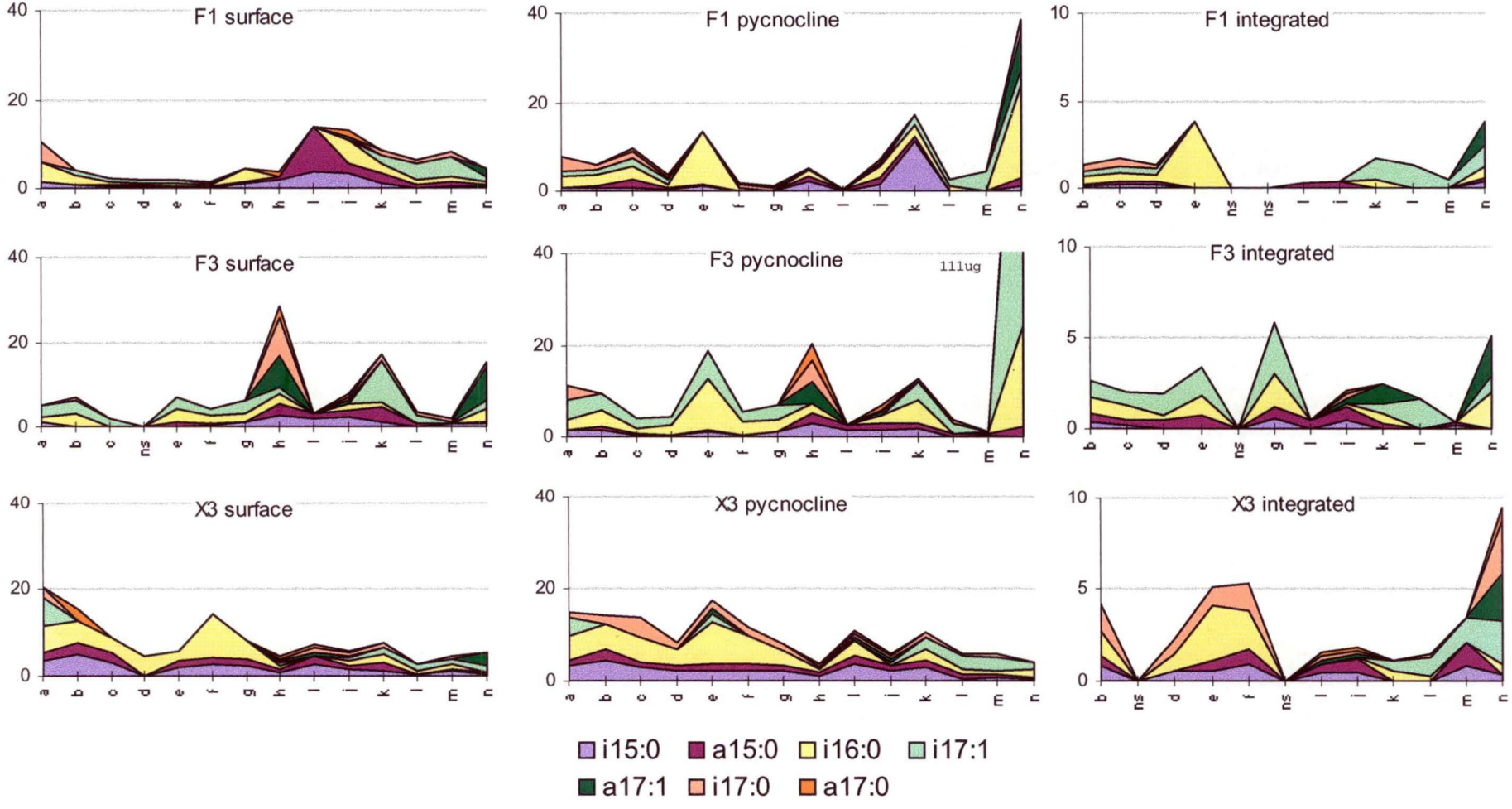


Figure 5.12 Variation of BCFA concentration in the Huon River at 3 sampling sites and 3 depths during 1998-99. Y axis: µg/l, X axis: Sampling dates (a:12/11/98, b:26/11/98, c:10/12/98, d:21/12/98, e:7/01/99, f:14/01/99, g:21/01/99, h:4/02/99, i:18/02/99, j:4/03/99, k:18/03/99, l:31/03/99, m:22/04/99, n:13/05/99, ns: not sampled).

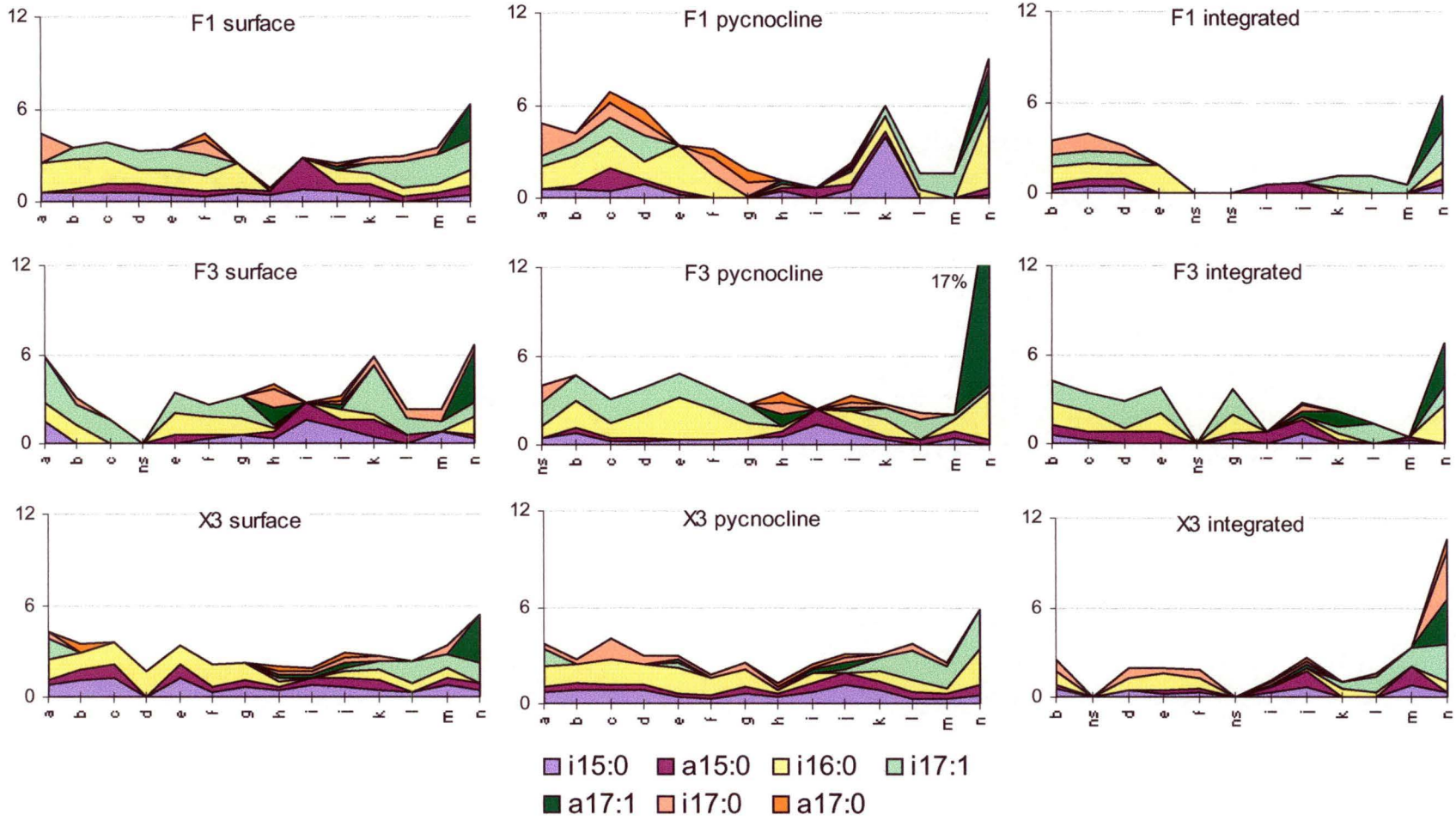


Figure 5.13 Variation of BCFA percentage in the Huon River at 3 sampling sites and 3 depths during 1998-99. Y axis: % BCFA, X axis: Sampling dates (a:12/11/98, b:26/11/98, c:10/12/98, d:21/12/98, e:7/01/99, f:14/01/99, g:21/01/99, h:4/02/99, i:18/02/99, j:4/03/99, k:18/03/99, l:31/03/99, m:22/04/99, n:13/05/99, ns: not sampled).

indicating that the average cultured bacteria from the Huon Estuary contained 42% BCFA (section 5.6). This proportion would be higher for this sample as the microbial community on May 13 was dominated by CFB (chapter 7). The use of these fatty acids to estimate bacterial number has not been previously performed for samples from marine waters.

Although high proportions and concentrations of BCFA were observed at some sites and on some dates, the same proportions or fatty acid types did not necessarily occur throughout the water column. However, BCFA profiles were more comparable for sample depths than sites (Figure 5.12 and 5.13). For example, on Feb 18, samples from all site F3 depths and the surface and integrated sample from site F1 were dominated by i15:0 and a15:0 (Figure 5.12). This indicates that the same community was present in all samples and was similar to bacteria found in the family *Flavobacteriaceae.*

BCFA also increased at the pycnocline for site F1 on Mar 18. This was because of the decay of the *Pseudonitzschia* spp. bloom during this period. The high proportion of i15:0 (Figure 5.12) indicates that the bacterial community present at this time probably belonged to the CFB cluster. This finding agrees with the observation that the *Pseudonitzschia* spp. bloom was declining and that members of the CFB are generally associated with particulates.

During the period between Feb 4 and Mar 4, all sites showed more diverse and complex fatty acid profiles than on other dates (Figure 5.13). This occurred when the summer bloom of *G. catenatum* was declining and the *Pseudonitzschia* spp. bloom was commencing. Sites F3 and X3 were very similar for their respective depths and fatty acid profiles, whereas site F1 was not as diverse. The similarities and respective simultaneous changes in the fatty acid profiles for sites F3 and F1 reflect a period of diversification in the bacterial species present. BCFA profiles also changed from more complex (6 BCFA) to simpler profiles (2 BCFA) during this period (Figure 5.13). BCFA profiles for the Feb 4, Feb 18 and March 4 samples at sites F3 and X3 were the most complex across all depths.

In contrast, the greatest diversity in BCFA for site F1 was during December before the summer *G. catenatum* bloom at the pycnocline. Although fatty acids proportions in mid March increased and changed during the decline of *Pseudonitzschia* spp. bloom, they were not as complex as those observed for sites F3 and X3.

Over the entire sampling period, site F3 contained the highest concentrations of BCFA. The BCFA concentration for site X3 remained comparatively constant throughout the year, even during algal blooms when BCFA at the other two sites increased dramatically. Site X3 also contained the lowest concentrations of BCFA overall. Integrated depth samples for all sites also had lower proportions and concentrations of BCFA in comparison to the surface and pycnocline samples where algal blooms were most intense.

5.8.5 Principal components analysis and hierarchical cluster analysis of BCFA

Principal components analysis (PCA) of BCFA

Principal components analysis reflected the change in BCFA for each of the sample sites and depths (Figure 5.14). All three depths demonstrated similar changes in BCFA during the season. Before the summer *G. catenatum* bloom, surface integrated and pycnocline samples were associated with the vectors i17:1, and a17:0. The sampling dates before the autumn *G. catenatum* bloom had a similar association with these vectors, although greater correlation with the branched C_{15} fatty acids was also observed. During the summer *G. catenatum* bloom, i15:0 was the dominant vector. Throughout the *Pseudonitzschia* spp. bloom, clustering was clearly evident and the major vector was primarily i15:0 and to a lesser extent a17:1 and a15:0. The fatty acid a17:1 was associated with the dates of the autumn *G. catenatum* bloom decline for surface samples at sites X3 and F3. The fatty acid i16:0 was associated with integrated samples for most of the season for site F1 indicating different communities present at the integrated and photic depths for this

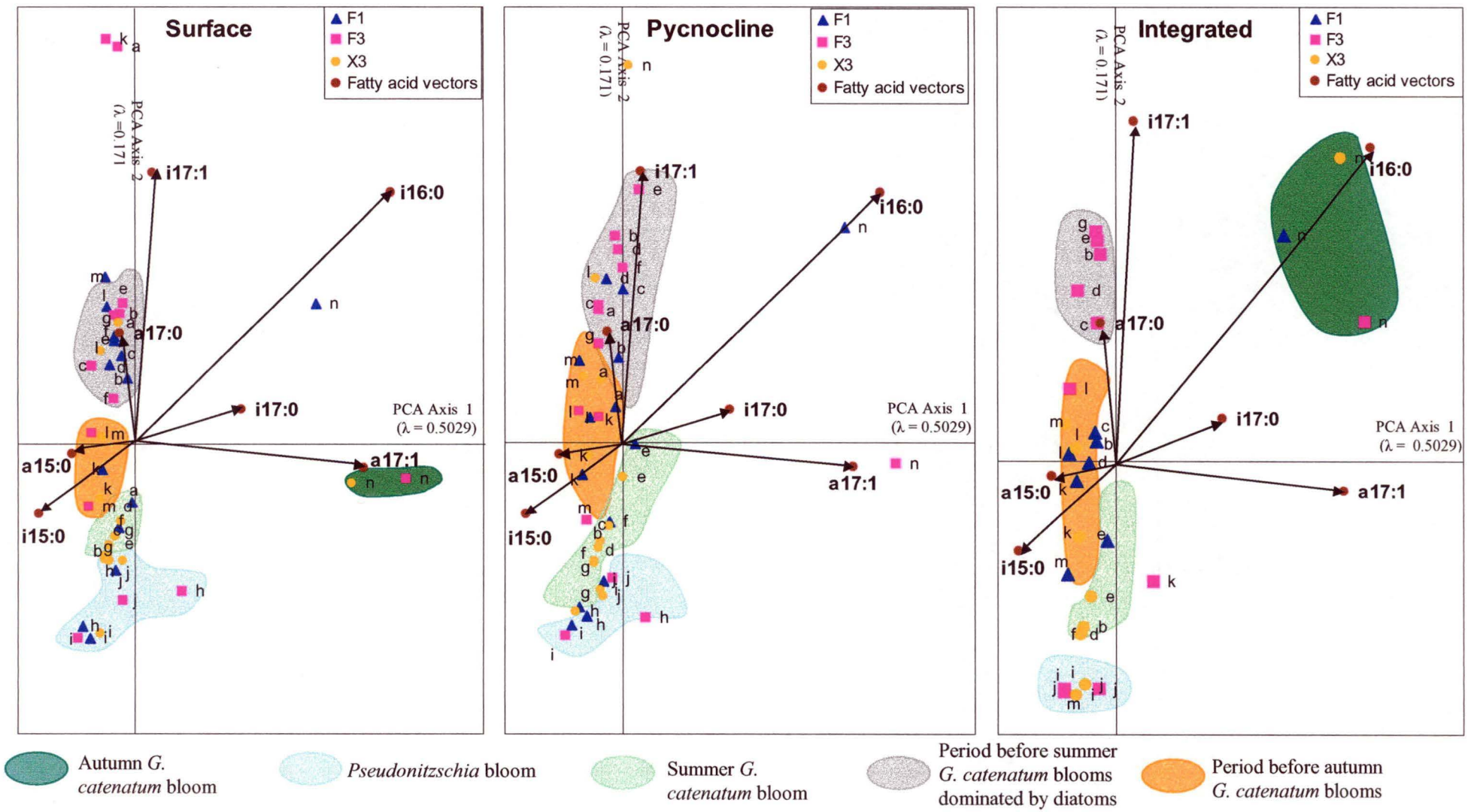


Figure 5.14 Principal components analysis of BCFA fatty acid variables and their relationship with sample sites and dates for the integrated surface and pycnocline. Date Legend (a:12/11/98, b:26/11/98, c:10/12/98, d:21/12/98, e:7/01/99, f:14/01/99, g:21/01/99, h:4/02/99, i:18/02/99, j:4/03/99, k:18/03/99, l:31/03/99, m:22/04/99, n:13/05/99)

site. The i17:1 and a17:0 vectors correlated with samples from site F3. This was caused by the absence of a17:0 in these samples and the high proportion of i17:1. The i15:0 vector was strongly associated with samples from site X3.

Hierarchal cluster analysis of BCFA

Hierarchical cluster analysis grouped the individual BCFA of the three sites and depths based on similarities in their presence, absence and proportions in the water column. Trends in the fatty acid profiles of these groups clarified and demonstrated the temporal and spatial movement through the water column of the bacterial community containing BCFA (Figure 5.15).

The dominant BCFA during the early diatom bloom and merging into the summer *G. catenatum* bloom were i15:0, i16:0 and i17:0 which are indicative of the CFB cluster. For site F1, these fatty acids occurred at the surface on Nov 12. During the following four sample dates, the same fatty acid profile moved to the lower depths (Figure 5.15). A change in BCFA profiles for all sites and depths occurred during the decline of the summer *G. catenatum* bloom and the start of the *Pseudonitzschia* spp. bloom (Feb 4, Feb 18, Mar 4). The BCFA profile observed on Feb 18 at site F3 for all depths [i15:0, a15:0], occurred between the more complex fatty acid profile [i15:0, i16:0, i17:1, i17:0 and a17:0] that was observed for the previous and following samples Feb 4 and Mar 4.

The autumn *G. catenatum* bloom (May 13) coincided with a high proportion and concentration of i17:1, a17:1 and i16:0. These are major fatty acids in bacteria from the CFB cluster.

Community	BCFA that dominate the community	Community	BCFA that dominate the community
1	i15:0, i16:0 and i17:0	6	all BCFA except a17:1
2	i16:0, i17:1, i15:0, a15:0	7	i15:0, a15:0
3	i16:0, i17:1	8	i17:1, a17:1, i16:0
4	only i17:1	9	i15:0, a17:0, i16:0, i17:1
5	only i15:0		

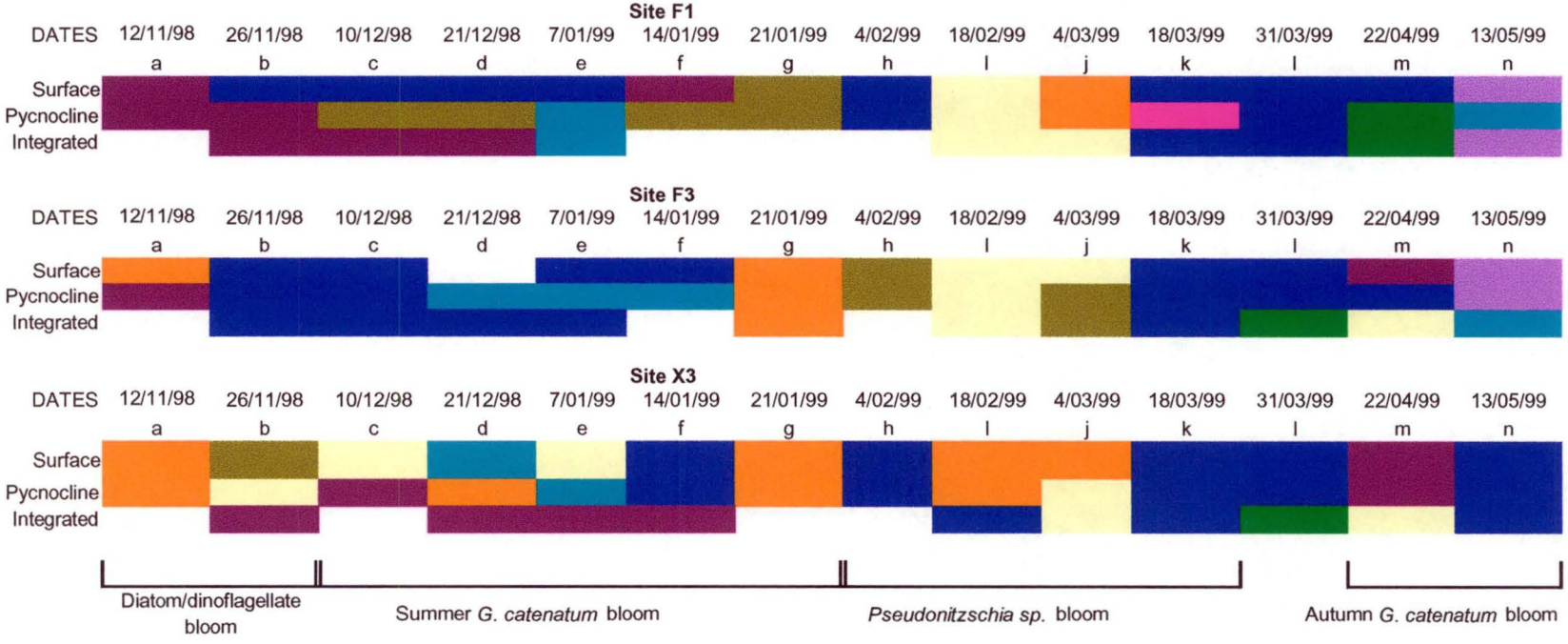


Figure 5.15 Cluster analysis showing distribution through the water column of BCFA and demonstrating changes in these profiles over the sampling season. Colours represent similarities in the observable BCFA profiles for a specific date (see legend above).

5.8.6 Principal components and hierarchical cluster analysis of total fatty acids

Principal components analysis

The 33 fatty acid vectors demonstrated changes over the season with relation to sample depth using PCA and hierarchical cluster analysis. Figure 5.16 represents PCA of the three sites and all depths.

The integrated samples generally clustered between the two SFA vectors, 16:0 and 18:0 (Figure 5.16). Pycnocline samples clustered with the diatom (20:5(n-3)) and dinoflagellate (22:6(n-3)) derived fatty acids and respectively. Surface samples clustered with the MUFA 16:1(n-7), 18:1(n-7), and the BCFA, i15:0 (Figure 5.16). Surface samples did not cluster as tightly as the integrated and pycnocline samples.

Cluster analysis of the 33 fatty acids was performed for the individual sample depths and dates (Figures 5.17 and 5.18). Surface samples did not cluster with any one fatty acid vector and no correlation with date or site was observed for these samples (Figure 5.17). These results reflect the dynamic nature of the surface layer.

Pycnocline samples clustered for particular dates that were correlated with the occurrence of the major algal blooms (Figure 5.17). The fatty acids 20:5(n-3) and 22:6(n-3) correlated with dates of the first diatom bloom, and the initial stages of summer *G. catenatum* bloom respectively. Sampling dates for the pycnocline samples during the autumn *G. catenatum* bloom correlated with MUFA vectors 18:1(n-9), 15:1 and the C_{17} fatty acids. These fatty acids indicate bacterial influence. This does not indicate that these fatty acids were dominant only that they were influential in differentiating the autumn *G. catenatum* bloom. Similar bacterial influence was observed for the integrated samples during the autumn *G. catenatum* bloom (Figure 5.18) when sampling dates correlated with the vectors 22:6(n-3), 16:1(n-5), a17:1 and i16:0 demonstrating the associated bacterial species at this time. Integrated samples clustered for particular dates associated

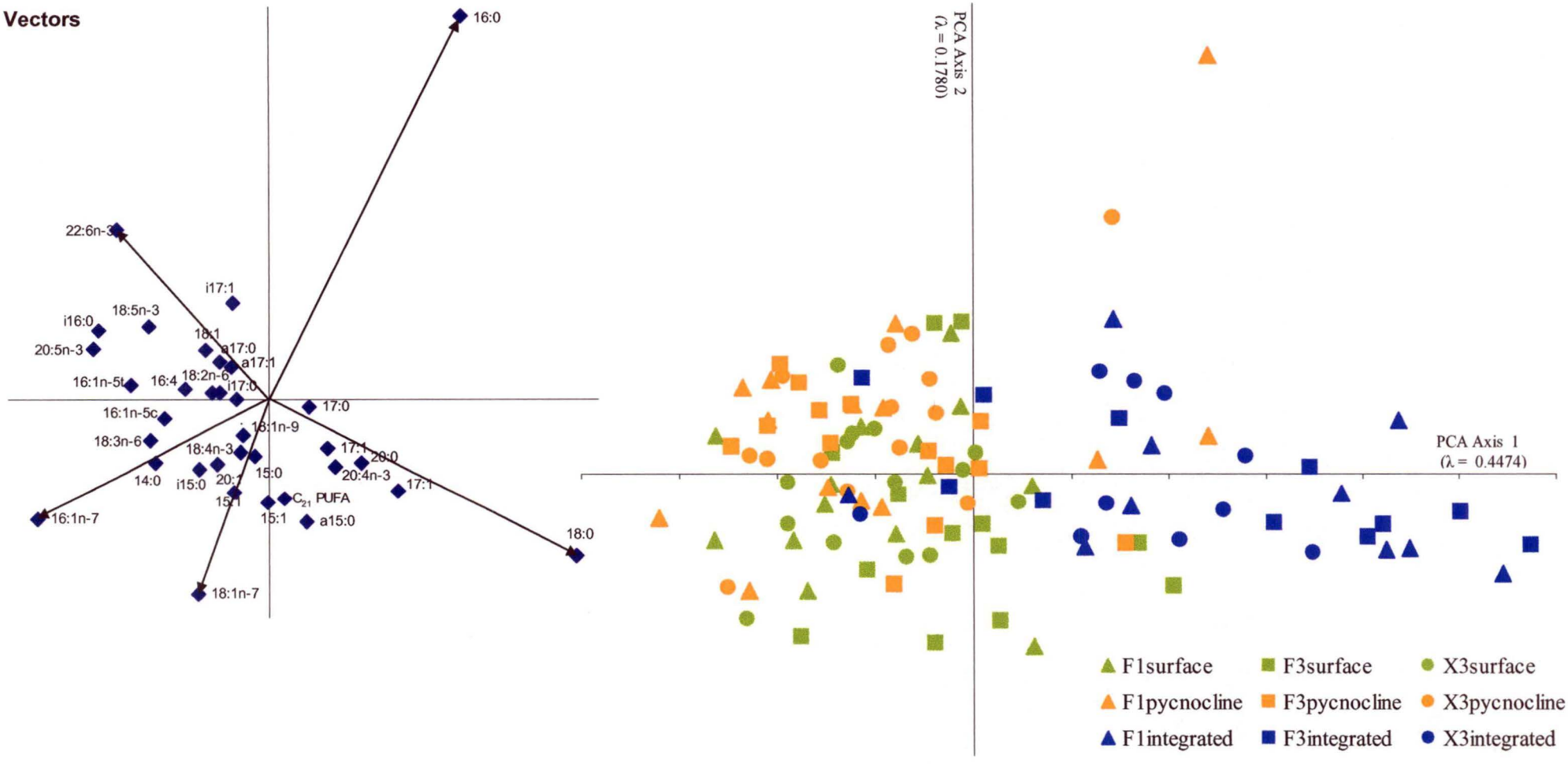


Figure 5.16 Principal components analysis of 33 total fatty acid variables and their relationship with sample sites (F1, F3 and X3) and all depths (surface, pycnocline and integrated). Vector diagram is shown with limited vector arrows due to congestion

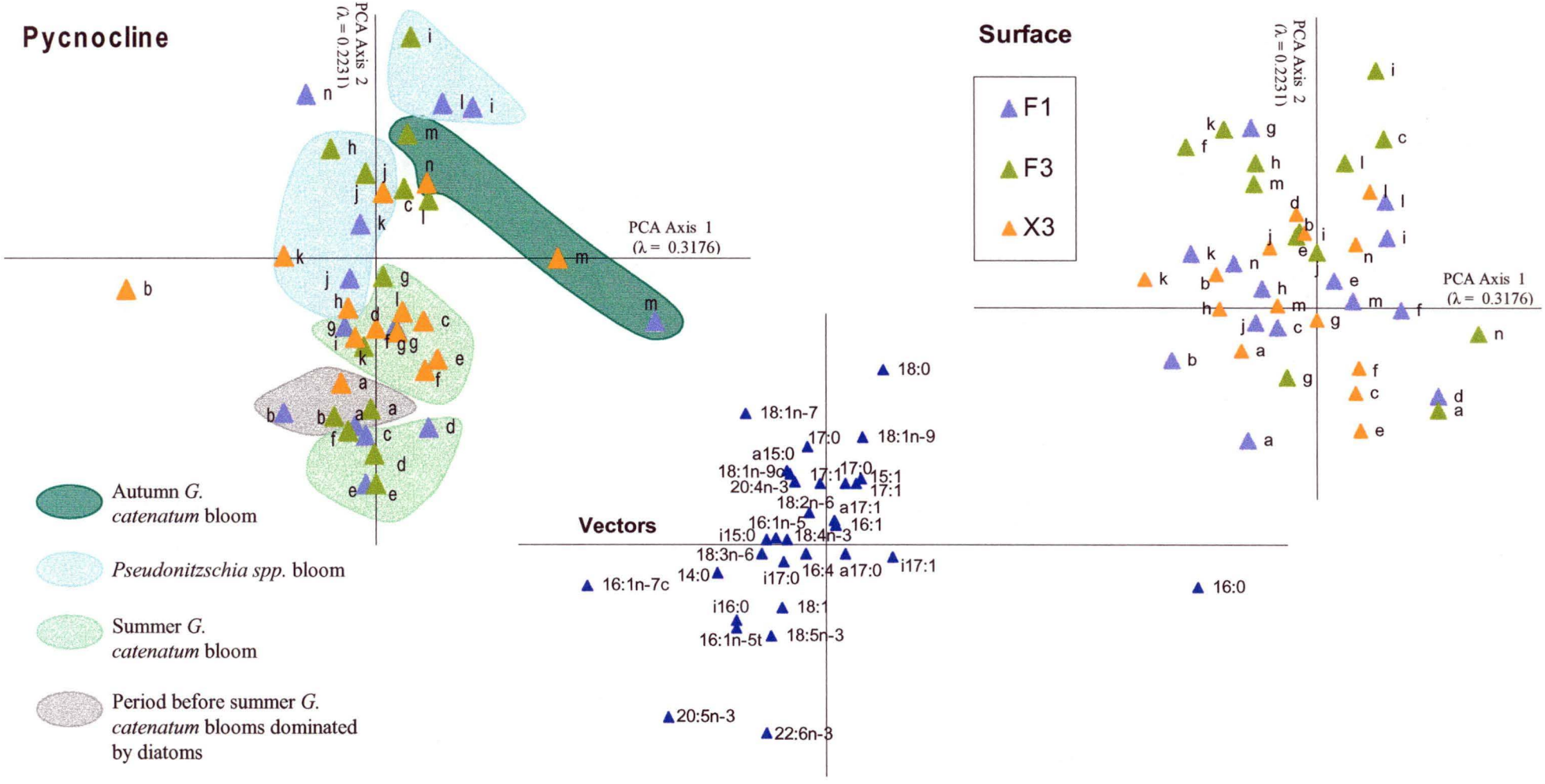


Figure 5.17 Principal components analysis of 33 total fatty acid variables and their relationship with sample sites and dates for the pycnocline and surface samples. Vector diagram is shown without arrows due to congestion. Date Legend (a:12/11/98, b:26/11/98, c:10/12/98, d:21/12/98, e:7/01/99, f:14/01/99, g:21/01/99, h:4/02/99, i:18/02/99, j:4/03/99, k:18/03/99, l:31/03/99, m:22/04/99, n:13/05/99)

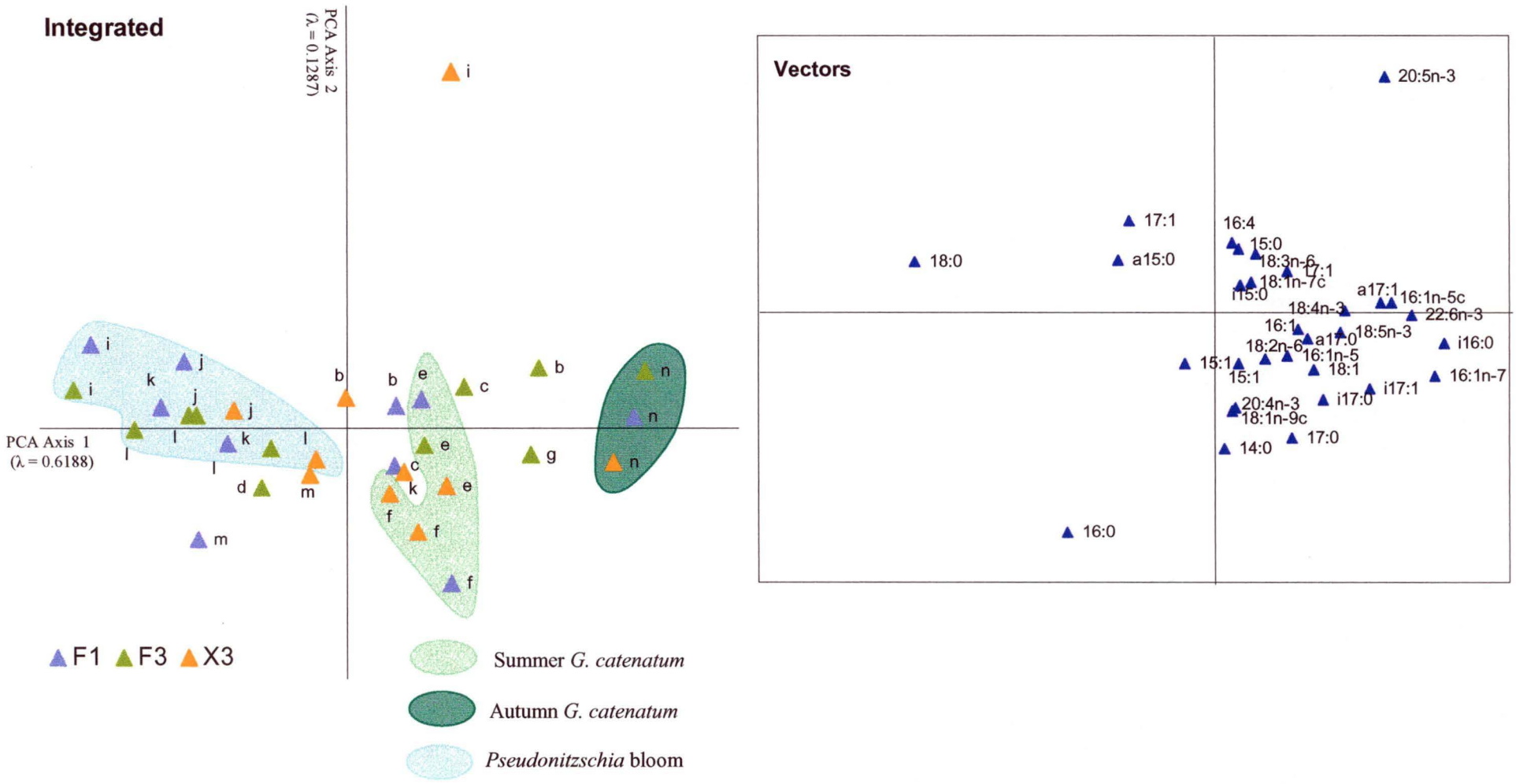


Figure 5.18 Principal components analysis of 33 total fatty acid variables and their relationship with sample sites and dates for the integrated sample. Vector diagram is shown without vector arrows due to congestion. Date Legend (a:12/11/98, b:26/11/98, c:10/12/98, d:21/12/98, e:7/01/99, f:14/01/99, g:21/01/99, h:4/02/99, i:18/02/99, j:4/03/99, k:18/03/99, l:31/03/99, m:22/04/99, n:13/05/99)

with the major algal blooms (Figure 5.18). Greater correlation with the 18:0 vector was observed for integrated samples for the period of the *Pseudonitzschia* spp. bloom. A high diversity of fatty acids correlated with the dates of the summer *G. catenatum* bloom.

Hierarchal cluster analysis

The distribution of the algal blooms through the water column can be observed using hierarchical cluster analysis. The analysis was simplified to 4 different clusters. In Figure 5.19, each colour represents a change in the fatty acid profile that involved a change in the bacterial and algal communities. The most striking observation for all three sites was the difference between the diatom and dinoflagellate blooms. The period before the autumn *G. catenatum* bloom demonstrated a greater correlation with BCFA for all sites.

The profiles indicate that diatom fatty acids dominated for much of the year at site X3 while sites F1 and F3 were strongly influenced by dinoflagellate-derived fatty acids. The three sites show similar seasonal profiles.

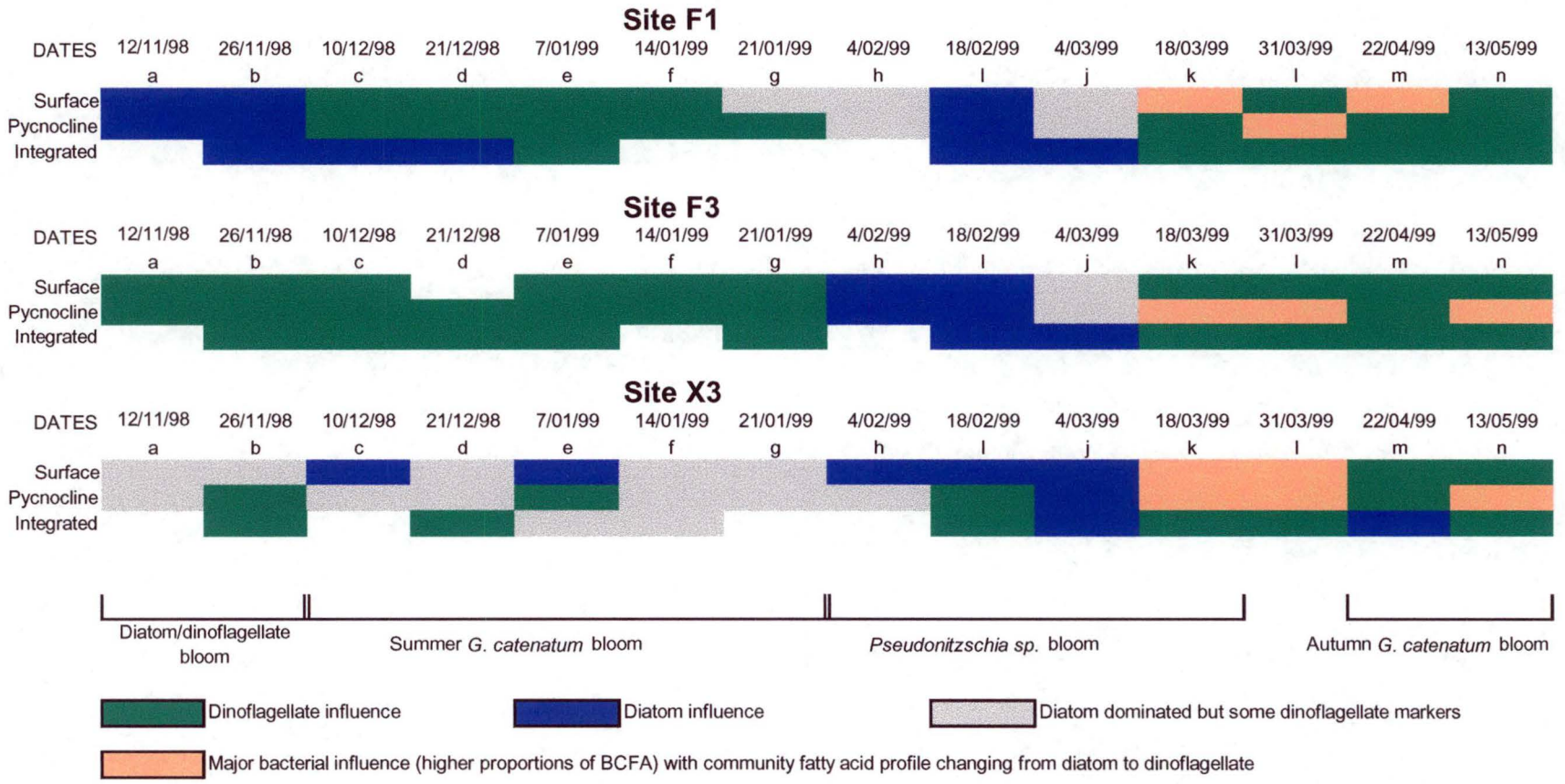


Figure 5.19 Hierarchical cluster analysis of TFA for individual sites and depths showing distribution through the water column of fatty acid markers over the sampling season. For each sampling period other organisms are also present.

Discussion

5.9 Fatty acid profiles of isolates cultured from the Huon Estuary

The 30 Huon Estuary isolates, represent a diverse range of bacterial classes that are often found in the marine environment. Analysis of their fatty acid profiles can be used to differentiate species in a manner similar to phylogenetic analysis. Fatty cid profiles also provide taxonomic information on the culturable species present. The profiles demonstrated sufficient contrast to separate bacterial classes to genus and species level for the 30 field isolates. Many culturable marine isolates contain predominantly BCFA (Table 5.1). This feature makes these species readily identifiable in environmental samples.

Many studies describing the bacterial species present in the marine environment, including those using modern molecular techniques, have shown that the dominant bacteria are generally from the γ and α proteobacteria subdivisions, and the *Cytophagales-Flavobacterium-Bacteriodes* cluster (CFB) (Pinhassi and Hagström 2000, Riemann *et al.* 2000, Glöckner *et al.* 1999, Crump *et al.* 1999, Delong *et al.* 1993).

Separation of the CFB within in the cluster analysis occurred because the CFB encompass a broad range of bacteria with distinctive fatty acid profiles. Difficulties are associated with separation of different genera within the CFB cluster and on occasions, morphological and phenotypic results do not correlate well with sequence analysis. The CFB cluster contains many inadequately characterised yellow or orange strains appearing as bacilli, filaments and other various shapes. This has lead to taxonomic problems resulting in CFB possessing a heterogeneous taxonomy (chapter 3, Jooste 1993). However, the fatty acids profiles of members of the CFB cluster are surprisingly similar and principally consist of either BCFA and/or hydroxy fatty acids (Homes *et al.* 1989, Reichenbach 1989). Despite their similarities, fatty acid profiles of members of the CFB cluster can be highly

distinctive to species level. The genus *Flavobacterium* has lower levels of i15:0 and βOH i17:0 than generally found in other members of the family *Flavobacteriaceae* in the CFB cluster (Bowman 2000, Bowman *et al.* 1998b, Shewan and McMeekin 1983) and fatty acid profiles within the other CFB genera are equally distinctive (Bowman 2000, Bowman *et al.* 1998b, Olsen 1994, Skerratt *et al.* 1991, Wilkinson 1988). These features enable this common marine cluster to be distinguished from other marine eukaryotes and bacterial classes. Within the CFB cluster, differentiation between genera using fatty acid analysis is possible, however too few strains have been analysed to date and so patterns are not obvious or consistent. In addition, species validation is also poor (John Bowman pers. com., Kaneda 1991). Members of the CFB cluster, as well as various gram-positive taxa (e.g. *Bacillus, Myxococcus, Arthrobacter* and *Streptomycetes*) possess high levels of BCFA. The validity of newly classified species in these genera can be better elucidated by examination of their BCFA profiles. The use of fatty acids to hierarchically group these bacteria is a complimentary method of separating genera and species within these difficult clusters.

Members of the α proteobacteria cluster are often specialized for oligotrophic environments (Abraham *et al.* 1999) and so can be common in the marine environment (Pinhassi and Hagström, 2000). Although most commonly found in terrestrial environments, *Sphingomonas* spp. (α proteobacteria) are another common aquatic bacterial genus (VanCanneyt *et al.* 2001). They are found in oligotrophic environments and contain hydroxy fatty acids and other straight chain fatty acids in similar proportions to the CFB cluster. The isolation of an α proteobacteria confirms the presence of this species in the Huon Estuary. The α proteobacteria, ACEM 29, contained 18:1 as 91% of the total fatty acid composition. Although many α proteobacteria contain high proportions of 18:1, it is unusual for a species to have such high proportions of only one fatty acid. Two species of *Octadecabacter* isolated from Antarctic and Arctic seawater and sea-ice both contained 18:1(n-7)c in excess of 70% of their fatty acids (Gosink *et al.* 1997). Phylogenetic analysis demonstrated that they were members of the α proteobacteria and were closely related to the genus *Roseobacter* (Gosink *et al.* 1997). *Roseobacter algicola* also has high proportions of 18:1 (91%) (Labrenz *et*

al. 1999). Thus, based on fatty acid and FISH analysis and the pink pigmentation of ACEM 29 it is likely that the isolate is related to *Roseobacter algicola.*

Another relatively widespread, easily culturable marine member of the α proteobacteria is the genus *Caulobacter* spp.. *Caulobacter* spp. contain up to 20% a17:0 and possess high proportions of 18:1 (Abraham *et al.* 1999). In particular, they produce an unusual branched 18:1 (cis-11-Me-18:1(n-11); 0.4-35%). The odd chain fatty acids 15:0, 17:0, and 17:1 are also principal components of this species.

Alcaligenes spp. (β proteobacteria) have high proportions of hydroxy fatty acids and/or cy17:0 and 18:1 fatty acids. β Proteobacteria do not generally exist in high numbers in marine waters. This is because NO_2/NH_3 oxidizers belong to different groups in marine waters (e.g. *Nitrospira* group).

Gram-positive species such as actinomycetes, although less common in marine environments, have been noted in samples taken from the Huon Estuary. Their fatty acids are also dominated by the BCFA; br15:1, a17:0, i15:0 and a15:0.

Commonly cultured marine and estuarine bacteria such as the γ proteobacteria, *Vibrio, Pseudoalteromonas* and *Alteromonas* species possess 16:0, 16:1 and 18:1 as dominant fatty acids. *Pseudoalteromonas* spp. contain SFA and MUFA as their fatty acids and do not contain high proportions of branched or hydroxy fatty acids. This genus is difficult to detect in the marine environment using fatty acid profiles. SFA and MUFA are common in eukaryotes so bacterial fatty acids are easily overwhelmed in the considerably higher proportion of these fatty acids derived from algal and detrital biomass. However, many commonly found marine bacterial species contain a proportion of BCFA that allows their differentiation from the rest of the marine microflora.

Common fatty acids of *Pseudomonadaceae* are 16:0, 16:1 and 18:1, however, they also contain hydroxy fatty acids (major hydroxy fatty acids: 12:0, 14:0, 14:1, 16:0, i13:0 and i15:0) and BCFA (Wilkinson 1988). *Aeromonas* have similar fatty acids to *Vibrio* except they also have high proportions of BCFA (i13:0, i15:0, i15:0, i17:0) and hydroxy fatty acids (βOHi17:1(n-9)c) (Wilkinson 1988). Most *Vibrio*

species contain at least 1-14 % of the total fatty acids as BCFA. The presence of high concentrations of 18:1(n-9) (Wakeham 1995) at site X3 may further signify that bacteria other than CFB dominate the community at this site (such as α or γ proteobacteria). It may indicate greater micro-heterotrophic activity (section 5.13) although this was not obvious under microscopic examination.

The diverse genera observed using morphological study of colony forming units (CFU), and the dominance of CFB and γ proteobacteria corroborates the findings of other ecological studies where the presence of similar genera were demonstrated using molecular techniques (Hagström *et al.* 2000, Rehnstam *et al.* 1993, chapter 7). The identification of characteristic fatty acids of the culturable bacterial population was effectively achieved. Hierarchical cluster analysis of their fatty acid profiles clustered the individual bacteria isolates into similar groups as those observed using 16S rDNA sequencing.

5.10 Variation in total lipid composition

Variation in lipid class composition was similar for sites X3 and F1. The result reinforces earlier findings (chapter 2) that a high level of homogeneity exists in the lower estuary due to similar environmental conditions prevailing at all sites.

Total lipid class composition of the algal bloom samples over the season reflected the physiological status of these blooms and demonstrated how the environment influences cell physiology. The change in relative levels of polar lipid, TG and FFA reflect the physiological condition of the algae. These changes demonstrated that a peak in algal numbers was not necessarily the growth peak of the species. Despite the observed apparent health of the algae, the periods where algal numbers were highest occurred once the algae was commencing stationary phase and forming storage and degradation products. Polar lipid, which is the membrane component indicative of growth, was high in all algal blooms examined until the peak of the bloom. The alga then turned to storing TG. TG is generally regulated by nutrient availability and can also be affected by temperature. In this study, water temperatures were cooling (chapter 2) during the period of highest TG proportions. Once the algal blooms declined, an increase in FFA was observed

caused by breakdown of polar lipid and TG. This finding is indicative of bacterial recycling and degradation of the algal community. This sequence of events would allow the subsequent algal bloom to benefit from increased nutrient levels. During the commencement of the next algal bloom, polar lipid increased again to pre-bloom conditions as algal cell numbers increased.

Although FFA in algal cultures and field samples can be a sampling artefact caused by degradation of TG and polar lipid, it can also indicate a natural decline in an algal community. Logarithmic phase cultures of some algae contain up to 30% FFA and up to 50% TG (Dunstan *et al.* 1994). In previous estuarine studies, riverine samples contained high FFA proportions when compared to open marine environments (Dachs *et al.* 1999). Higher proportions of FFA were also observed in the particulate fraction collected near river mouths (Derieux *et al.* 1998). In Arctic studies, a shift from early to late bloom conditions caused a shift from polar to neutral lipids (TG and FFA) (Smith *et al.* 1993). In the Antarctic, high FFA proportions were present in sea-ice and pelagic phytoplankton blooms as salinity fell and temperature increased (Skerratt *et al.* 1997, Green *et al.* 1992). In the Huon Estuary, observations of particulate samples with elevated FFA are therefore indicative of natural algal degradation. This may be partly because of the temperature at which the alga blooms during summer. The water column can reach up to 19°C (chapter 2) which is past the optimum temperature for this alga (14-16 °C, Hallegraeff *et al.* 1995b). The high proportion of FFA in these samples is unlikely to be an artifact of sample degradation. It is likely to be an indicator of the physiological status of the microbial community present in the water column in response to temperature, algal physiology, life-stage and salinity variations. As a result, the algae were undergoing a natural process of degradation before remineralisation for future algal blooms.

Although only low proportions of TG were noted in pure cultures of *G. catenatum,* field samples of previous *G. catenatum* blooms have also demonstrated high proportions of TG and FFA (Hallegraeff *et al.* 1991). In the earlier study a *G. catenatum* bloom was sampled at the peak of the bloom when it had commenced storing TG. In this study at the height of the autumn *G. catenatum* bloom, the

proportions of degradation products and storage lipid were much lower than those observed during the summer bloom.

Some diatom and dinoflagellate species produce hydrocarbons (Budge and Parrish 1998). These include populations where *Ceratium* spp. are dominant, (Green *et al.* 1992), and algal sources in marine environments including estuaries (Cooke *et al.* 1998, Hayakawa *et al.* 1996). The lack of petrochemical markers and the presence of these hydrocarbon-containing algal species was consistent with the occurrence of natural marine source of hydrocarbons.

Lipid analyses of the two *G. catenatum* blooms and the diatom blooms identified differences in the physiological status of each bloom during the summer and autumn *G. catenatum* blooms and *Pseudonitzschia* spp. bloom. The lipid compositional data for both blooms, when examined with physical data, shows that the temperature and salinity of the water column appears to affect the physiology of *G. catenatum.* Higher temperatures and lower salinity influence the progression of the bloom, and subsequent formation of TG and FFA.

5.11 Differentiation of *G. catenatum* and *Pseudonitzschia* spp. algal blooms using fatty acids

The highest fatty acid concentrations occurred at the pycnocline in summer and autumn, during the peak bloom periods of *G. catenatum* and in summer for *Pseudonitzschia* spp. (section 5.8.1). Fatty acid analyses demonstrated that algal blooms occurred at certain depths in the water column and moved to different depths at later dates. The majority of algal blooms occurred close to the pycnocline to maximise light requirements. At the pycnocline algae would also benefit from higher nutrient levels available from the deeper marine waters (chapter 2).

Blooms of *G. catenatum* contained high concentrations of cellular lipid owing to the large size of this alga compared to diatom species (chapter 2). Estimates of algal cell numbers of *G. catenatum* were obtained during mono-species blooms.

Variations in and changing ratios of PUFA can differentiate between dinoflagellates and diatoms (Volkman *et al.* 1998, Cripps and Hill 1998, Leveille *et al.* 1997, Skerratt *et al.* 1995). The fatty acids 20:5(n-3), 16:1(n-7) and C_{16} PUFA are generally indicative for diatoms and 16:0, 18:5(n-3), 20:5(n-3) and 22:6(n-3) are the most common fatty acids found in many dinoflagellate species. Some dinoflagellates and flagellates may contain relatively high proportions of 20:5(n-3). These flagellates can be from a number of genera and can therefore affect the proportions of these two main PUFA. Some prymnesiophytes such as coccolithophores contain similar proportions of 22:6(n-3) although they have higher proportions of the C_{18} PUFAs and lower proportions of 16:0 (Pond *et al.* 1998) than most dinoflagellates.

The major diatom bloom in the Huon Estuary during the study was *Pseudonitzschia* spp. (predominantly *P. pseudodelicatissima*) (chapter 2). Fatty acid profiles of members of the genus *Pseudonitzschia* are similar to each other as well as to those in the family *Bacillariophyceae*. Analyses of three *Pseudonitzschia* species indicate that they contain high proportions of 16:1(n-7) (32-38%) and C_{16} PUFA (12-21%), moderately high proportions of 20:5(n-3) (10-16%) and very low proportions of C_{18} PUFA and 22:6(n-3) (4%) (Budge and Parrish 1999, Zhukova *et al.* 1998, Whyte *et al.* 1996). The major difference between the above-mentioned *Pseudonitzschia* species is the variation in C_{16} PUFA; these include 16:2, 16:3 and two isomers of 16:4. Fatty acid profiles from the Huon Estuary samples reflected the presence of the *Pseudonitzschia* spp. bloom from Feb 18 to Mar 18 with high proportions and concentrations of 16:4 and 20:5(n-3). The fatty acid profiles also demonstrated the presence and absence of the bloom at certain depths in the water column. Changes in proportions of specific C_{16} or C_{20} PUFA also allowed differentiation between diatom and dinoflagellate blooms. However, for most sites 22:6(n-3) was present throughout the *Pseudonitzschia* spp. bloom indicating that other species were also contributing. Further evidence for this observation are the high proportions of C_{18} PUFA observed during the *Pseudonitzschia* spp. bloom. Members of the genus *Pseudonitzschia* do not contain C_{18} PUFA. This suggests other unidentified

flagellates such as prymnesiophytes or heterotrophic species that contain high proportions of C_{18} PUFA were also present.

G. catenatum contains 22:6(n-3) (16-26%) as the major fatty acid as well as 20:5(n-3) in similar proportions to *Pseudonitzschia* (11-15%) and 16:0 (27-33%). *G. catenatum* also has relatively high proportions of 18:2(n-6) (3-5%) (Hallegraeff *et al.* 1991). The presence of 18:2(n-6), during periods other then when *G. catenatum* was present, suggests the presence of other unidentified sources including flagellates that contain this fatty acid. However, 18:2(n-6) was at highest concentrations during the *G. catenatum* blooms. Together with the high proportions of 22:6(n-3) and 22:5(n-3), a distinct profile for this species was demonstrated in samples taken during the two *G. catenatum* blooms. These two blooms also demonstrated an increase in specific dinoflagellate-derived sterols (section 5.7). The lower concentrations and proportions of 22:6(n-3) and 20:5(n-3) during the autumn *G. catenatum* bloom were consistent with the lower intensity of the autumn bloom compared to the summer *G. catenatum* bloom (chapter 2).

5.12 BCFA profiles and the bacterial community

Fatty acid indicators for specific bacterial classes or genera have been successfully used in many marine studies (Navarrete *et al.* 2000, Haack *et al.* 1994, Nichols *et al.* 1993). These studies showed that bacteria contain particular fatty acids or fatty acid profiles that enable them to be identified within their respective communities. The use of the total proportion of BCFA within a community has been previously described (e.g. Skerratt *et al.* 1997, Boon *et al.* 1996, Wakeham 1995). Separating bacterial populations present in environmental communities based on fatty acid analyses has been undertaken with soils (Okabe *et al.* 2000, Ibekwe and Kennedy 1998, Zelles *et al.* 1995). However, most soil studies separate bacteria into two categories, gram-positive species represented by BCFA and gram-negative species represented by MUFA and hydroxy fatty acids. These categories are too coarsely defined for the marine environment, as many marine bacteria do not fit into this simplified categorisation.

The variation in BCFA over the season in the Huon Estuary demonstrated changes in the dominance of bacterial species. The occurrence of a high proportion of *anteiso*-BCFA such as a15:0, and a17:0 in some of the Huon Estuary samples could be from *Bacillus*, *Planococcus* and gram-positive species. The increase in i15:0 was likely to be from species in the CFB cluster. The occurrence of the highest proportion of BCFA at the pycnocline indicates that the BCFA were most closely associated with algal-derived particulate matter. This is because BCFA were at their highest concentration in the upper photic zone and at the chlorophyll maximum (chapter 2).

Lower concentrations of BCFA at site X3 during periods of algal bloom, in comparison with the other two sites, may be due in part to the shellfish farm near site X3. The mussel farm may serve to filter the water in this area (Graham Jones, Tas Blue Mussels, pers. comm.; Ed Butler, CSIRO, pers. comm.).

Over the season, integrated samples had the lowest concentrations and proportions of BCFA. The low proportions of BCFA in the integrated samples and for the samples taken during the *Pseudonitzschia* spp. bloom indicates that the bacteria at these depths contain a similar profile to the particles they are degrading, a finding consistent with the presence of γ proteobacteria (chapter 7). Bacterial numbers obtained through the year, using CFU and epifluoresence microscopy (chapter 2 and chapter 7), were slightly higher for integrated samples than at the pycnocline and surface. However, BCFA were at higher concentrations and proportions in the surface and pycnocline sites. The upper photic zone therefore contained bacteria from the CFB cluster (evidenced by BCFA content) and were associated with algal blooms and detritus recycling.

In general, the BCFA were significant components of the lipid profile in the Huon Estuary considering their comparative biomass with respect to that from eukaryotic sources. Variations between the BCFA demonstrate bacterial species change with respect to algal blooms. The concentration of BCFA during a period when bacteria from the CFB dominated the algal community estimated bacterial cell numbers with a surprising degree of accuracy.

5.13 Fatty acid biomarkers for heterotrophic species

Grazing by herbivorous heterotrophs removes fatty acids derived from a diatom or dinoflagellate diet. Heterotrophs selectively remove polyunsaturated components from the water column and preserving the saturated components and occasionally 18:1(n-9) (Wakeham 1995, Neale *et al.* 1986).

Highest SFA and 18:1(n-9) proportions and concentrations were observed in the integrated samples throughout the *Pseudonitzschia* spp. bloom and during the autumn *G. catenatum* bloom. Although the presence of 18:1(n-9) can be indicative of specific bacterial groups, the concentration of this biomarker was very high in comparison to the expected biomass if bacteria were the primary source. These findings could be explained by herbivorous grazing. This interpretation is consistent with previous observations of the fatty acid compositions of particles in which SFA increased as ocean depth increased (Wakeham *et al.* 1995, Saliot *et al.* 1982). High proportions of SFA in the integrated samples were therefore interpreted to be indicative of intense herbivorous grazing as the particulate matter passed down the water column to lower depths. PUFA and MUFA were being preferentially degraded by heterotrophs such as bacteria or other processes. Heterotrophic species such as zooplankton and bacteria may contribute greatly to the lipid distribution in the integrated samples by the breakdown of unsaturated fatty acids. The particles have had more time to be broken down as they progress through the water column than those at the surface or pycnocline. The breakdown of unsaturated fatty acids within such a short depth range is indicative of a highly active microbial community.

The increase in C_{18} PUFA after the *Pseudonitzschia* spp. bloom may also indicate the presence of heterotrophic grazing, or may be attributable to the presence of other algal species that contain C_{18} PUFA. Heterotrophs can contain lower proportions of 14:0, and similar or greater proportions of 18:0, 18:1(n-9) and C_{18} PUFA than many diatom and other algal species.

Heterotrophic grazing was also a possible cause of high proportions of 20:4(n-6) in the integrated samples. The integrated samples contain higher proportions of SFA

from degraded unsaturated fatty acids. Heterotrophs remove diatom-derived fatty acids, such as 20:5 from the water column by grazing. An alternative explanation for the presence of 20:4(n-6) in the integrated samples, is the contribution from species that provide direct input. Pennate diatoms can contain 20:4(n-6) (3.5-5.6% 20:4, Dunstan *et al.* 1994). Red algae can also contain high proportions of 20:4(n-6) and C_{18} PUFA (Fuentes*et al.* 2000, Sajiki and Kakimi 1998). For the pycnocline and surface samples, these species may have been present on Apr 24 during the start of the autumn *G. catenatum* bloom at site F3 when this fatty acid was present in high proportions at both these depths. However, direct contribution of 20:4 from these species to the integrated samples is less likely given that 80-90 % of the integrated sample was water from below the photic zone.

5.14 Bacterial-algal associations

Variation in total fatty acid profiles provided an overall picture of the changes in algal and bacterial species. The approach can be used to observe the movement of these species through the water column and their occurrence at different sites over time. Fatty acid analysis illustrated that the microbial community was highly dynamic within the water column.

The fatty acid profiles indicate that certain bacterial groups were associated with the degradation of algal communities. These species differ from those lower in the water column, and from those that occur before a bloom. In particular, BCFA profiles demonstrate that the CFB cluster is principally associated with algal blooms and their decline. Gliding bacteria, such as those in the CFB, can follow their food source more easily than other bacterial genera as gliding allows rapid movement. CFB would be expected to be more abundant in the area of the water column that has the highest proportion of algal cells because of their predatory nature and association with algal blooms.

High proportions of BCFA during the peak of the autumn *G. catenatum* bloom indicated that these bacteria were coexisting with the alga and then benefited from their decline. The presence of BCFA occasionally preceded the algal blooms in the water column. For site F1, this sequence occurred before the summer *G.*

catenatum bloom and again before the *Pseudonitzschia* spp. bloom. During the summer *G. catenatum* bloom, BCFA were at low concentrations in comparison to later in the season once this bloom had declined. The second highest BCFA concentration occurred after the summer *G. catenatum* bloom finished and before the *Pseudonitzschia* spp. bloom commenced. For this period, the BCFA profile differed from those observed during early summer and the autumn-winter period. This was also the time when the greatest diversification of BCFA occurred (section 5.8.4, Figure 5.13), possibly indicating that a number of bacterial species were present simultaneously. Thus, this period was the time of greatest transition for both bacterial and algal communities.

An advantage of fatty acid analysis is that it allows a large proportion of the community to be analysed simultaneously for the identification of bacterial and algal species. Information concerning bacterial community structure, the physiological status of species in the water column, and the ability to view the species composition of the entire microbial community (both bacterial and algal) was obtained. Signature lipid analysis acts as a complementary technique to classic microscopy and molecular methods. The total lipid class and fatty acid profiles over the sampling period provided an indication of the physiological status of the algal blooms and gave insight to changes in the bacterial groups present. Lipid analysis proved to be an effective tool in this study. When used in conjunction with other methods lipid analysis can give a better understanding of community interactions and relationships.

6. Bacterial PUFA: Winter nutrient source?

Summary

Two novel *Shewanella* strains, ACEM 6 and ACEM 9, were isolated from the Huon Estuary. The two strains contain the highest proportions of eicosapentaenoic acid (EPA) yet reported for a temperate *Shewanella* species. EPA proportions for both strains were similar to Antarctic and barophilic polyunsaturated fatty acid (PUFA) producing bacteria. However, the Huon Estuary strains are capable of faster growth and equivalent EPA production at higher temperatures. PUFA proportions varied inversely with temperature for both isolates. The highest proportion of eicosapentaenoic acid (EPA) was produced by both ACEM 9 and ACEM 6 at 4 °C (20 % and 19 %). Growth was more vigorous above 10 °C (10 °C; 14 % and 15 % EPA respectively). EPA continued to be a significant fatty acid at higher temperatures (17 and 20 °C: 10-14 % EPA). Growth at differing salinities reflected the marine nature of the isolates. EPA was highest for ACEM 9 at 20 psu NaCl (13 % EPA) and ACEM 6 at 33 psu (14 % EPA). We propose that the maximum relative levels of EPA produced by these bacteria would occur in winter as typical winter and summer water column temperatures in the estuary are 10 °C and 17 °C respectively. These results are evidence that the presence of EPA in the genus *Shewanella* is not always associated with the level of cold adaptation. Our results indicate that temperate *Shewanella* species can produce high levels of EPA including at temperatures above 15 °C. Given the widespread distribution of the genus *Shewanella* in nature we suggest that these species may represent a source of PUFA in marine and estuarine ecosystems greater than hither to realised.

Introduction

Only a limited number of bacteria produce PUFA and many of the described species are in the genus *Shewanella* (Bowman 2001, Russell and Nichols 1999). *Shewanella* species that produce high proportions of EPA have been isolated from

marine, cold or pressurised environments (polar psychrophiles and barophiles)(Bowman 2001, Bowman *et al.* 1997, Delong and Yayanos 1986). To my knowledge, *Flexibacter polymorphus* (Johns and Perry 1977) and *Shewanella peleana* (Russell and Nichols 1999) are the only other non-barophilic or cold water isolates that produce relatively high levels of EPA. High proportions of EPA in *Shewanella* spp. are predominantly associated with a cold adaptation mechanism (Bowman *et al.* 1997) and are highest at the lowest growth temperatures of the bacteria. The review by Russell and Nichols (1999) examines the situation regarding PUFAs and their involvement with cold adaptation. They state that MUFA remain fluid at temperatures below zero just as effectively as PUFA. However the advantage of membranes containing PUFA instead of MUFA is that the packing order of molecules is more effective.

Salinity has also been shown to have an effect on PUFA in *Shewanella* spp. with high levels of NaCl shown to reduce EPA (Russell and Nichols 1999, Nichols *et al.* 2000). Estuaries can fluctuate markedly in salinity so the relative level of PUFA for members of the genus *Shewanella* may be expected to vary at differing salinities.

Data presented describes two temperate PUFA producing *Shewanella* strains isolated from the Huon Estuary, ACEM 6 and ACEM 9. Objectives of the research reported in this chapter were to:

- determine the effects of temperature and salinity on their fatty acid profile over the range these strains tolerate in their estuarine environment;
- assist in the taxonomy of these bacteria, knowledge of the fatty acid profiles and resultant changes with key environmental parameters;
- understand their contribution to natural relative levels of PUFA in the estuary and in a broader context, aspects of the physiology of PUFA biosynthesis.

Methods

6.1 Isolation

The Huon Estuary in Tasmania, Australia flows unregulated through a predominantly native forest water catchment and contains a number of finfish and shellfish farms. For isolation of bacterial isolates, water column samples were taken from below the pycnocline during February 1998. Salinity was 28 psu at the time of collection.

The water samples were initially plated onto modified marine agar (800 ml 0.2 μm filtered seawater; 200 ml distilled water; 5 g Bacteriological Peptone, 1 g Yeast extract, (Difco Laboratories, Detroit Michigan)). Fifty random colonies with differing morphologies were isolated and purified by streak plate technique. Two isolates were chosen for this study after fatty acid analysis revealed the presence of EPA (chapter 5).

6.2 Effects of Temperature and Salinity

Analysis of the effects of temperature used the above mentioned media. Plates were incubated at 2, 4, 10, 15, 20, 25, 30 and 36 °C respectively. NaCl was used to modify salinity and NaCl concentrations were 0, 8, 20, 33, 66 and 99 psu respectively. Plates were incubated at 22 °C.

6.3 Phylogenetic analysis

Genomic DNA was extracted and purified from cells using the procedure of Marmur and Doty (1962). The 16S rRNA genes from these strains were amplified by PCR using the primers 1492rM13r and 10F. Conditions used for PCR are described in chapter 3. The 16S rDNA sequences determined for the strains were compared to the sequences in the GenBank nucleotide database using the BLAST search program of the National Center for Biotechnology Information (NCBI) website http://www.ncbi.nlm.nih.gov. Analyses of the 16S rDNA sequences datasets utilized PHYLIP version 3.57c (Felsenstein 1993). DNADIST was used to determine sequence similarities using the maximum-likelihood algorithm option.

Phylogenetic trees were constructed with the neighbour-joining method by using the program NEIGHBOR. Bootstrap analysis was performed with SEQBOOT and CONSENCE using 250 resamplings of the dataset, using both DNADIST and NEIGHBOUR as well as the program DNAPARS, which constructs trees based on the maximum-parsimony method.

6.4 Lipid analysis

Replicate isolates were harvested for lipid analysis after 24 hours growth (10, 15, 20, 25 °C and all salinities) or 72 hours (2, 4 °C). Lipid analysis was completed by a whole cell methanolysis procedure. Samples were scraped off plates and placed in individual precleaned screw cap test tubes. Methylating reagent (5 ml of 10:1:1, MeOH:$CHCl_3$:HCL) was added and the air above the sample evacuated with N_2 gas. The sample was placed at 90 °C for 60 minutes to produce fatty acid methyl esters (FAME). The test tube was cooled, 4 ml H_2O was added followed by 2 ml of 4:1 C_6H_{14}:$CHCl_3$. After mixing, the mix was centrifuged separating the organic and aqueous layers. The C_6H_{14}:$CHCL_3$ layer containing FAME was transferred to a vial in preparation for analysis. Fatty acid profiles were determined using a Hewlett Packard 5890 Gas chromatograph (GC) equipped with a 7673a autosampler and 50 m x 0.32 mm i.d. crosslinked HP5 methyl silicone fused silica capillary column (Hewlett Packard) and Fisons GC-mass spectrometer (GC-MS) with conditions as described elsewhere (Gutierrez *et al.* 1999, Skerratt *et al.* 1998). Fatty acids were identified by GC-MS analysis and by comparing retention time data with that obtained for authentic and laboratory standards. The GC data was compiled and analysed with Millennium software (Waters).

Fatty acid nomenclature: Fatty acids are designated as number of carbon atoms: number of double bonds followed by the position of the double bond from the aliphatic end of the molecular. The prefixes, i, a and cy indicate *iso*, *anteiso* and cyclopropyl containing fatty acids, respectively. Abbreviations for fatty acid groups are: Polyunsaturated fatty acids (PUFA); monounsaturated fatty acids; (MUFA); saturated fatty acids (SFA); branched chain fatty acids (BCFA).

Results

Both strains form pink-tan pigmented colonies 2 to 4 mm in diameter following 1 to 3 days incubation. Growth was observed from 2-25 °C with best growth at 20 °C. Colony growth at 2 and 4 °C was slower (4 days) and less prolific when compared to plates at 10 to 25 °C. Fast growing, (1 day) numerous colonies, were produced by the isolates grown at 10 to 25 °C. Lipid biomass produced by both strains for temperatures between 10 and 25 °C were higher when compared to other PUFA producing *Shewanella* species. No growth was observed for either strains at 30 or 37 °C. However, weak growth was observed for ACEM 9 at 30 °C when grown in marine agar broth. Strains required Na^+ for growth and grew between 20-66 psu NaCl, with best growth at 20-33 psu. See chapter 3 for phenotypic data for the two strains.

6.5 Total fatty acid composition

The highest relative level of PUFA for both strains was observed at 20 psu or 33 psu NaCl (Figure 6.1). At higher salinities, there was a drop in the proportion of PUFA and a rise in the relative levels of MUFA and SFA. The temperature changes reflected that although growth was slower at 2 and 4 °C, PUFA percentages were highest at these temperatures for both strains (Figure 6.2). Both ACEM 9 and ACEM 6 had higher PUFA at 4 than at 2 °C (Figure 6.2). The proportion of MUFA increased at 10 °C for ACEM 9 which corresponded to a drop in PUFA. Growth of the two cultures at different temperatures was shown to alter PUFA composition by up to 20 %. At the higher temperatures, both strains showed an increase in BCFA at the expense of PUFA and MUFA.

6.6 Variation of individual fatty acid composition

The dominant individual fatty acids of ACEM 6 and ACEM 9 were 16:0 (12-22 %), i15:0 (7-24 %), i13:0 (6-14 %), 16:1(n-7) (12-25 %) and 20:5(n-3) (EPA) (2.5-20 %) (Table 6.1). Other PUFAs observed in low proportions (≤ 1.5 %) were 20:4(n-3), 20:3(n-6) and 22:6(n-3).

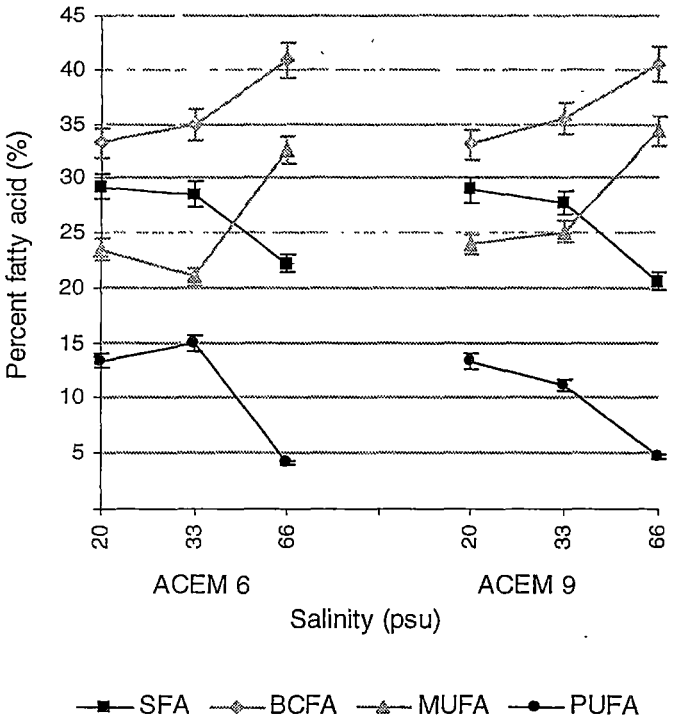


Figure 6.1 Variation of fatty acid composition with increasing NaCl for ACEM 6 and ACEM 9

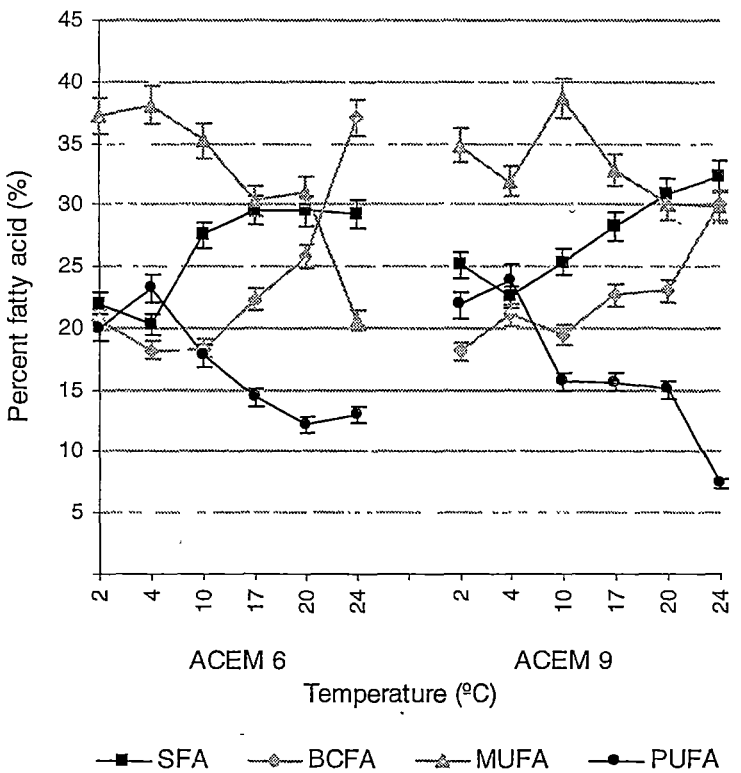


Figure 6.2 Variation of fatty acid composition with increasing temperature for ACEM 6 and ACEM 9

Table 6.1 Fatty acid compositions of *Shewanella* strains ACEM 6 and ACEM 9

	ACEM 6									ACEM 9								
	Temperature (ºC)						*Salinity (psu)*			*Temperature (ºC)*						*Salinity (psu)*		
Fatty acids	2	4	10	17	20	24	20	33	66	2	4	10	17	20	24	20	33	66
12:0	1.8	1.8	2.5	2.9	2.7	3.4	3.0	2.7	2.6	2.0	2.1	2.4	2.7	3.3	4.3	3.0	2 3	2.2
13:0	0.8	0.6	0 8	0.8	0.9	1.9	1.3	1.6	1.9	0.5	0.8	0.5	0.9	0.9	0.7	1.3	1.4	1.3
14:0	3.9	2.9	4.2	3.4	3.2	3.3	3.3	3.4	2.8	5.6	4.0	4.8	4.1	3.7	3.0	3.2	3.0	2.7
15:0	2.3	2.4	2.6	2.5	2 6	5.4	4.6	5 0	5.4	2.6	2.7	1.5	2.7	2.4	2.0	4.6	4.3	3.9
16.0	10	10	14	16	16	12	14	13	7	14	11	15	16	18	18	14	14	8
17:0	0 9	1.5	1.4	2.2	2.3	2.1	2.3	2.2	1.4	0.6	0.9	0.6	1.2	1.4	1.9	2.3	1.7	1.3
18:0	0.9	0.7	1.3	1.8	2.1	0.7	0.8	0.7	0.5	0.1	0.7	0.9	0.9	1.5	2.4	0.8	0.7	0.8
22.0	1.1		0 3															
Sum SFA	22	20	28	30	30	29	29	28	22	25	23	25	28	31	32	29	28	21
i13:0	6	7	6	6	5	10	10	10	14	6	7	6	6	6	6	10	10	10
i14:0	0.5	0.8	0.8	1.1	1 3	2.4	1.3	1.8	2.1	0.8	1.0	1.1	1.0	0.9	2.6	1.3	2.0	1.1
i15:0	8	8	10	12	14	20	18	19	21	7	9	9	13	13	15	18	20	24
a15:0	1.4	1.2	1.2	1.9	2.3	2.7	1.5	1.7	2.6	3.4	2.9	2.7	1.8	1.9	2.9	1.5	2.0	2.7
i16:0	0.2	0.2	0.3	0.5	0.5	0.4	0.2	0.3	0.1	0 1	0.2	0.2		0.1	0.6	0.2	0.2	0.3
i17:0	4.7	0.6	0.7	1.4	2.0	1.4	1 5	1.5	1 8	0.3	0.6	0.8	1.0	1 3	2.4	1.5	1.7	2.4
Sum BCFA	21	18	18	22	26	37	33	35	41	18	21	19	23	23	30	33	35	40
13:1				0.1	0.1					0.2	0.1	0.1				0.1		
14:1	0.4	0.4	0.3	0.2	0.2	0.2		0.2	0.3	0.4	0.3	0.3	0.3	0.2	0.2	0.2	0.1	
15:1	0.3	0.5	0 2	0.2	0.2	0.2	0.2	0 3	1.1	0.2	0.3	0.2		0.1	0.2	0.2	0.3	0.8
16:1n-7	22	25	22	20	17	12	14	13	18	25	21	28	22	20	20	14	17	16
17:1	1.4	1.5	2.3	3.4	3 8	4.3	4.3	3.7	7.2	1.2	1.4	1.2	2.4	2.4	2.5	4.2	3.4	2.5
17:1	0.8		0.8	0.6	0.6	0.3		0.3	0.7	0.4	0.6	0.3	0.4	0.3	0.2	0.4	0.4	6.5
18:1(n-9)	0.8	0.6	1.2	1.8	2.3	1.1	1.0	1.0	1.8	0.7	0.6	1.5	1.5	1.9	3.2	1.0	1.3	2.2
18:1(n-7)	8	10	8	4	6	2	3	2	3	6	7	7	6	5	2	3	2	4
20:1	0.9	0.2	0.3	0.2	0.3	0.2			0.5	0.8	0.1	0 2	0.7	0.3	0.6		0.2	2.5
22:1	3.0		0.9															
Sum MUFA	37	38	35	30	31	21	23	21	33	35	32	39	33	30	30	24	25	34
C_{18} PUFA	1.6	1.2	0.8	0.2	0.3	0.2	0.3	0.3	0.8	0.9	1.0	0.5	0.6	0.3	0.1	0.3	0.2	0.8
20.4(n-3)	0.2	0.3	0.3	0.2	0.3	0.2	0.2	0.2		0.3	0.2	0.2	0.2	0.3	0.2	0.2	0.2	
20:5(n-3) "EPA"	**16**	**19**	**15**	**12**	**10**	**11**	**12**	**14**	**3**	**19**	**20**	**14**	**14**	**13**	**6**	**12**	**10**	**3**
20.3(n-6)	0.3	0.3	0.4	0.4	0.5	0.3	0.1	0.2	0 1	0.2	0.3	0.2	0.2	0.3	0.2	0.1	0.2	
C_{20} PUFA	1.5	2.4	1.3	1.0	0.9	0.6	0.5	0.6	0.6	1.5	2.1	0.8	0.7	0.7	0.5	0.5	0.5	0.4
22.6(n-3) "DHA"	0.3	0 4	0.3	0.3	0.3	0.2	0.2		0.1	0.4	0.5	0.3	0.2	0.3	0.2	0.2	0.2	
Sum PUFA	20	23	18	14	12	13	13	15	4	22	24	16	16	15	7	13	11	5
cy19:0	0.1	0.1	1.0	3.4	1.5	0.2	0.7	0.7	0.2	0.1	0.5	0.8	0.5	1.1	0.5	0.7	0.6	

6.7 Effect of salinity on individual fatty acids

For high salinities, an increase in saturated BCFA (i15:0: 18-24 % ACEM 6 and 18-20 % ACEM 9) and MUFA (17:1,16:1(n-7)) were observed for both strains (Table 6.1). At the highest salinity range (66 psu), PUFA and 16:0 decreased and i15:0 and 17:1 increased. The highest proportion of EPA was noted in ACEM 6 at 33 psu (13.6 %) (Table 6.1). Interestingly i13:0 remained the same within the temperature and salinity experiments but differed between the two experiments.

6.8 Effect of temperature on individual fatty acids

The highest overall PUFA (Figures 6.1 and 6.2) and EPA (18.6 % ACEM 6: 20 % ACEM 9) proportions were at 4 °C (Table 6.1). Growth was not as vigorous at this temperature for either strain. At 10, 17 and 20 °C the proportion of EPA remained relatively constant at 13.5 % for ACEM 9 and 10 to 15 % for ACEM 6. PUFA remained high for both strains even at 24 °C (11 % for both ACEM 6 and ACEM 9). The changes in fatty acid proportions revealed an increase in saturated BCFA with increasing temperature. An inverse relationship of BCFA to MUFA occurred (Figures 6.1 and 6.2). At 17 °C, cy19:0 increased to 3.4 % of the total fatty acids for ACEM 6.

6.9 Phylogenetic placement

The two *Shewanella* strains possessed similar 16S rRNA sequences. The strains are phylogenetically novel strains that form an outlying lineage with a group of *Shewanella* species that have a non-halophilic, psychrotolerant ecophysiology (*S. frigidimarina*, *S. baltica* and *S. oneidensis*). The Huon Estuary strains are distinct from the cluster of psychrophilic Na^+-requiring *Shewanella* species in which PUFA synthesis is a common trait (Figure 6.3).

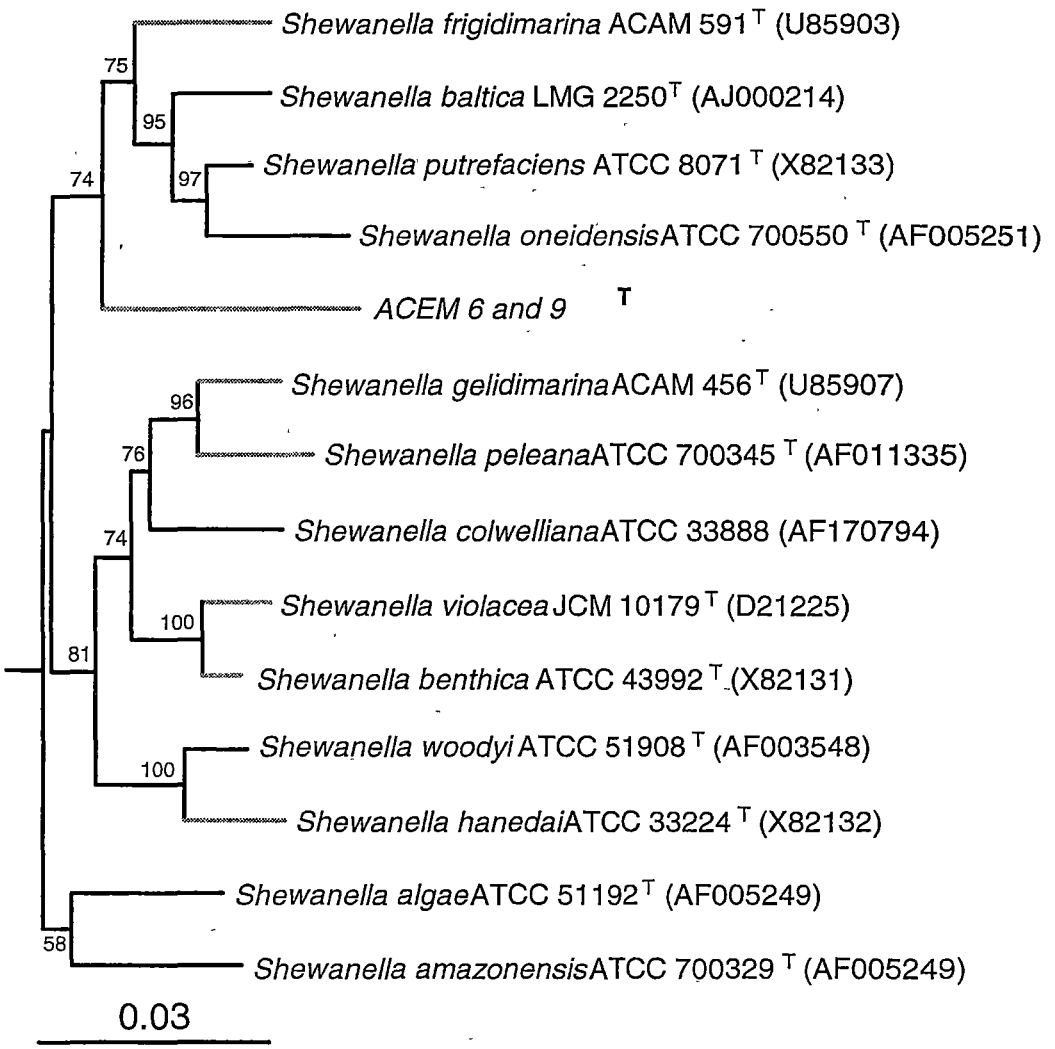


Figure 6.3 Unrooted phylogenetic tree based on 16S rRNA comparisons showing the taxonomic positions of ACEM 6 and ACEM 9 within the genus *Shewanella*. The branching pattern was generated by the neighbour-joining method. Bar equals sequence dissimilarity. Light lines indicate known PUFA producing species. Black lines indicate species that do not contain PUFA (adapted from Bowman *et al.* 2001, Russell and Nichols 1999). PUFA production in *S. woodyi, S. colwelliana, S. oneidensis* is unknown.

Discussion

Many *Shewanella* species have been isolated from sea-ice environments where they produce PUFA as a protection mechanism against freezing. *Shewanella* species that produce high levels of EPA have until recently only been isolated from these cold and/or barophilic environments. However, estuarine environments can be viewed as systems with similar characteristics to melting sea-ice (Nichols *et al.* 1995) as they have highly variable salinity and distinct pycnoclines where salt and fresh water layers meet.

Temperature variation may also play a role in the presence of PUFA and *Shewanella* spp. in the estuary. This is because of the changes that occur from the brackish to marine environments. The fresh water layer has input from mountain snow in winter yet can rise to 19 °C in summer due to the pigmentation in the water (chapter 2). Fatty acid profiles of the two *Shewanella* strains indicate that they would contain maximum PUFA at temperature conditions commonly occurring in this brackish water environment. However, both strains are capable of high proportions of EPA production at higher temperatures (up to 25 °C). This is unusual when compared with other *Shewanella* strains studied to date.

The importance of salinity as a factor in bacterial production of PUFA in addition to temperature has been raised recently (Nichols *et al.* 2000, Nichols *et al.* 1994). Our study on the two *Shewanella* strains further confirms that salinity can play a role in bacterial PUFA production. The Huon Estuary typically has salt levels in the range where the endemic *Shewanella* population produces high proportions of PUFA. The inverse relationship between PUFA proportion and BCFA and MUFA with respect to salinity (Nichols *et al.* 2000) was also observed in this study. Similarities can be drawn again between sea-ice and estuaries with dramatic changes in salinities being a common feature of both systems.

The two strains fall most closely into a section of the genus *Shewanella* which includes species adapted to brackish aquatic ecosystems. *S. baltica*, a psychrotrophic species which does not contain EPA (Nichols *et al.* 2000) and *S. frigidimarina* which does produce low proportions of EPA (2-7 %) (Bowman *et*

al. 1997) are close relatives of ACEM 6 and ACEM 9. Other members of the genus *Shewanella* containing high concentrations of EPA, are the barophilic or psychrophilic species such as *S. gelidimarina* (12-16 % EPA), *S. benthica* (16-19 % EPA) and *S. hanedai* (19-22 %) (Bowman *et al.* 1997). The two strains from the Huon Estuary are consequently capable of producing nearly the same proportions of EPA despite not requiring this for cold or pressure adaptation. They also produce these high PUFA proportions at higher temperatures, yet equivalent biomass, when compared with the barophiles and psychrophiles in the genus.

Bacteria are generally considered to be minor producers of PUFA in the marine environment in comparison to algal species. However, during winter periods it is possible that when algal biomass is low, these bacteria have the ability to provide essential fatty acids for the higher trophic levels in this environment. It is also possible that the biosynthesis of high proportions of EPA capable of being produced at rapid growth rates and high temperatures gives this species potential for biotechnological exploitation.

7. Interactions of bacteria with algal blooms in the Huon Estuary using molecular and morphological techniques

Summary

Bacteria were closely associated with the three main algal blooms in the Huon Estuary during 1999. Species from the CFB and gamma (γ) proteobacteria clusters were most strongly associated with algal blooms and their decline. Algicidal bacteria were regularly observed in the estuary at all depths and sites sampled. *Pseudoalteromonas* was a dominant genus during the *Pseudonitzschia* spp. bloom as indicated by FISH analysis. The CFB cluster was common throughout the water column during blooms of the dinoflagellate *G. catenatum*. Alpha (α) and γ proteobacteria were widespread in the estuary but beta (β) proteobacteria were not. Unidentified bacterial species were present that did not bind with the universal bacterial probes tested. These may have been from the gram-positive cluster or from the *Planctomycetales* or may have been bacteria with low levels of rRNA. Between 5 and 15 colony types were present for each depth sampled. Variation in colony morphotype was greater between depths than sites. Bacteria present in the integrated water column samples were characterised by higher numbers of white colonies at all sites. Surface and pycnocline samples were dominated by pigmented isolates. Changes in the dominance of different bacterial species or communities with respect to blooms of different algal genera were clearly observed. The microbial community was dominated by different bacterial species related to prevailing environmental conditions. Overall, similar microbial trends were observed using traditional or molecular techniques.

Introduction

Advances in molecular phylogeny have enabled the study of bacterial populations using culture-independent approaches such as fluorescence *in situ* hybridisation (FISH) (Amann and Ludwig 2000, Amann *et al.* 1995, Amann 1995). The 16S rRNA molecule and to some extent the 23S rRNA molecule are important tools in molecular phylogenetic studies. Comparison of 16S rRNA sequences of different bacteria can reveal variability indicative of evolutionary relationships between species. Small 20 base pair segments (probes) of either the 16S or the 23S rRNA molecule are generally sufficiently distinctive to allow identification of particular clusters, genera or species of bacteria. Flurophores are attached to these probes and using FISH, the bacterial group of interest in a sample can be identified.

Successful use of fluorescent bacterial oligonucleotides to study sewerage, activated sludge and biofilms has been reported (MacDonald and Brozel 2000, Bond *et al.* 1999, Wagner *et al.* 1996, Amann *et al.* 1995). More recently, fluorescent probes have successfully identified bacterial community structure in marine environments (Cottrell and Kirchman 2000, Eilers *et al.* 2000, Glöckner *et al.* 1999, Giuliano *et al.* 1999, Lebaron *et al.* 1997, Glöckner *et al.* 1996).

There are difficulties associated with using FISH in samples from estuaries, rivers or oceans as bacteria in these environments have a limited and variable nutrient supply so are unable to maintain high levels of growth and production (Amann *et al.* 1995). Low nutrient levels result in fewer copy numbers of 16S rRNA within the cell for the fluorescent probe to attach to. The probed species will thus fluoresce less than those found in a nutrient rich environment. Reporting on a laboratory study, Oda *et al.* (2000) noted "In habitats with growing, non-growing and starving bacteria, data on quantitative detection of populations based on 16S rRNA-targeted probing should be used with extreme caution as detection of the individual cells is strongly influenced by their physiological history and current physiological state".

In addition, the proportion of dissolved organic matter can greatly affect fluorescence levels in oligonucleotide probe hybridisations (Alm *et al.* 2000), which is an important factor in a humic rich estuary. This is because a decrease in the probe signal response occurs as humic compounds increase in the extracts. The humic compounds saturate the hybridisation membrane resulting in a lower amount of probe bound to the target rRNA in the cell (Alm *et al.* 2000).

For the estuarine samples in this study, a fluorescent oligonucleotide specific for the genus *Pseudoalteromonas* was used to identify this genus given its high algicidal potential. The use of a fluorescent oligonucleotide specific to the CFB was also of interest because of the association of this group with algal bloom decline and algicidal activity.

Objectives of the research reported in this chapter were to:

- examine the water column from 1998 to 1999 at sites F1, F3 and X3 for the presence of algicidal species;
- examine the bacterial community and their interactions in the Huon Estuary using traditional morphological and molecular techniques (FISH);
- identify changes in the dominant bacterial communities during the *Pseudonitzschia* spp. bloom and the summer and autumn *G. catenatum* blooms;
- identify if the PUFA producing *Shewanella* species are dominant in the water column and, if so, at what depth and which part of the season;
- observe changes in other distinctive bacterial morphotypes at different depths and sites for each date. Morphological changes over the seasons for the four different depths and three sites are described using principal components analysis (PCA);
- compare morphological and molecular techniques as tools for observing changes in the bacterial community These two techniques complement the community analyses by lipid and fatty acid profiles in chapter 5.

Methods

Complementary results for the same sampling sites, dates, and depths as described in this chapter are discussed in chapter 2 (physical, chemical and algal abundance), chapter 3 (morphology) and chapter 5 (identification of algal and bacterial genera using lipid biomarkers).

7.1 Sample collection, growth conditions

Bacteria were isolated from the water column and sediment of the Huon Estuary. Sites were F1, F3, and X3 (chapter 2). As temperature plays a strong role in controlling estuarine bacterial dynamics (Shiah and Ducklow 1995), water samples were incubated at *in situ* river temperatures (12-15 °C) until they were plated onto media 1 to 2 hours after sampling. This was to ensure that bacterial activity was not stimulated or retarded relative to their natural environment (Shiah and Ducklow 1995).

Subsamples (10-50 µl) from sites and depths for each date were plated and incubated in triplicate on modified marine agar at two different salinities (28 psu and 7 psu). The media contained 14 g agar, 4 g bacteriological peptone (Oxoid), 1 g yeast extract (Oxoid) and 1 l, 0.7 µm filtered Huon Estuary river water with the salinity adjusted to 28 psu or 7 psu using artificial sea salts. Two salinities were used as salinities in the estuary range from 5 psu to 35 psu. Colony pigment formation on media containing river water and distilled water was compared and pigmentation was found to be enhanced using river water. Enhanced pigmentation allowed easier identification of the isolates in comparison to those samples grown on media just using distilled water and sea salts.

Bacterial abundance was assessed by counting colony-forming-units (CFU) for the 3 sites and at 4 different depths (Figure 7.1, Table 7.1). Algicidal bacteria and *Shewanella* species had visually obvious colony morphologies and their specific numbers were enumerated by CFU.

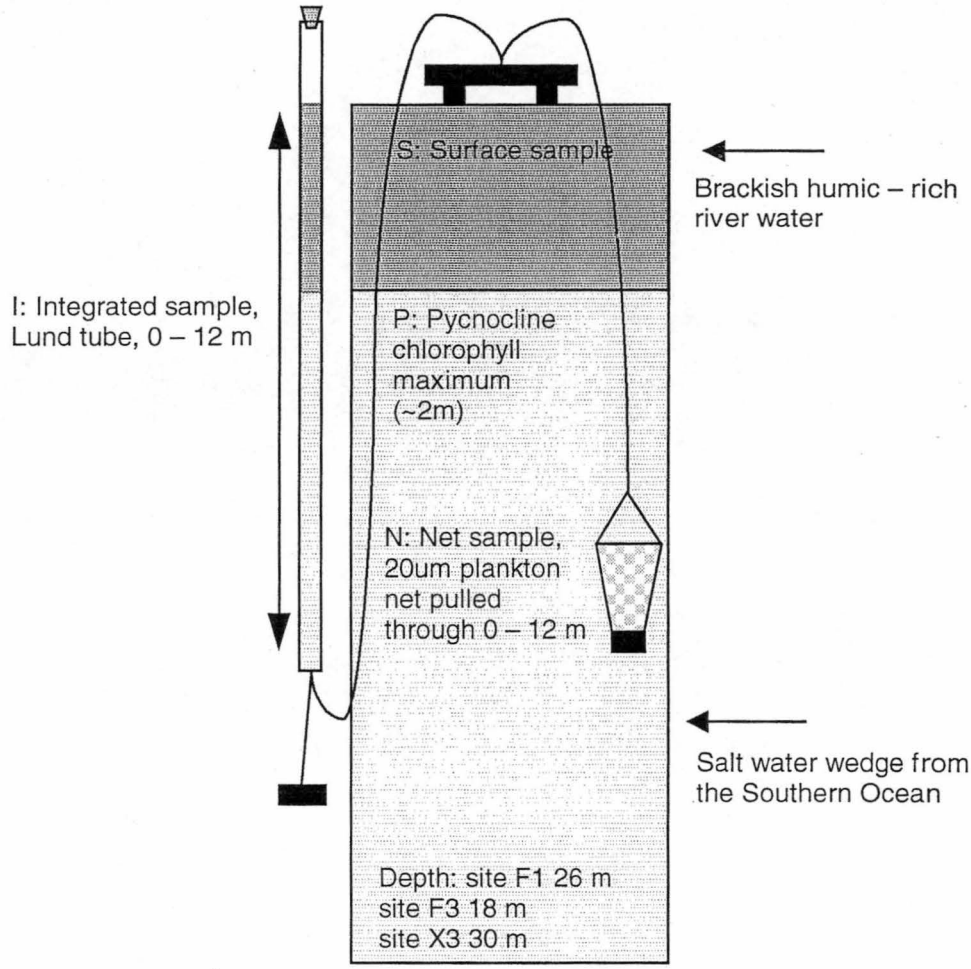


Figure 7.1 Schematic representation of the sample types taken during the field study and depths of the three sites. Stratification of the brackish and salt water is indicated. (Repeat of Figure 2.2)

Table 7.1 Sampling methods for collection of samples from sites F1, F3 and X3. (Repeat of Table 2.1)

Depth	Sample type	Collection device	Depth	Sampling procedure
I	Integrated	Lund tube (weighted 2.5 cm x 12 m silicone tubing ~ 5 l)	0 – 12 m	Water collected in tube, emptied into plastic container, mixed and sub sampled
S	Surface	5 l Niskin	0 – 0.2 m	Niskin inverted 3 times
P	Pycnocline /Mid depth	5 l Niskin	2 m or at the pycnocline which was calculated with a salinometer	Niskin inverted 3 times
N	Net/particulate fraction	20 µm net	0 – 12 m (F3N 0 – 9 m)	Plankton net sampled at ~1 m/sec

For most sample dates, the integrated sample (0-12 m) from site F3 was also analysed using the fluorescent stain 4'6'-diamidino-2-phenylindole (DAPI, Sigma) to identify total bacterial numbers for comparison with total CFU (section 7.4). Data for two years were collected from one site (X3) but only data for the second year is presented in this chapter (chapter 2 contains bacterial numbers for X3 during 1997-98). Traditional morphological techniques enabled identification of the algicidal bacteria, which were the focus of the field study.

Although the standard incubation method for CFU in environmental samples is 3-5 days, many isolates in this study did not develop until 7 or more days had elapsed. Cultures were therefore counted after incubation at 22 °C for 10-14 days. Colony groups were categorised into 41 different colony types that had been noted as common isolates in prior research (J. Skerratt, unpublished data). A majority of bacteria were categorised as appearing white-cream, this category included a number of different colonial morphotypes that could not be reliably distinguished and as a result, this category was assumed relatively diverse in species. Colonies that did not fit into the 41 described morphologies were categorised "other". Some of the 41 identifiable morphotypes were similar to the ACEM isolates that were isolated from the Huon Estuary water column and sediment (chapter 5).

7.2 Statistical analysis

Principal components analysis (PCA) was used to describe the results of the morphotypes present in the Huon Estuary samples. PCA is an ordination technique used to reduce the dimensionality of multivariate data sets and enable graphical presentation of the relationships between factors. Prior to statistical analysis, environmental data were screened to reject any variables where samples were not collected for all depths and sites. Relationships between dates and bacterial colonies were examined using PCA (PCA CANOCO Version 3.12; Ter Braak 1998, 1990). Forty morphotypes were included in the analysis of 273 samples obtained for different sites and depths. Statistical analysis was also completed using ANOVA (SAS) to test the statistical significance of the differences between the obtained means of the different bacterial morphologies

from the sites and depths. Statistical analyses of data for ANOVA were performed using the 'General Linear Models Procedure' of the software package 'SAS System for Windows v 6.12' (SAS Institute Inc. USA). Differences between individual means were deemed to be significant at $\alpha = 0.08$. ANOVA analysis demonstrated that communities were less similar between depths than between sites throughout the season.

The 41 bacterial morphotypes were separated into 11 different vectors. Separation and grouping of these 11 different vectors was based on correlation with initial PCA clustering using all 41 bacterial morphotypes, or the morphotypes interest value (i.e. *Shewanella* species). Colonies morphologically categorised as 'pinpoint white or cream' dominated the bacterial communities at certain depths and at specific times of the year rather than large pigmented or unpigmented colonies. These colonies were therefore grouped in their own category for the final statistical analysis as they clustered away from the other morphotypes in the initial PCA of the 41 colony morphotypes.

ACEM 1, ACEM 21 and ACEM22 dominated colonies categorised as "algicidal". These three algicidal bacteria clustered together in the initial analysis of the 41 colony morphotypes. ACEM 4 only appeared for two sampling dates during the season. The algicidal morphotype excluded the yellow-orange morphotype that may include the algicidal species ACEM 20 and some ACEM 1 colonies, as many yellow-orange morphotypes were not algicidal.

Colony morphotypes categorised as 'tan' may have included *Shewanella* species as the genus *Shewanella* do not always have distinctive pink-tan colonies or high proportions of PUFA.

After the initial PCA of the 41 colony morphotypes categorised the morphotypes into general clusters, the final 11 vectors were as follows:

1. pinpoint white or cream colonies (< 1mm)
2. cream and white colonies (>1mm did not include colonies from morphotype 1)
3. algicidal (included morphotypes typical of ACEM 1, ACEM 4, ACEM 22 and ACEM 21)

4. bright or translucent orange
5. mucoid orange, gram-negative
6. yellow-orange, gram-negative
7. pink
8. tan (may include some *Shewanella* species)
9. clear or with halo
10. *Shewanella*-like (gram-negative tan-pink colonies)
11. yellow gram-positive cocci.

7.3 FISH

7.3.1 Oligonucleotide probes

Oligonucleotides used in this study were specific for common marine bacterial classes. Probe specificities were for Eubacteria, CFB cluster and the α, β and γ subdivisions of proteobacteria and *Pseudoalteromonas-Colwellia* (Table 7.2) (Amann *et al.* 1990, Wallner *et al.* 1993, Manz *et al.* 1992, Neef 1997, Manz *et al.* 1996, Neef *et al.* 1998, Stahl and Amann 1991, Giuliano *et al.* 1999, Pukell *et al.* 1999). Species detected by the *Pseudoalteromonas-Colwellia* probe would be expected to be dominated by the genus *Pseudoalteromonas* given the highly psychrophilic nature of the genus *Colwellia* (Bowman *et al.* 1998a). Probes were purchased from GenSet (South Australia) and labelled with the indocarbocyanine fluorescent dye Cy3 (Amersham Pharmacia Biotech Ltd; Absorbance Max 552 nm Emission Max 570 nm). FISH probes were prepared and stored as a concentrated standard (200 ng/µl) in 1 x trisEDTA (Sigma) RNAse free water (Sigma) and then diluted to a working standard (50 ng/µl). Hybridisation solutions were filter sterilised and frozen as 1.5 ml aliquots at -80 °C.

Table 7.2 Oligonucleotide probes used for FISH in samples from this study† and other marine studies.

Probe	Probe sequence (5'-3')	Specificity	% Formamide in buffer	Original reference
†Eub338	gctgcctcccgtaggagt	Eubacteria	0	Amann *et al.* 1990
†Non338	actcctacgggaggcagc		0	Wallner *et al.* 1993
†Gam42a	gccttcccacatcgttt	γ subclass of Proteobacteria	35	Manz *et al.* 1992
†Bet42a	gccttcccacttcgttt	β subclass of Proteobacteria	35	Manz *et al.* 1992
†Alpha968	ggtaaggttctgcgcgtt	α subclass of Proteobacteria	20	Neef 1997
†CF319a	tggtccgtgtctcagtac	*Cytophaga-Flavobacterium* cluster of CFB Phylum	30	Manz *et al.* 996
Pla886	gccttgcgaccatactccc	Planctomycetales	35	Neef *et al.* 1998
Arch915	gtgctcccccgccaattcct	Archaea	35	Stahl and Amann 1991
†*Pseudoalteromonas*	catcttctagcaagctagaaatg	*Pseudoalteromonas* and *Colwellia* species	35	Pukall *et al.* 1999
†*Vibrio* (G V)	aggccacaacctccaagtag	*Vibrio*	35	Giuliano *et al.* 1999

7.3.2 Preservation and hybridisation

Methods for concentration and preservation varied. Generally triplicate 50-80 ml of water for all samples was filtered though 47 mm, 0.2 µm, black Isopore polycarbonate membrane Millipore filters and preserved and hybridised using the methods of Alfreider *et al.* (1996) and Glöckner *et al.* (1999). Essentially, after water samples were filtered, filters were placed on a 4 % paraformaldehyde (PFA) saturated filterpad for 3 hours at 4 °C (PFA: 6 g PFA in 100 ml preheated MilliQ (75 °C), 2M NaOH added dropwise until the solution was clear, 50 ml of 3 x phosphate buffered saline (PBS-see below for protocol) was added, cooled on ice and the pH adjusted to 7.2. PFA was stored at 4 °C and used within 24 hours or frozen in 14 ml aliquots at -20 °C). Filters were then air dried after which 2 ml of ice cold 50 % ethanol: 50 % 1 x PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4 pH 7. filtered through 0.2 um poly carbonate filters (Millipore) and autoclaved at 120 °C for 15 minutes) was passed through the filter. Filters were dried, folded and stored in foil at -20 °C.

For hybridisation, filters were cut into small sections (0.5 cm^2) and placed in a 35 mm sterilised petri dish (Iwaki). The hybridisation buffer was placed on top of the filter. Glöckner *et al.* (1996) demonstrated that this method removes less than 10 % of cells from the filter due to the adhesion of the cells to the polycarbonate filter once preserved. The hybridisation buffer stringency was specific for the probe (Table 7.3) and included 32 µl of the buffer (Appendix) and 4 µl (50 ng/µl) of the required fluorescent probe. Petri dishes were sealed with parafilm, covered in aluminium foil and placed at 46 °C in a water bath for 1.5 hours.

Table 7.3 Hybridisation solutions for specific bacterial oligonucleotides

Hybridisation solution	*Pseudoalteromonas*, CFB, beta/gamma proteobacteria	Alpha proteobacteria	Eubacteria
NaCl	1.04 g	1.04 g	1.04 g
Tris HCl (0.04M, pH 7.4)	10 ml	10 ml	10 ml
10% SDS	200 µl	200 µl	200 µl
Deionised formamide	7 ml	4 ml	0 ml
Milli-Q water	2.8 ml	5.8 ml	9.8 ml

To determine bacterial abundance, filter sections were washed and stained for 15 minutes in the dark at 48 °C with the individual washing buffer specific for the probe (Table 7.4) and containing 2 µg/ml DAPI. Hybridisation wash solutions were filter sterilised and stored a +4 °C.

Table 7.4 Wash solutions for specified bacterial oligonucleotides

Wash solutions	*Pseudoalteromonas*, CFB, beta/gamma proteobacteria	Alpha proteobacteria	Eubacteria
NaCl	0.46 g	1.32 g	5.62 g
Tris HCl (1 M, pH 7.4)	2 ml	2 ml	2 ml
EDTA (0.5M) pH 8.0	1 ml	1 ml	1 ml
1 % SDS	1 ml	1 ml	1 ml
Milli-Q water	96 ml	96 ml	96 ml

Filters were mounted with Citifluor (Citifluor, Leicester) or Slowfade Light (Molecular Probes, Oregon) on glass slides and inspected with an epifluoresence microscope (Leica, Germany), equipped with a 50-watt HBO mercury bulb (Osram) and specific filter sets for DAPI and Cy3. Each microscopic field was first viewed with the Cy3 filters before switching to DAPI filters to avoid bleaching of the Cy3 probe during DAPI examination. Photobleaching (as distinct from quenching) of the Cy3 fluorophore occurred in less than 2 minutes (Molecular Probes states 100 seconds). Some problems occurred with antifade reagents as they reduced the total fluorescence emission. This can create a problem for marine samples that may not have an initial strong fluorescence. The mounting fluids Slowfade Light or Citifluor were adequate under these circumstances.

All probe specific cell counts are presented as the percentage of cells visualised by DAPI. This accounts for the underestimation of cell numbers using the EUB probe for some samples where the sum of the probes was greater than the measurement of EUB percentage against DAPI. The mean percentage abundances were estimated from counts of 15-20 random fields (20-40 for dates 13/5 and 24/5) on each filter section similar to the method of Lebaron *et al.* (1997). Samples analysed using FISH were run concurrently with positive and negative controls for the probes being used. The probes fluorescent signal for the results reported in this

thesis was distinct from the unmarked cells. Samples where probe signals were indistinct are not shown.

Samples from 20/11/98 to 13/5/99 employed additional protocols for FISH that involved different preservation and hybridisation methods. Water samples from surface pycnocline and integrated samples were filtered at low pressure onto white 0.2 µm polycarbonate filters (Millipore). Filters were immediately vortexed, briefly sonicated with 2mM Na-pyrophosphate (Velji and Albright 1986) and washed with filtered seawater into 15 ml centrifuge tubes.

Plankton net samples include particulates from the water column greater than 20 µm. These samples were further concentrated via centrifuging and then preserved. Preservation methods for the net samples and the above mentioned rinsed filter volumes were similar to those used for filtered samples. An equivalent volume of 4 % PFA was added to the sample and left 3 hours before centrifuging, removal of the supernatant (x 2) and storage in 50:50 ethanol: 1 x PBS at -20 °C. 2 µl of the samples were then spotted onto 6, 12 or 24 well hybridisation slides, dried and then dehydrated using an ethanol dehydration series (50 %, 80 % and 95 % ethanol, 3 min, 3 min, 3 min) and stored at -20°C (Appendix). Preserved net samples were also hybridised in solution in Eppendorf tubes rather than on slides.

7.3.3 FISH technique

Due to the relatively recent application of FISH in marine environments, the problematic nature of its use in these conditions will be discussed in section 7.8.6. Success with FISH is often gauged by the percent of eubacterial probe against the DAPI stain. EUB338 labelled between 15 and 65 % of DAPI for most samples. Samples where EUB338 hybridised less than 50 % of the DAPI stain for the majority of depths and sites at particular dates are not shown.

It was possible to use FISH successfully for a selection of dates and sites with results for ~30 % of the samples collected, preserved, hybridised or counted presented in this thesis.

Impact of humic compounds on FISH in samples from the Huon Estuary.

The levels of humic compounds in the Huon Estuary are high because of the extensive plains of native button grass (*Gymnoschoenus sphaerocephalus)* in the catchment. The chromophoric (or coloured) dissolved organic matter (CDOM) of the Huon Estuary is among the highest values recorded for Australian water bodies (Absorbance coefficient of the CDOM at 440 nm was 7-14 /m (HST 2000)). To test the impact of humic compounds on the efficiency of FISH, a short experiment was undertaken using two dilutions of river water from the upper reaches of the Huon Estuary (diluted by 2/3 with autoclaved distilled water). The samples were immediately analysed with the EUB338 probe and the dilution factor was taken into account for the enumeration of bacterial numbers. Without dilution, 59 ± 5 % of DAPI stained cells were labelled with the EUB338 probe. With 2/3 dilution the result was 72 ± 8 %, indicating that the humic compounds may have affected the binding of probe in some of the field samples.

The hybridisation solution in the method of Harmsen *et al.* (1997) was initially tested in order to lower interference from humic compounds. The method involves the addition of PolyU (Sigma) and Bovine Serum Albumin (BSA, Sigma) and was designed for hybridisation of soils containing humic compounds. For Huon Estuary water samples, no improvement was observed.

Results

7.4 Bacterial cell numbers for samples sites (CFU and DAPI)

Water samples from sites X3 and F3 contained higher cell numbers for all depths than those from site F1. Throughout the season, cell numbers for the integrated samples at all sites were approximately three times higher than for the surface or pycnocline samples (Figure 7.2). Cell numbers for the surface and pycnocline samples were similar at all sites. An exception occurred for site F1 on the 21/12 sampling date before the summer *G. catenatum* bloom. Cell numbers for the

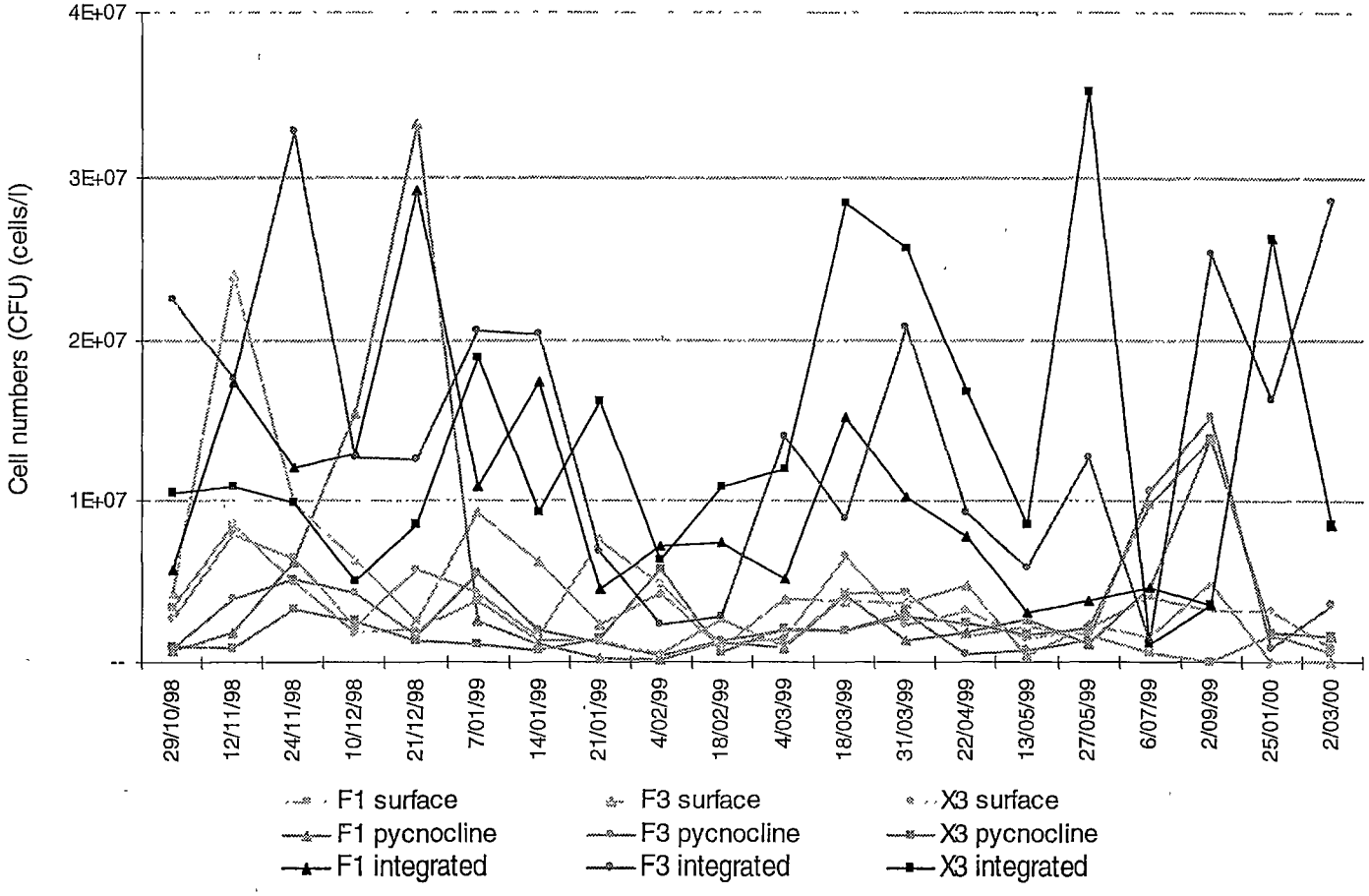


Figure 7.2 Bacterial cell number for sites and depths over the sampling season

surface and pycnocline samples were the same for this date except at site F1 when the pycnocline sample contained higher cell numbers than the integrated sample. Numbers of culturable isolates were therefore higher in integrated samples that contained water from below the photic zone than from surface or pycnocline samples. Sites F3 and X3 also had higher numbers of culturable cells than site F1.

Bacterial cell numbers enumerated using CFU accounted for ~10 % of the bacterial numbers detected using DAPI counts (Figure 7.3) with occasional exceptions, including on the 14/1 and the 24/11 where CFU results accounted for up to 15 % of DAPI count.

7.4.1 Variation in morphotype

ANOVA demonstrated that maximum colony morphotype variation occurred within the depth profile of the samples taken for each site rather than between the sites. The number of different morphotypes for each date and depth across the season were between 5 and 12 (Figure 7.4). No site demonstrated higher proportions of bacterial morphotypes. Net samples taken before and during the initial stages of the summer 14/1 *G. catenatum* bloom showed only 1 or 2 morphotypes.

Algicidal bacteria (as defined in section 7.2) were dominant morphotypes in early summer (Figure 7.5) for all four depths. Most algicidal morphotypes were present in samples taken after the summer *G. catenatum* bloom, and dominated the colonies from the net sample. This infers that these algicidal morphotypes were particle associated. Samples taken from different depths for this same period contained the highest algicidal bacteria concentration. Algicidal bacteria in the net samples appeared to be dominated by the CFB-like morphotypes.

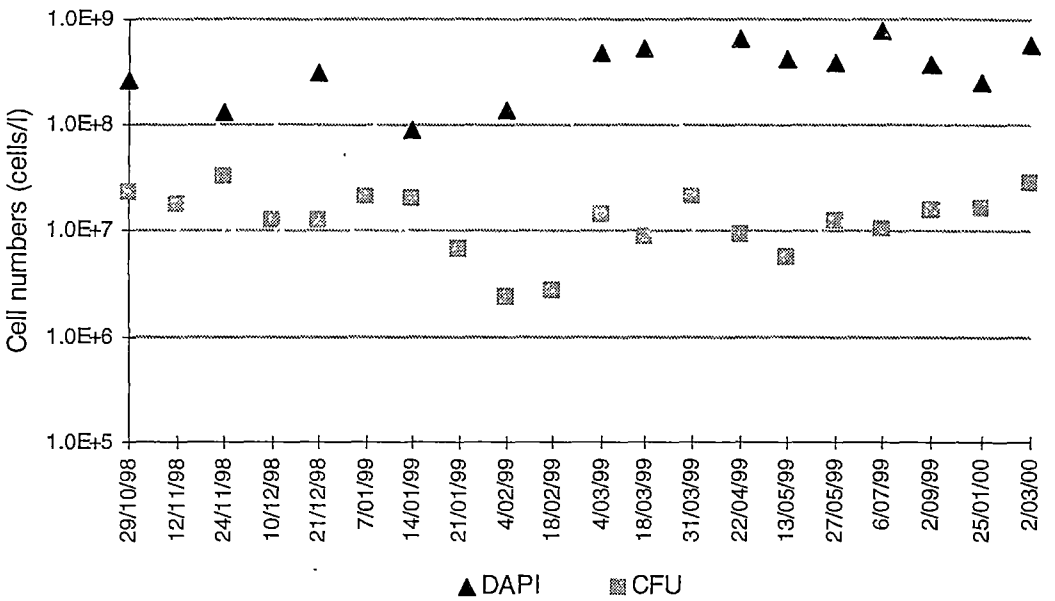


Figure 7.3 Variation of cell numbers over the field season using CFU and DAPI counts for the integrated sample (0-12m) at site F3.

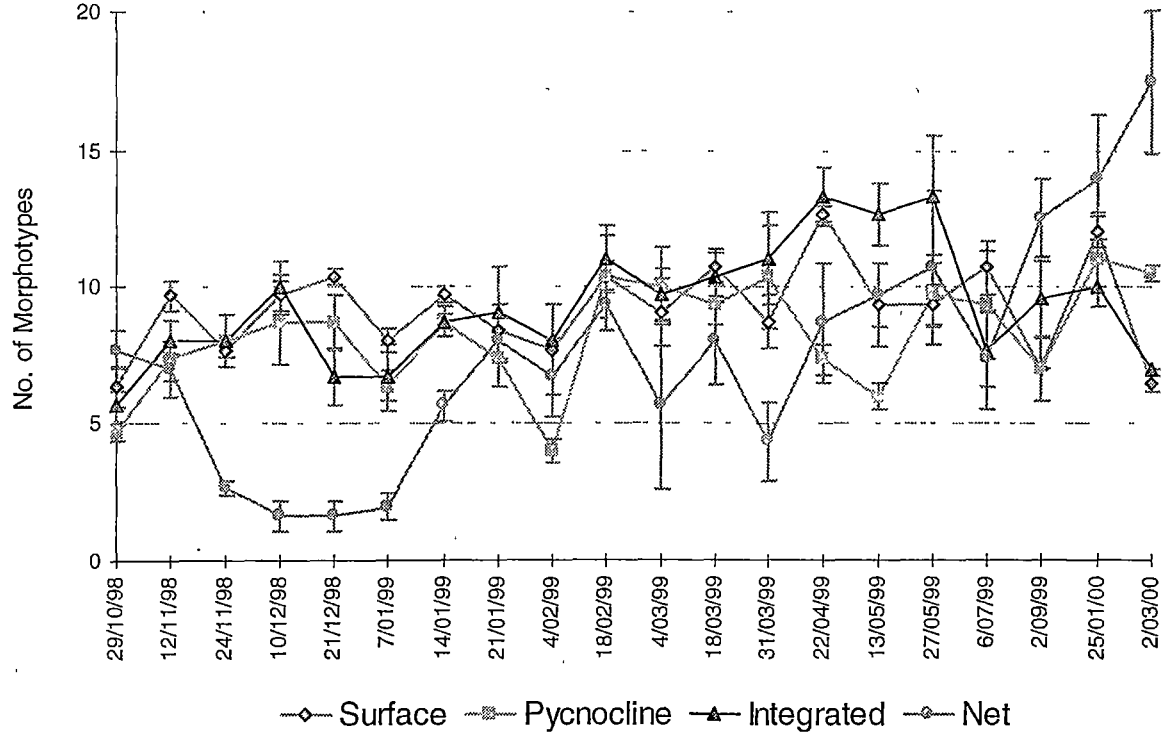


Figure 7.4 Bacterial morphological types for each depth and for the net samples (particulate fraction) over the field season.

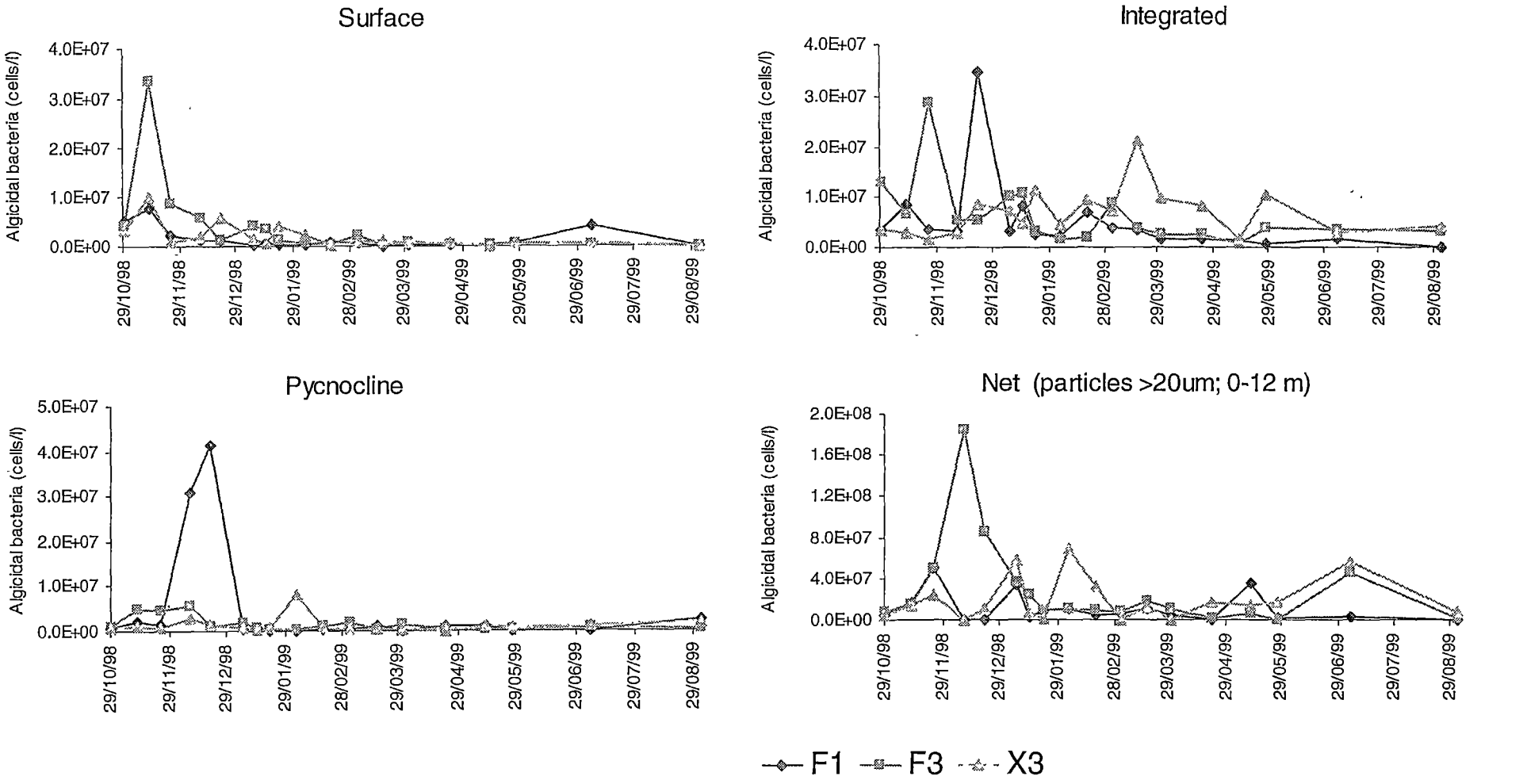


Figure 7.5 Variation in algicidal bacteria numbers (CFU) at 3 sites and depths over the field-sampling season.

7.5 Principal components analysis of morphotypes (PCA)

Although 41 colony morphotypes were identified, four vectors strongly contributed to the overall variation in the field samples. Surface, pycnocline and integrated samples at each of the sites were generally very similar. Differences were observed in morphotype between the depth profiles rather than between sites. This was also observed for environmental parameters and lipid components (chapters 2 and 5).

7.5.1 Cluster analysis of surface water samples

Cluster analysis of surface samples by site

Cluster analysis demonstrated that for the surface water samples, there was no relationship between site location and the occurrence of a particular morphotype during the season (Figure 7.6).

Cluster analysis of surface samples by collection dates

Cluster analysis demonstrated that there was a relationship between morphotypes and the dates that the samples were taken. The surface samples for the three sites clustered together for dates which coincided with both *G. catenatum* blooms and the *Pseudonitzschia* spp. bloom (Figure 7.6). During the peak of the summer *G. catenatum* bloom, the bacterial community was categorised by the dominance of tan morphotypes. Cluster analysis demonstrated that after the summer *G. catenatum* bloom, algicidal bacteria were dominant. The white-cream morphotype correlated with dates during the autumn *G. catenatum* bloom. Throughout this bloom period no correlation occurred between collection dates and the algicidal morphotype. Cluster analysis indicated that the presence of pinpoint white colonies correlated with samples taken during winter and after the autumn *G. catenatum* bloom. Throughout the November 1998 diatom bloom and during the *Pseudonitzschia* spp. bloom, the dominant vector was the algicidal morphotype.

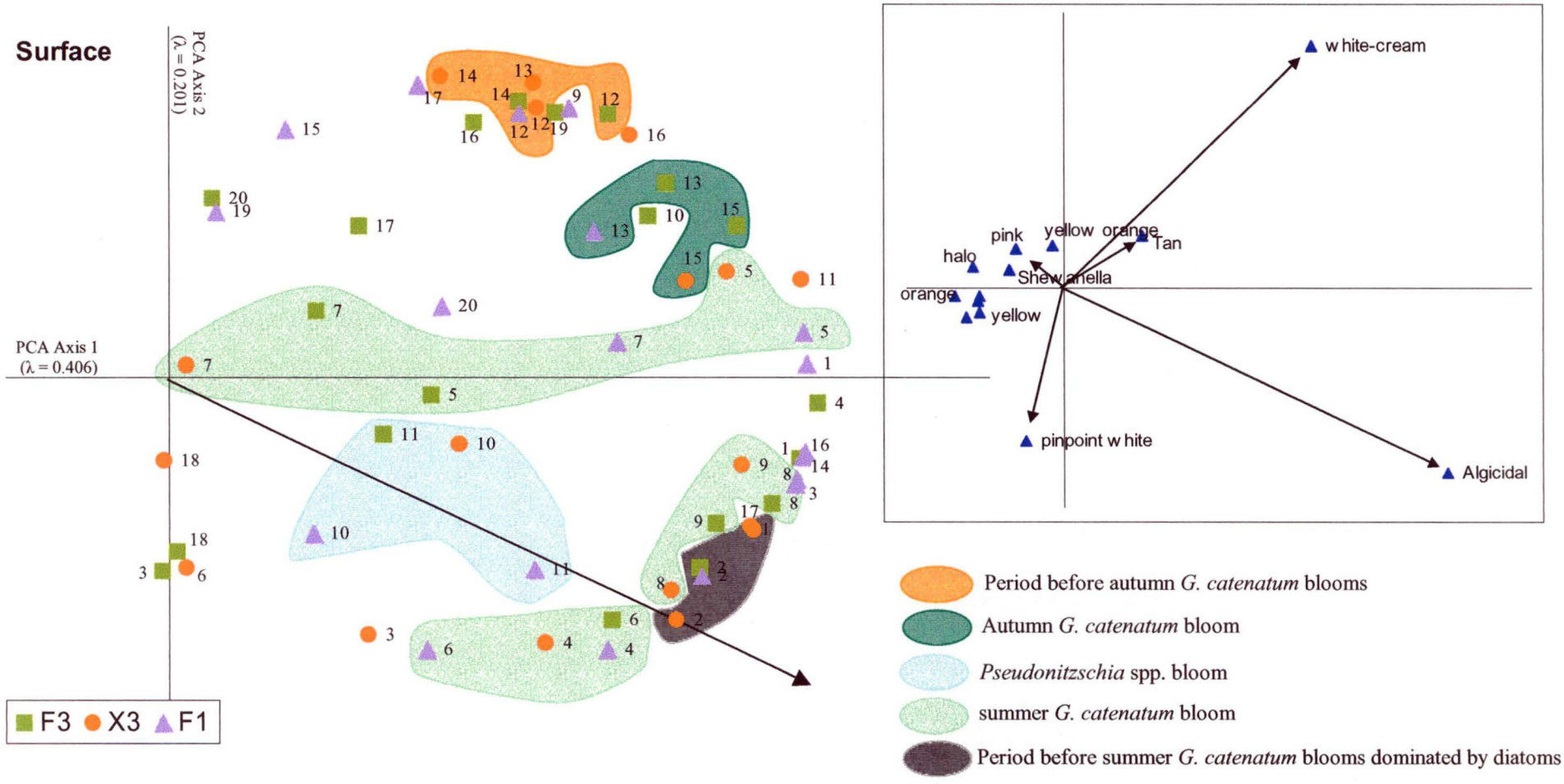


Figure 7.6 Principal component analysis of the 3 sites and 20 dates for the surface samples with the colony morphotype that influence their spacing in the Vector diagram above. Date key: 1=29/10/98, 2=12/11/98, 3=24/11/98, 4=10/12/98, 5=21/12/98, 6=7/01/99, 7=14/01/99, 8=21/01/99, 9=4/02/99, 10=18/02/99, 11=4/03/99, 12=18/03/99, 13=31/03/99, 14=22/04/99, 15=13/05/99, 16=27/05/99, 17=6/07/99, 18=2/09/99, 19=25/01/00, 20=4/2/00. Main PCA diagram has algicidal bacteria vector inserted as reference

7.5.2 Cluster analysis of pycnocline samples

Cluster analysis of pycnocline samples by sites

The algicidal morphotype was the dominant vector for the pycnocline samples for all sites during the November 1998 diatom bloom and before the autumn *G. catenatum* bloom (Figure 7.7). The algicidal morphotype was the dominant vector at site F3 before both the summer and autumn *G. catenatum* blooms.

Cluster analysis of pycnocline samples by collection dates

Cluster analysis of morphotypes showed that all sites clustered together for similar dates during the sampling season (Figure 7.7). The algicidal vector was the dominant morphotype during the earliest diatom bloom and again throughout the *Pseudonitzschia* spp. bloom (Figure 7.7). The algicidal and white-cream vectors were equally dominant vectors during the dates of the autumn *G. catenatum* bloom. A broad range of morphotypes that were not related to the three strongest vectors were observed during dates when the summer *G. catenatum* bloom occurred. Pinpoint white morphotypes were associated with the winter dates sampled in July and September.

7.5.3 Cluster analysis of Particulate (Plankton Net) samples

Cluster analysis of particulate fraction samples by sites

Cluster analysis, performed to detect correlations between sites and particulate samples, showed that there was no clear relationship. (Figure 7.8).

Cluster analysis of particulate fraction samples by collection dates

Clustering was distinct for the plankton net particulate samples for all dates (Figure 7.8). Again, the algicidal morphotype demonstrated a strong association with sampling dates coinciding with the *Pseudonitzschia* spp. bloom for sites F3 and X3. During this same period, dates for site F1 correlated more strongly with the white-cream morphotype. The summer *G. catenatum* bloom was associated with

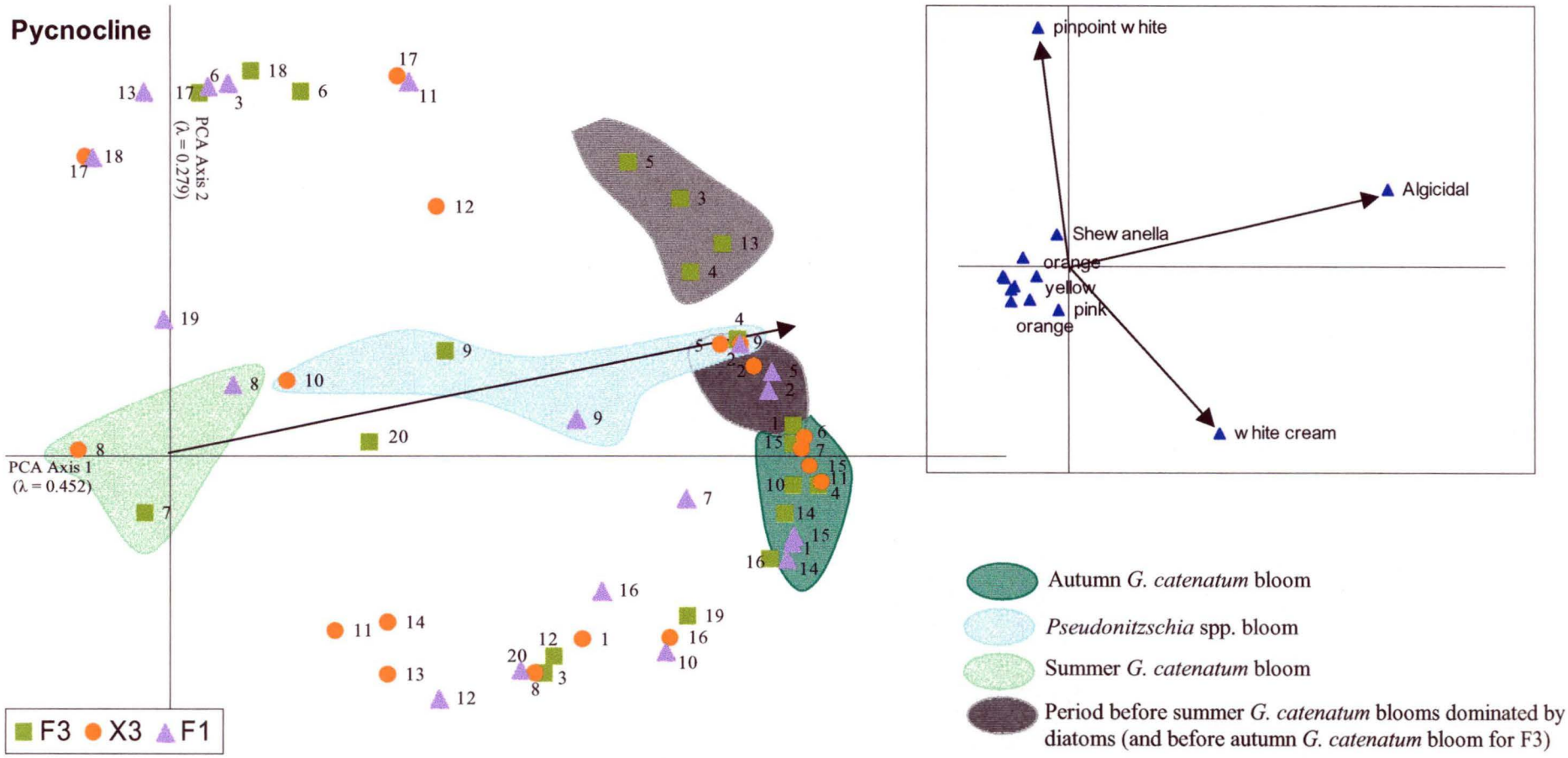


Figure 7.7 Principal component analysis of the 3 sites and 20 dates for the Pycnocline samples with the colony morphotype that influence their spacing in the vector diagram. Date key: 1=29/10/98, 2=12/11/98, 3=24/11/98, 4=10/12/98, 5=21/12/98, 6=7/01/99, 7=14/01/99, 8=21/01/99, 9=4/02/99, 10=18/02/99, 11=4/03/99, 12=18/03/99, 13=31/03/99, 14=22/04/99, 15=13/05/99, 16=27/05/99, 17=6/07/99, 18=2/09/99, 19=25/01/00, 20=4/2/00. Main PCA diagram has algicidal bacteria vector inserted as reference.

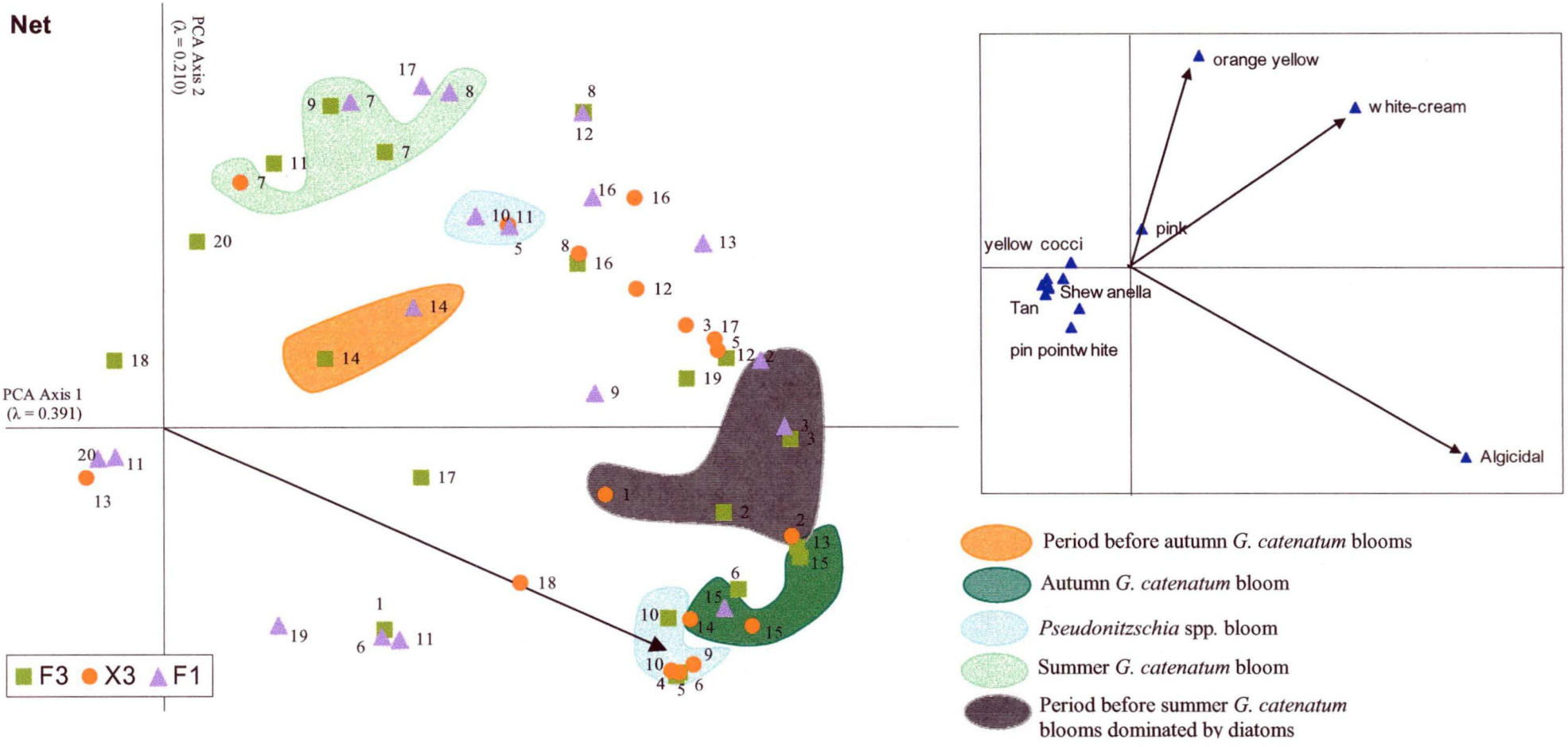


Figure 7.8 Principal component analysis of the 3 sites and 20 dates for the Net samples with the colony morphotype that influence their spacing in the Vector diagram above. Date key: 1=29/10/98, 2=12/11/98, 3=24/11/98, 4=10/12/98, 5=21/12/98, 6=7/01/99, 7=14/01/99, 8=21/01/99, 9=4/02/99, 10=18/02/99, 11=4/03/99, 12=18/03/99, 13=31/03/99, 14=22/04/99, 15=13/05/99, 16=27/05/99, 17=6/07/99, 18=2/09/99, 19=25/01/00, 20=4/2/00. Main PCA diagram has algicidal bacteria vector inserted as reference

the yellow-orange morphotype for all three sites during the bloom maximum. White-cream and algicidal morphotypes equally dominated dates during the early 1998 diatom dominated bloom. During the autumn *G. catenatum* bloom, the white-cream vector was associated with sampling dates at the peak of the bloom for sites F1 and F3. Conversely, algicidal bacteria morphotypes at site X3 were associated with dates throughout the autumn *G. catenatum* bloom. Algicidal morphotypes were associated with dates during the decline of the *G. catenatum* bloom at both sites F1 and F3.

7.5.4 Cluster analysis of Integrated Samples

Cluster analysis of integrated samples by sites

The integrated sample demonstrated the tightest clustering of sites when compared to other depths (Figure 7.9). The algicidal vector was strongly associated with sites F1 and X3 for the integrated water samples.

Cluster analysis of integrated samples by dates

Unlike the other depths, the integrated samples did not show a relationship between the algicidal vector and the dates of the *Pseudonitzschia* spp. bloom (Figure 7.9). Both the yellow-orange and the white-cream morphotypes were associated with the dates of the *Pseudonitzschia* spp. bloom. The white-cream vector strongly clustered with the dates of the autumn *G. catenatum* bloom. All sites and dates clustered tightly for the autumn *G. catenatum* bloom. The dates of the summer *G. catenatum* bloom were associated with the pinpoint white colony morphotypes. White-cream colony morphotypes dominated the integrated samples for most dates during the season. The algicidal and pigmented morphotypes showed less association with dates.

7.5.5 Cluster analysis of bacterial colony size by date

Over the field season colony size also varied. Small colonies dominated in late winter (July-September) and large colonies dominated in late summer. Large

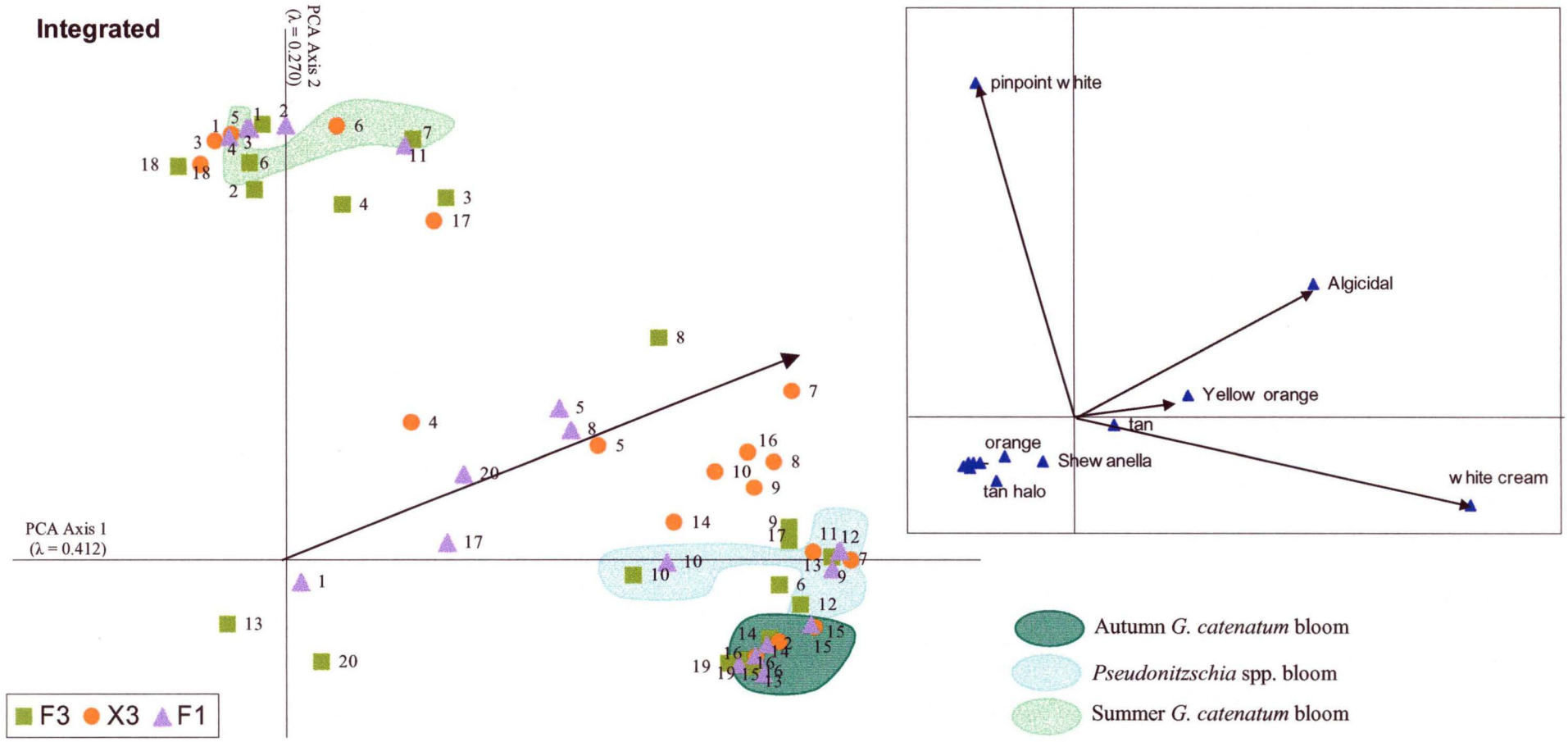


Figure 7.9 Principal component analysis of the 3 sites and 20 dates for the Integrated samples with the colony morphotype that influence their spacing in the Vector diagram above. Date key: 1=29/10/98, 2=12/11/98, 3=24/11/98, 4=10/12/98, 5=21/12/98, 6=7/01/99, 7=14/01/99, 8=21/01/99, 9=4/02/99, 10=18/02/99, 11=4/03/99, 12=18/03/99, 13=31/03/99, 14=22/04/99, 15=13/05/99, 16=27/05/99, 17=6/07/99, 18=2/09/99, 19=25/01/00, 20=4/2/00. Main PCA diagram has algicidal bacteria vector inserted as reference.

colonies were cultured more regularly from the pycnocline and surface samples and smaller colonies were cultured more regularly from the integrated samples (Figure 7.9).

7.6 *Shewanella* species distribution in the Huon Estuary

Isolates of the genus *Shewanella* were identified based on morphological characteristics (gram-negative, pink-tan colonies) and were confirmed at 3 points though the sampling period using fatty acid analyses of field colonies. *Shewanella* spp. colonies were mainly cultured from the integrated samples and dominated in the cooler waters (integrated and pycnocline) during summer (Figure 7.10).

A probe previously designed for *Shewanella putrefaciens* (DiChristina and Delong 1993) was modified to be less specific. The abbreviated probe sequence (ctgtgacgttacctacagaagaa) permitted labelling of the psychrotrophic *Shewanella* species including ACEM 6 and ACEM 9, as well as *S. baltica, S. frigidimarina, S. putrefaciens* and *S. oneidensis.* During trials with pure cultures of ACEM 6, ACEM 9, *S. putrefaciens* and *S frigidimarina* all strains demonstrated good probe binding efficiency with 15% formamide in the hybridisation buffer. Laboratory cultures of *E coli and S. gelidimarina,* and environmental isolates ACEM 21, ACEM 32 and ACEM 22 did not bind. Unfortunately according to the GenBank sequence database, the probe is also specific for two species of the genus *Oceanospirillum* (*O. multiglobuliferum* and *O. beijerinckii* subspecies: *pelagicum* and *beijerinckii*). The original *S. putrefaciens* probe was also specific for *O. multiglobuliferum.* Differences between these two species may be observable via microscopy as the genus *Oceanospirillum* is helical in shape while the genus *Shewanella* is rod-shaped. The *Shewanella* probe was not used in the samples from the Huon Estuary, but in future should allow better identification of psychrotrophic *Shewanella* species in the environment.

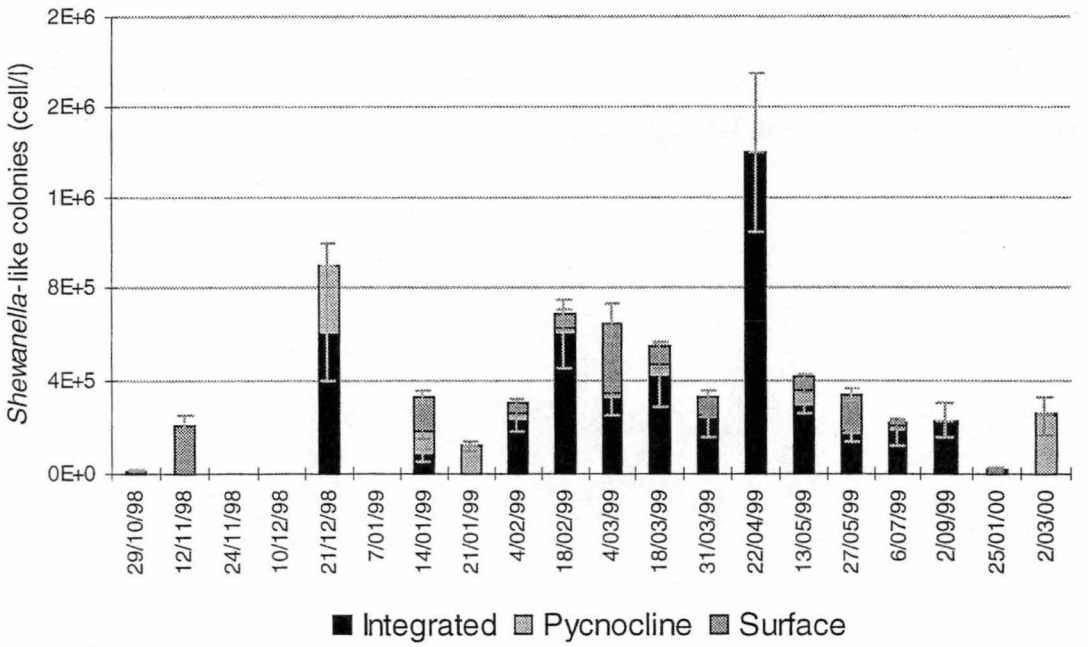


Figure 7.10 Variation in *Shewanella* species over the field season at the three different sites and 4 dept

7.7 Fluorescence *in situ* hybridisation (FISH)

7.7.1 Field samples

Results from FISH analysis of the different sites and depths during the *Pseudonitzschia* spp. and *G. catenatum* algal blooms are shown in Figure 7.11. Examples of pure culture and field experiment results using FISH oligonucleotides can be seen in Figure 7.12. During the summer *G. catenatum* bloom, the dominant groups were CFB, α proteobacteria and "other" which may have included some gram-positive or *Planctomycete* species as these can occur in marine waters. A decrease in α proteobacteria and an increase in the genus *Pseudoalteromonas* and other γ proteobacteria was observed during the decay of the summer *G. catenatum* bloom. During the autumn *G. catenatum* bloom, the proportion of *Pseudoalteromonas* species during bloom decay was low compared to the summer *G. catenatum* and the *Pseudonitzschia* spp. blooms. The CFB cluster was dominant at all sites at the beginning of the autumn *G. catenatum* bloom. At this time, the microbial community class profiles for sites F3 and X3 were very similar. The CFB cluster was still dominant at the decline of the autumn *G. catenatum* bloom at site F3, while sites F1 and X3, which had lower numbers of *G. catenatum*, were dominated by α and γ proteobacteria and *Pseudoalteromonas*.

The genus *Pseudoalteromonas* was a dominant member of samples from all three sites at the beginning of the *Pseudonitzschia* spp. bloom and during the decline of the *G. catenatum* bloom. The proportion of this genus for all sites and depths was very high and indicates that it can numerically dominate the microbial community.

Site X3 demonstrated the highest continuous occurrence of the genus *Pseudoalteromonas* in all samples and depths with the exception of 18/2 when numbers were higher at site F1. Results from the plankton net sample show that during the height of the *Pseudonitzschia* spp. bloom the community altered and high proportions of γ proteobacteria (excluding the genus *Pseudoalteromonas*)

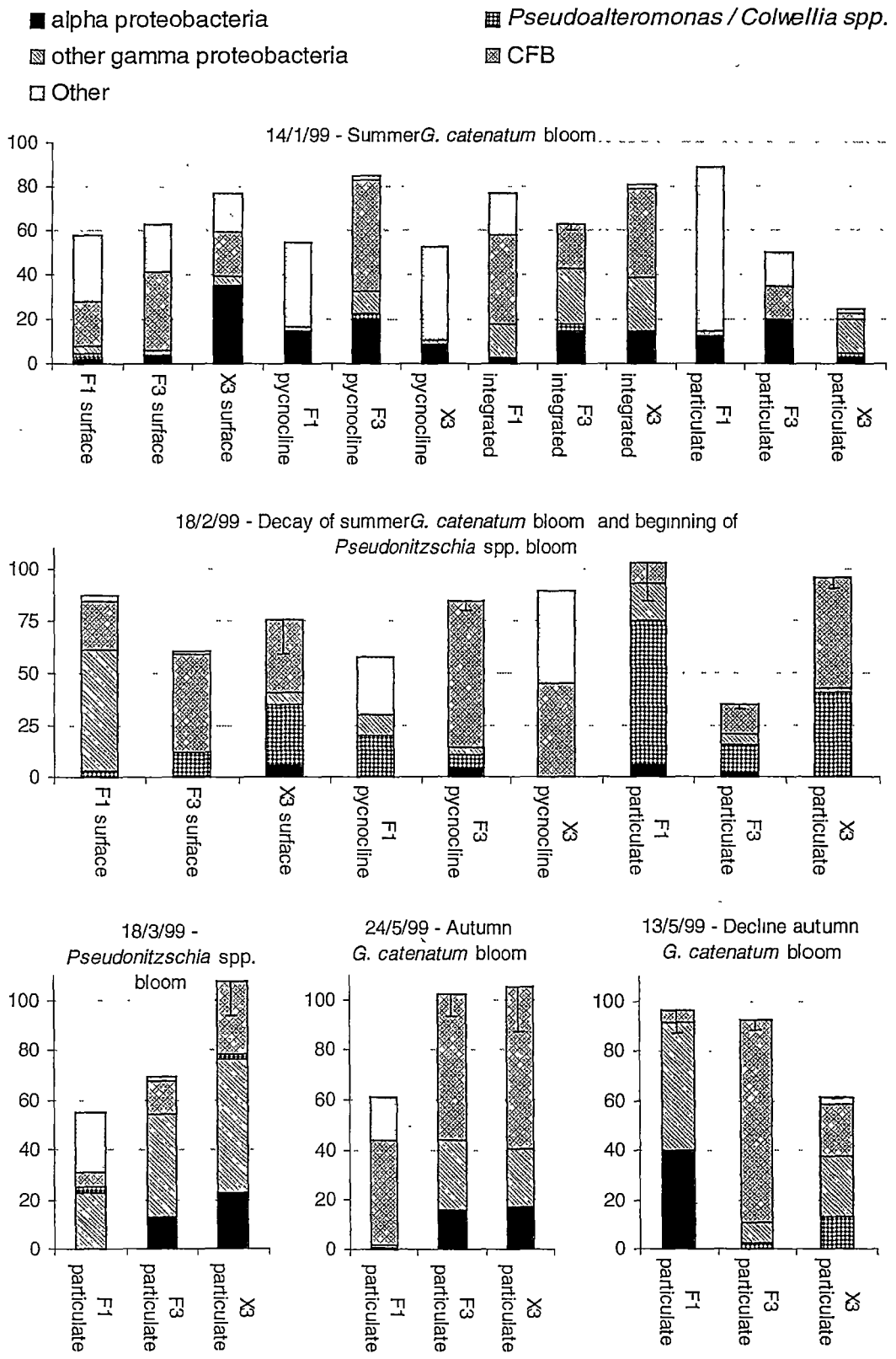


Figure 7.11 Variation in bacterial community composition using FISH during the two major *G. catenatum* blooms and the *Pseudonitzschia* spp. bloom. Data are presented as a proportion of DAPI stained particles.

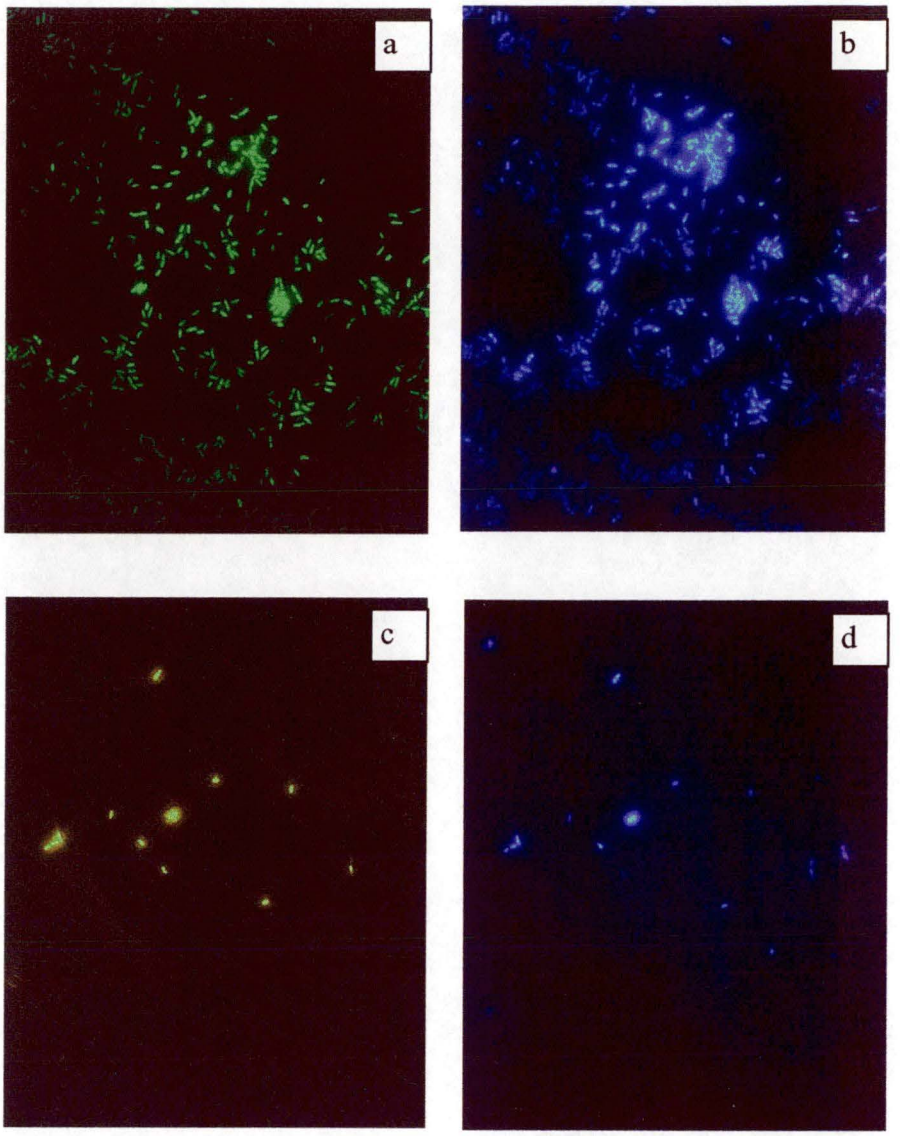


Figure 7.12 a) Pure culture of ACEM 1 with *Pseudoalteromonas/Colwellia*-Cy3 probe.

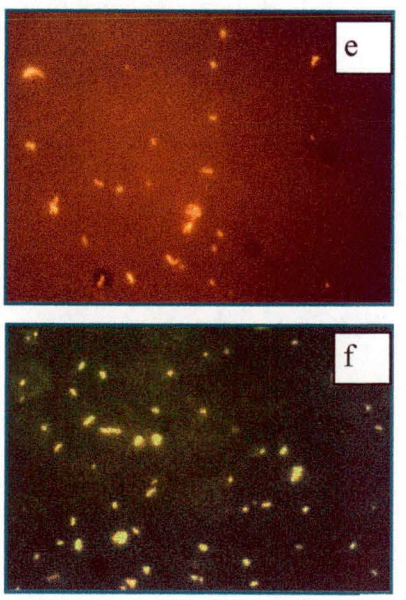
b) DAPI stain of a).

c) Field sample from the Huon with γ proteobacteria-Cy3 probe.

d) DAPI stain of c).

e) and f) Field samples from the Huon using the probes gamma and EUB respectively. Background of e) is typical of what is observed when humics have interfered in the reaction.

Pictures a)-d) were taken with a Leica digital camera (DC 200). Pictures e) and f) were taken with a Leica MPS60 camera, both were mounted directly to the microscope. Thanks to Walshes Optics for loan of the digital camera.



were present at all three sites. Because of this result, many of the bacteria isolated from the Huon Estuary were tested for specificity using the *Pseudoalteromonas* probe (section 7.8.2).

The CFB, EUB338, α proteobacteria and γ (with β-unmarked) proteobacteria probes were all successful in environmental samples where nutrients were higher during periods of algal blooms. Both ACEM 1 and ACEM 4 cultures produced bright fluorescent signals with the *Pseudoalteromonas* probe. The *Vibrio* spp. probe was unsuccessful in field samples, but trials with *V. alginolyticus* and an environmental *Vibrio* sp. strain also failed repeatedly. Therefore *Vibrio* species may have been present in the estuary despite the negative result when using this probe. The gram-positive probe was not used as part of the community analysis because it was initially thought that the occurrence of these species in the marine environment was unlikely. It is probable that gram-positive bacteria may account for the "other" bacterial species that were not detected by the 5 main probes but were detected with the EUB338 probe. Support for the presence of gram-positive species is strengthened by the presence of gram-positive algicidal species and the occurrence of a number of common gram-positive species in the cultured isolates (chapters 3 and 5). *Planctomycetales,* or to a much lesser extent δ proteobacteria, may also be included in this group as they have occasionally been noted in molecular analyses of marine samples. The β proteobacteria probe was used with the γ proteobacteria unmarked probe for three samples but results were all under 2 % (14/1: 0 % for all depths and sites apart from X3 surface 0.8 %, 18/3: F3N 0.9 %, X3N 0%, F1N 0 %, 13/5: X3N 0.5 %, F3N 0%, F1 0 %).

Discussion

7.8 Culture independent techniques

The FISH results indicated that the composition of the microbial community was usually similar for the three sites and throughout the water column for any one date. However, between dates, dramatic changes occurred.

7.8.1 CFB

Members of the CFB are a dominant group in marine and estuarine environments (see chapters 1 and 5). As genera in the CFB are recognized for their abilities to degrade large macromolecules, the abundance of humic compounds in the Huon Estuary would lead to a highly suitable habitat for this cluster. Other marine studies using FISH to identify bacterial species have found that this family can dominate marine bacterioplankton communities (Cottrell and Kirchman 2000). The CFB community played a major role in the Huon Estuary microbial community during the period studied. Nearly all samples analysed using FISH indicated the presence of this cluster. It was very strongly associated with algal blooms and with the particulate fraction.

7.8.2 Gamma proteobacteria

γ Proteobacteria are a common class of bacteria found in marine and estuarine environments, although their high growth efficiency on solid media is thought to have over-emphasised their importance in the marine environment (Eilers *et al.* 2000). γ Proteobacteria can dominate in river systems as nitrogen fixers (Affourtit *et al.* 2001). They were present throughout the Huon Estuary at high proportions in many samples analysed using FISH. The results from the *Pseudoalteromonas-Colwellia* specific probe in particular indicated that one genus could dominate. During the *Pseudonitzschia* spp. bloom, FISH analysis indicated that very high proportions of *Pseudoalteromonas* were present at all three sites. The binding efficiency of the *Pseudoalteromonas* probe was very high and was often more

fluorescent than the EUB338 probe in pure cultures. It was also highly specific for the genus when tested against a laboratory strain of *E. coli* and a number of other unrelated environmental bacteria from the estuary. The ease with which this genus can be cultured by traditional techniques indicates that an easily cultured group can play an important role in the microbial community. In a recent study, *Pseudoalteromonas* and *Alteromonas* were dominant in a temperate marine community during early autumn (61%, Kelly and Chistoserdov 2001) paralleling the results of my research where *Pseudoalteromonas* species were found to be common in the Huon Estuary in the same season. Kelly and Chistoserdov (2001) speculated that *Pseudoalteromonas* was involved in the observed demise of the toxic brown alga *Aureococcus anophagefferens.*

7.8.3 Beta proteobacteria

β Proteobacteria can be relatively common in certain coastal and fresh water environments. They are generally comprised of methylotrophic bacteria (Rappé *et al.* 2000). Traditional and molecular studies to date have detected only very low numbers of this class in the open ocean environment (Hagström *et al.* 2000, Glöckner *et al.* 1999, Pinhassi *et al.* 1997). Members of this class are more commonly found in river systems (Crump *et al.* 1999) and can be numerically dominant in higher river reaches and fresh water lakes (Böckelmann *et al.* 2000, Methe *et al.*1998). β Proteobacteria (*Nitrosomonas* sp.) have also been observed as part of an estuarine bacterial community based on denaturing gradient gel electrophoresis (DGGE) analysis (de Bie *et al.* 2001). The estuary in question was eutrophic as it contained high levels of domestic and industrial waste products. In samples from the Huon Estuary study and other samples where probe efficiencies were poorer, very low proportions of β proteobacteria were observed. This indicates that this cluster was not numerically dominant at these three marine dominated samples sites in the Huon Estuary.

7.8.4 Alpha proteobacteria

Until the introduction of molecular techniques, α proteobacteria were not thought of as central marine species. This was owing to difficulties in their cultivation.

However, they can be a dominant bacterial class in the upper reaches of estuaries (González and Moran 1997) and oceans (Pinhassi and Hagström 2000). Pinhassi and Hagström (2000) identified *Sphingomonas* and *Caulobacter* as the dominant bacteria present during summer in the Baltic Sea when bacterial production was low. They also found low levels of the genus *Roseobacter*. González and Moran (1997) found *Roseobacter* to be a relatively widely spread genus in the upper fresh water reaches of estuaries examined. In another study by the same researchers the genus *Roseobacter* represented over 20 % of the 16S rDNA in the heterotrophic bacterial community sampled (González *et al.* 2000). An advantage that α proteobacteria would have in the Huon Estuary community would be their ability to breakdown humic compounds as some *Roseobacter* species produce enzymes that are capable of degrading aromatic ring structures (Buchan *et al.* 2000). The presence of α proteobacteria in estuaries may be as a result of the high levels of dissolved organic matter (DOM) in rivers containing high molecular weight compounds that are produced by plants. Covert *et al.* (1999) reported that α proteobacteria decrease towards the oceanic end of estuaries as a response to the change in the source of DOM from higher plant to marine. Other studies have demonstrated that in the offshore environment (North Atlantic), α proteobacteria can sometimes dominate during blooms of dimethylsulfoniopropionate (DMSP) producing algae (González *et al.* 2000). Various α proteobacteria have been studied with respect to DMSP degradation and the potential of this group for cycling organic sulphur compounds in the estuarine or marine environments (Ansede *et al.* 2001, Ledyard *et al.* 1993). α Proteobacteria and CFB have previously been associated with particulate matter and high growth rates of diatoms in mesocosms (Riemann *et al.* 2000). FISH analysis in this study clearly demonstrated that α proteobacteria were present in the estuary at the end of the *G. catenatum* bloom. At site F1, α proteobacteria accounted for up to 40 % of the DAPI stained particles. The contention that α proteobacteria are associated with algal particulate matter is therefore supported.

7.8.5 Other bacterial groups

The incidence of gram-positive bacteria was not investigated in this study. More recently, they have been shown to be an important part of the species composition in estuarine and oceanic environments (Crump *et al.* 1999, Rappé *et al.* 1997). In a study of the ability of isolated estuarine bacteria to degrade humic compounds and comparing enrichment cultures with the natural community, 6 of 18 isolates from the enrichment cultures were α proteobacteria, three were γ proteobacteria, and nine were gram-positive bacteria (Esham *et al.* 2000). The natural bacterial community showed 28 % degradation of humic compounds whereas the isolated bacteria only achieved 3-8 % degradation together or as single strains. Esham *et al.* (2000) suggested that the majority of the species able to degrade humic compounds were non-culturable (although not necessarily gram-positive).

Planctomycetales could also have been present in the Huon Estuary samples, but were not included in the FISH analysis. They have been reported on river snow aggregates (2% FISH, Böckelmann *et al.* 2000) and fresh water and ocean samples (< 7% FISH Glöckner *et al.* 1999, bacterial clones Rappé *et al.* 1997).

Another bacterial cluster not included in this study was the δ proteobacteria which has been observed via FISH as the dominant bacteria associated with the marine sponge *Aplysina* (Friedrich *et al.* 1999). Evidence from Coates *et al.* (1998) demonstrated that *Geobacteraceae* (δ proteobacteria) are also capable of breaking down humic compounds. However, in other estuarine and coastal studies these bacteria have so far been less frequently reported in the water column.

Clones of *Verrucomicrobiales* have been found in enrichment cultures of the prymnesiophyte *Phaeocystis* and were related to the degradation of complex algal polymers (Janse *et al.* 2000). However, this division is generally most prolific in soils and anoxic environments and occurs infrequently in studies of the marine environment.

7.8.6 FISH techniques

The success of the FISH technique varied. Probe binding efficiency was highest for samples taken during peak bloom periods of dinoflagellates and diatoms. Particle-attached bacteria in plankton net samples also provided good results. Binding efficiency of the EUB338 probe was up to 95 % of the DAPI stain for samples collected during algal bloom periods but was lower at other times of year. The low success for FISH analysis in many of the other samples may be the result of number of factors. Possibilities include: the cell wall was not permeable to the probe (i.e. gram-positive); low abundance of rRNA because of cooler or less productive periods; the rRNA was not accessible to the probe; some of the cells were Archaea; interference from humic compounds; high levels of autofluorescence from particulate matter.

Background fluorescence, although observed occasionally was not a problem in samples where FISH was successful. This is due to the intensity of Cy3 flurophores and the ability to compare between the DAPI and Cy3 filters. In a few samples some of the river particles fluoresced, causing the background to be more fluorescent than would be ideal. This generally occurred with samples taken during periods of high rainfall.

Eriksen (2000) and Campbell *et al.* (1997) report that a coating effect caused by riverine humic compounds can protect cells (of salmon and algae) from the effects of high copper or heavy metal concentrations. Humic compounds in Macquarie Harbour, Tasmania are at lower concentrations than those found in the Huon Estuary. These compounds have been shown to protect fish in this environment from copper poisoning by coating the biological surfaces (i.e. cell walls, gill membranes etc) (Eriksen 2000). This prevents the copper travelling into the cells where they could exert an effect (Campbell *et al.* 1997). This coating effect may also have interfered with probe efficiency in the use of FISH in the Huon Estuary.

Amplification techniques and newer, more fluorescent flurophores have assisted in the use of FISH in the marine environment. A number of amplification techniques were trialed with the samples. The greatest increase in fluorescence was gained by

using the flurophore Cy3. Other flurophores such as FITIC, Rhodamine, and fluoresein were not as intensely fluorescent as Cy3 even when a number of probes for the same class were attached. It was observed that one Cy3 probe equated to the same fluorescence as 5 fluoresein probes specific for Eubacteria. Cy3 is also over 10 times more photostable than FITC (Amersham Life Science 1995, Wessendorf and Brelje 1992).

Pre-boosting the field sample with an RNA amplification technique has demonstrated improved signal intensity (tyramide: Lebaron *et al.* 1997, chloramphenicol and/or suitable liquid media: MacDonald and Brozel 2000, Ouverney and Fuhrman 1997). However, some amplification compounds are large (e.g. tyramide linked with horseradish peroxidase), so difficulties occur in fitting a number of the amplified flurophores in the cell (Lebaron *et al.* 1997). Tyramide and horseradish peroxidase (Cerylid, Australia) were trialed but discarded after no improvement was noted.

The Huon Estuary is generally a system with low levels of ammonium and nutrients. Such conditions can cause a decrease in hybridisation signals because cells are nutrient deprived (Konuma *et al.* 2001). The use of chloramphenicol (MacDonald and Brozel 2000, Ouverney and Fuhrman 1997) and a suitable marine media improves fluorescence in these oligotrophic environments by increasing nutrients but stopping cell division. After the field study, chloramphenicol and marine broth were shown to increase probe efficiency if used in combination, especially for samples with < 50 % of EUB338 as a proportion of DAPI. Water samples were filtered and overlaid with 1 ml of the 100 ng/ml chloramphenicol and liquid media (Huon Estuary river water media section 7.1). Use of either chloramphenicol (Ouverney and Fuhrman 1997) or marine broth alone was not as effective as combined used. Combined use increased probe efficiency by ~10 %, but more importantly, allowed easier enumeration by increasing the fluorescence intensity of existing labelled bacteria. Although temperate coastal waters demonstrated an enhancement of signal, attempts to use this amplification method with Antarctic water samples demonstrated no improvement. Poor results with Antarctic samples may be explained by the choice of media. A more suitable

(dilute) media may prove to be more successful. This highlights that media type is very important when using this amplification method.

Ascorbic acid was found to be an effective addition to slow quenching. Ascorbic acid acts as a free radical scavenger that extends fluorescence emission (McBain Instruments 2001). It did not appear to change the signal intensity but did decrease the quenching time. Quenching differs from photobleaching, which occurs by reduction (or in some cases, enhancement) of fluorescence intensity by competing factors such as temperature, high oxygen concentrations, and molecular aggregation in the presence of salts or halogenated compounds (Molecular Expressions 1995). Quenching occasionally occurred in some Huon Estuary samples, when high levels of humic compounds caused an increase in background fluorescence and an adverse affect on FISH probing efficiency. This has also occurred in other FISH studies (Alm *et al.* 2000).

All techniques have weaknesses. In the Huon Estuary and in winter Antarctic waters (unpublished data) FISH performed relatively poorly. Greater success with other Tasmanian coastal water samples was achieved after the sampling period of this thesis, although the technique can still be highly variable. The use of radioactive oligonucleotides would have increased the sensitivity of the technique. Despite this, molecular techniques such as FISH are very powerful and show great potential. They have considerably advanced knowledge of the microbial community and are setting the standard by which other techniques are now gauged.

7.9 Culture dependent techniques

All algicidal bacteria that were isolated possessed distinctive colonial morphologies. The occurrence of algicidal species in the estuary may not guarantee the presence of biocidal exudates, but it is possible to observe the algicidal potential of these genera in the water column using morphological or molecular means. The use of CFU to identify the algicidal bacteria was practical, as growth of the algicidal *Pseudoalteromonas* and *Cellulophaga* species was not inhibited on marine agar.

The summer *G. catenatum* bloom demonstrated that the algicidal bacteria morphotype was dominant just before the height of the algal bloom. This may be because blooms predominate in the upper 2 m and the integrated sample incorporates a broader depth.

Morphotype characterisations from 1998 are not included as allotment of the 41 different colony morphotypes was being established during this period. Species overlap within these 41 morphotypes is possible, as morphotype variation is not necessarily indicative of species. However, overall variation between the dominant and algicidal morphotypes (excluding ACEM 20) was readily identifiable. Bias can occur in favour of species such as *Pseudoalteromonas* and other γ proteobacteria when using CFU (Eilers *et al.* 2000), but other genera were easily isolated. Isolation of a number of diverse morphotypes, information from chapters 4 and 5, and molecular analysis indicates that the method was not solely representing one or two genera. After three years of field observations, it was evident that a high number of white-cream isolates occurred for integrated samples. These isolates would be from genera that grow easily on plates, such as *Pseudoalteromonas, Bacteriodes, Vibrio* or *Pseudomonas.* Surface and pycnocline samples contained greater morphotype diversity and pigmented isolates were more common. Integrated samples also contained higher proportions of small colonies. The observation of colony size variation with respect to both the integrated samples and samples taken during colder periods may be an indication of the physiology of the isolates. Small colonies occurring in the surface and pycnocline depths in winter periods may indicate a physiological response to cooler waters.

Morphotype variation for the different depths maybe linked to environmental factors. Surface waters were less saline when compared with the pycnocline and integrated samples which were generally at typical seawater salinities. The differences observed between pycnocline and integrated samples were related to the greater water column depth obtained by the integrated samples.

7.10 Comparison of analysis techniques

A number of recent studies have concluded that many culturable bacteria are yet to be isolated, however, Hagström *et al.* (2000) established that a large proportion of cultured isolates are similar to environmental clones. FISH analyses of a picoplankton enrichment culture were dominated, in order, by γ- β- and α proteobacteria (Uphoff *et al.* 2001). Among the 410 isolates in this picoplankton study, a high degree of diversity was observed, with respect to both colony pigmentation and morphology and genetic diversity. Lebaron *et al.* (2001) reported that 14 % of marine bacteria were also culturable. In a study of the bacterial community in the Elbe River, application of specific oligonucleotide probes indicated that culturable populations of bacteria were dominant members (Böckelmann *et al.* 2000). Another study demonstrated that α proteobacteria were numerically dominant and easily cultured despite being new or unculturable isolates with respect to the GenBank database in temperate coastal seawater (Gonzalez and Moran 1997). Pinhassi and Hagström (2000) demonstrated that the majority of the isolates from their study in the Baltic Sea had low similarity to GenBank sequences indicating that the bacterial diversity able to grow on marine agar was relatively unexplored. The lack of sequence data from bacteria in culture collections has been given as the reason that many clones are new, unculturable bacteria (Pinhassi and Hagström 2000, Hagström *et al.* 2000, Hagström *et al.* 1997). In chapter 4 six new species were identified from culturable isolates using 16S rDNA sequencing. This supports that finding new species by the use of molecular techniques such as by cloned 16S rRNA sequences does not necessarily mean that all these new species are non-culturable.

Studies using molecular techniques such as clone libraries and DGGE have revealed that a limited number of species consistently represented the majority of a diverse bacterial community and that these dominant species were present in equal densities in the water column (Pinhassi and Hagström 2000, Rehnstam *et al.* 1993). Clone libraries obtained for most marine studies have demonstrated that there are between 6 and 15 species present in the oceanic or marine waters at any one time. These figures are also similar to the number of different bacterial morphotypes that

were cultured at any one sampling depth or site in this study of the Huon Estuary (2-15). If at any one time, the number of species dominating a microbial community is between 5 and 15, it follows that the presence of 9 morphologically different isolates indicates that the majority of the species types may be culturable although they may not be represented in their correct proportions. In some marine environments, traditional morphological techniques using suitable media may therefore be almost as successful in determining the species present in the population as FISH (even if not able to correctly determine their given proportions).

Isolates that form CFU are also active, even if they do not dominate the marine community under normal environmental circumstances. Morphotypes are not necessarily indicative of different species, however this finding does support reports of the dominance of only a few genera or species numbers at any one time. Interestingly, research papers often report FISH results that classify 25-55 % of DAPI stained cells with the assumption that the rest of the community is similar. In comparison, culturable cells enumerated only 1-10 % of DAPI stained cells. The DGGE procedure can be used to identify microorganisms via sequence analysis rather than their abundances, which it cannot quantify. Thus, the identification of a number of bacterial genera in the community does not automatically signify that they are a major or active species, only that they are present. FISH cannot be specific at species level but can identify the dominant viable populations. It cannot demonstrate activity of the cells. However where rRNA levels are high enough to be observed using FISH the population probably plays an important role in the microbial community at that particular time as cells contain high rRNA copy numbers.

The ability to observe the bacterial population in the water column is of major ecological interest, although at present only a very small fraction of the community can be viewed at any one time. Microscopy typically involves a 50 ml water sample on a 4.5 cm filter of which 10-30 fields of view are generally counted providing an effective volume of 60 µl. Techniques such as DGGE convey the bacterial species present except they are not a quantitative measure of the bacterial numbers or their importance in the environment. FISH uses similar volumes to

microscopy and gives proportions and bacterial types in the environment based on this volume. Culture dependent methods involve an effective volume of 60 µl and only enumerate 1-15 % of the community present. Fatty acid analysis uses larger volumes and a larger sample of the overall population is obtained. It has the ability to analyse the algal and heterotrophic community coexisting with the microbes although it cannot distinguish bacteria that do not have distinct fatty acid constituents. Therefore, all these methods have clear drawbacks. A combination of these methodologies will, however, create a more complete understanding of the community as a whole.

7.11 *Shewanella* species distribution in the Huon Estuary

Although most other bacterial isolates were closely associated with algal blooms, the *Shewanella* isolates occurred in integrated samples and their occurrence in the water column did not appear to be related to periods of algal blooms. The importance of *Shewanella* in the marine environment has not been fully explored and this genus may play a pivotal role in the nutritional ecology of the estuary during periods when algal blooms are absent. The presence of this genus throughout the winter season is of interest because of the nature of the essential nutrients it is capable of producing. During winter, water temperatures were lowest as was algal biomass (chapter 2). The presence and apparent increase of the genus *Shewanella* during cooler periods may increase the supply of the essential polyunsaturated fatty acid, eicosapentaenoic acid (EPA, 20:5(n-3)) to the water column during winter. The cooler winter water temperatures would maximise the EPA production in the *Shewanella* species (see chapter 6 and 2).

The use of oligonucleotide probes for this genus would be worthwhile. The *Shewanella* probe, which successfully binds with the psychrotrophic species of the genus, would be of considerable value in understanding the role of this species in many marine environments. Future research of this genus would benefit by the design of another oligonucleotide for the psychrophilic species of the genus.

7.12 Comparison of morphology and FISH results

Examination of colony types demonstrated marked variation and change between depths and dates indicative of bacterial community change over the season. A key feature of the results was the relationship of algicidal morphotypes to diatom blooms rather than dinoflagellate blooms at 3 depths (excluding integrated). As algicidal bacteria do not kill diatom species, superficially, this would then appear to benefit diatoms. It could be hypothesized that the algicidal bacteria are repressing the dinoflagellate bloom and nurturing the diatom bloom, although this is presently conjectural. However, this research suggests there is a community shift between the two bloom periods and that algicidal morphotypes occurred throughout the photic depths during the *Pseudonitzschia* spp. bloom.

The integrated samples were more likely to contain non-pigmented colonies, while isolates from the pycnocline and surface water samples were more likely to be pigmented morphotypes. Given that many of the CFB are coloured, and many of the γ proteobacteria are white, this supports the findings of the fatty acid profiles in chapter 5. These results suggest the photic depths, where algal blooms occurred, had higher proportions of BCFA (and thus CFB). Conversely, integrated samples showed fatty acid profiles that were indicative of bacterial species whose fatty acids merged with the eukaryotic signature (no BCFA). That is, integrated samples contain higher proportions of the bacterial genera that contain straight chain and monounsaturated fatty acids. Supporting this argument is knowledge that the integrated samples also contained the highest bacterial numbers when compared with surface or pycnocline samples, despite having the lowest concentrations of BCFA. γ Proteobacteria such as *Pseudoalteromonas* and *Vibrio* species are non-pigmented and do not form BCFA. The morphotype results indicate that some of the unpigmented colonies occurring in the integrated sample may belong to these genera.

Success in the use of FISH for integrated samples was generally poor. However during the summer *G. catenatum* bloom, the γ proteobacteria were highest for the integrated samples when compared to all other depths. The morphological evidence supports this finding with the integrated samples being more closely

related to those in the white-cream morphotype. Interestingly, during both *G. catenatum* blooms, algicidal species were present but were not algicidal, and the dominant bacterial cluster was CFB. Conversely, during the *Pseudonitzschia* spp. bloom, algicidal morphotypes were present, *Pseudoalteromonas* was a dominant genus, and isolates of algicidal bacteria demonstrated algicidal properties at this time (chapter 4). This indicates that species, strain and environmental factors can effect the algicidal properties of a microbial community.

Due to the complexity of ecology, comparison between diverse techniques such as CFU and FISH is difficult. However, generalised similarities can be seen between the two techniques for many the sites. During the summer *G. catenatum* bloom period, the dominant morphologies were yellow-orange and the major FISH classes were "other", α proteobacteria and CFB. The bacterial species that did not hybridise with any of the probes used may have been gram-positive species. The pycnocline sample at site F3, in particular for the summer *G. catenatum* bloom, was dominated by pink colonies and FISH analysis demonstrated the dominance of α proteobacteria. For this same date, the F1 pycnocline was dominated by algicidal colonies and by the CFB cluster. Site X3 for the summer *G. catenatum* bloom was also dominated by the algicidal vector and contained a high proportion of CFB. Site X3 also contained a high proportion of eubacterial species that did not hybridise with any of the probes used.

Throughout the diatom bloom, the dominant cultured morphotype at site F1 formed white cream colonies. FISH analysis demonstrated that γ proteobacteria and the genus *Pseudoalteromonas* were the major classes. The other two sites contained higher proportions of algicidal morphotypes, had different FISH profiles, and were dominated by CFB and the genus *Pseudoalteromonas*.

Between the summer *G. catenatum* bloom and the *Pseudonitzschia* spp. bloom the pycnocline at site F1 contained mainly white cream colonies. FISH analysis indicated that this sample was dominated by *Pseudoalteromonas* and other γ proteobacteria.

During the autumn *G. catenatum* bloom, sites X3 and F3 were increasingly influenced by the algicidal morphotype over the bloom period. The CFB cluster dominated at both sites.

The field study in the Huon Estuary over the three major blooms in 1999 showed that the bacterial community can be dominated by genera with algicidal properties. Changes were observed in algicidal genera with relation to diatom and dinoflagellate blooms. The genus *Pseudoalteromonas* was demonstrated to closely associate with the *Pseudonitzschia* spp. diatom bloom. The genus *Shewanella* could be contributing to the presence of 20:5n-3 in the estuary in winter when algal blooms are less frequent and temperatures are suitable for higher production of this fatty acid. In combination, FISH fatty acid analysis and morphological techniques demonstrated that no one bacterial species dominated and there were seasonal transitions in the bacterial community composition similar to those that occur in the algal community.

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8. Antibiotic compounds in *Pseudoalteromonas*

Summary

The *Pseudoalteromonas* isolate ACEM 1 secretes a number of structurally related, brominated metabolites that are not associated with the algicidal activity of the bacteria. These metabolites are antimicrobial and inhibit selected environmental and pathogenic bacteria but have no effect on others in the same genus. The molecular weights of these new metabolites are smaller than antimicrobial peptides, and larger than small antimicrobial metabolites, produced by sponges or other marine bacteria (including other *Pseudoalteromonas* species). These results further demonstrate the bioactive nature of compounds produced by the genus *Pseudoalteromonas*.

Introduction

In 1936, Zobell stated that antibiotic producing marine bacteria contributed to the bactericidal action of seawater (Zobell 1936). Antibiotic production was thought to enhance survival of bacteria under nutrient limited conditions, enabling them to out-compete other microbes. Proof of this nutrient limitation hypothesis includes the observation that many antibiotic and secondary metabolites formed by bacteria are produced in late log and early stationary phase when required nutrients become depleted. Bacteria synthesise low levels of secondary metabolites when grown under optimal conditions whilst greater levels are produced when they become growth limited. In a study by Austin (1989), antibiotic-producing marine bacteria were more common in winter than summer because of the cooler temperatures in winter causing growth limitation of the cells.

Many of the antibiotic metabolites produced by bacteria are halogen based. Many antibiotic-producing bacterial species have been isolated from sponges (Bultel-

Poncé *et al.* 2000) and other invertebrates and it is thought that antibiotic compounds play a role in the defence mechanisms.

The genus *Pseudoalteromonas* has shown a high degree of bioactivity and the strain ACEM 1 has demonstrated algicidal properties (chapter 4). The brominated metabolites produced by this genus are further indication of the bioactivity of *Pseudoalteromonas* and also an example of the seemingly multiple roles that bacteria can have in the marine environment.

Objectives of this chapter were to:

- investigate the anti-microbial activity of ACEM 1 on environmental and pathogenic bacterial species;
- confirm if bactericidal compounds were the algicidal compounds;
- partially elucidate the structure of the compound/s involved;
- compare brominated compounds with other antimicrobial components and secondary metabolites of similar molecular weight produced by the genus *Pseudoalteromonas* and other marine organisms.

Methods

8.1 Concentration and elucidation of brominated compounds

To produce biomass for antibiotic extraction, ACEM 1 was inoculated and grown in 1600 ml of liquid marine media (Sea salts (Sigma) 28 g Yeast extract (Difco) 1 g, Bacteriological Peptone (Difco) 4 g) in a 2 l flask for 1 week at 22 °C. The medium was supplemented with approximately 1 g/l of KBr. Sea salts were replaced with 28 g/l NaCl to monitor the effect of the presence or absence bromine. KBr was also increased to 4 g/l to investigate the effect of additional bromine.

The ACEM 1 culture was partially filtered through GFF filters (Whatman) and was left to extract for 12 hours with dichloromethane and occasional vigorous shaking.

The crude extract was rotary evaporated under vacuum at 35 °C. This extract was used for proton and carbon NMR and HPLC-MS and FAB-MS analysis. NMR analysis required milligram quantities of brominated compounds. The volume of culture (1600 ml) precluded filtration through 0.2 µm filters. A small subsample from this extract was used for HPLC-MS-MS analysis. This was centrifuged and filtered through 0.2 µm polycarbonate filters (Millipore) prior to analysis.

Examination of antibiotic components was initially completed in conjunction with the elucidation of the algicidal compounds. An HPLC (Waters Alliance 2690) was coupled with a photo diode array, a reverse phase C_{18} column (Nova-Pak C_{18} 3.9 x 150 mm) and a Finnigan LCQ mass spectrometer with APCI source-vaporizer 450, capillary 170, sheath gas 60, aux gas 15, source current 5 µamps, (or Finnigan LCQ with Electrospray source, capillary 200, sheath gas 90, aux gas 15, ESI needle 5 KV). The scan range was m/z 100 to m/z 1200 (or m/z 100 to m/z 2000 for the Electrospray source). Data-dependent MS/MS scans were collected from the most intense ions. The elution gradients included a gradient of water-2 % acetic acid-methanol at 0.8 ml/min or a 50:50 methanol:water gradient at 0.8 ml/min finishing with 90 % methanol at 25 minutes. Sample fractions were collected every 5 minutes during the HPLC run. Later, when elution of the algicidal components was not required this method was altered to a methanol:water gradient finishing with 90% methanol. This shortened the elution time for the brominated compounds from 40-45 minutes to 20-25 minutes. Some peak definition was lost in this process, but the use of the photodiode array and specific mass fragmentation permitted compound and isomer separation and identification.

^{13}C and ^{1}H NMR spectra in $CDCL_3$ were recorded at room temperature in 5 mm o.d. tubes on a Bruker ACF 300 or DMX 500 spectrometer using the deuterium signal of the solvent as the lock. The chemical shifts were read from the residual protonated solvent. One-dimensional ^{1}H NMR experiments were carried out on a Bruker ACF 300 spectrometer with a spectral width of ~ 3500 Hz, a 45° pulse angle, 8K data points and a repetition delay of 3 seconds. One-dimensional ^{13}C NMR spectra were recorded in the pulsed Fourier transform mode (16K data points for the FID) at 298 on a Bruker ACF 300 spectrometer operating at 75.47

MHz or a Bruker DMX 500 spectrometer operating at 125.77 MHz (Maximilien *et al.* 1998).

8.2 Testing of antibiotic properties

ACEM 1 was prepared for the well diffusion assay by inoculating 15 ml of Marine Agar Broth with ACEM 1. This was placed on a shaker table and incubated at 24 °C for 6 hours.

Lawn cultures were prepared on marine agar plates with laboratory strains of *Bacillus subtilis, Escherichia coli, Listeria monocytogenes, Staphylococcus aureus* and *Salmonella typhimurium*, and the environmental strains, ACEM 4, ACEM 20, ACEM 22, ACEM 32, ACEM 21, *P. tunicata* and *P. ulva.* Bacterial suspensions were spread across the plates with a bent glass rod. These were allowed to dry before wells were punched into each plate using a sterile 5mm diameter plastic drinking straw. Sub samples (50 µl) of ACEM 1 were deposited by sterile pipette into the wells and allowed to diffuse into the agar. The plates were incubated at 25 °C for 36 hours and any zones of inhibition were recorded. The diameter of the well and zone of inhibition were measured and the diameter of the annulus recorded. Zones were bactericidal if no growth occurred and bacteriostatic if growth was merely limited.

Well diffusion assays were also completed with the HPLC extract fractions (48 hour old culture) for *L. monocytogenes*, *S. typhimurium* and *E. coli.*

Well diffusion assays were also completed on the environmental strains with a white colony isolated from ACEM 1 to determine if antibiotic activity was related to pigment production.

The *Pseudoalteromonas* species, *P. luteoviolacea* and *P. piscicida,* were tested for the presence of brominated compounds. The supernatant of *P. luteoviolacea* was also assayed for well inhibition, as it was the *Pseudoalteromonas* species that had produced most of the previously described, bioactive compounds for this genus.

Results

The HPLC fractions were tested for algicidal activity. The fractions containing the brominated compounds demonstrated no algicidal activity. The algicidally active compounds eluted very early in the run. In contrast, the brominated compounds were non-polar and eluted during the methanol gradient late in the run. Strains of *P. luteoviolacea* and *P. piscicida* did not contain any brominated compounds.

8.3 Stability of brominated compounds

Antibiotic properties of the compounds were not affected if left at room temperature for five days but the components did not tolerate autoclaving, or 10 minutes at 80 °C. The compounds were produced at substantially lower levels if ACEM 1 was cultured in bromine free media (with NaCl replacing sea salts) (Figure 8.1). Growth of ACEM 1 was slightly inhibited with 4 g/l KBr and no antibiotic compounds were identified. A slight increase of the brominated compounds was observed using the LCMS-MS if 1 g/l KBr was added.

8.4 Inhibition of environmental and pathogenic strains

Inhibition zones of between 4-10 mm were observed when the ACEM 1 compounds diffused from the wells into the lawn cultures of *E. coli, L. monocytogenes, Staphylococcus aureus, Salmonella. typhimurium*, ACEM 4, *Pseudoalteromonas ulva*, *Pseudoalteromonas tunicata* and ACEM 21. Slight inhibition or a bacteriostatic effect of the ACEM 1 compounds was observed for ACEM 22 but no inhibition was apparent for ACEM 20, ACEM 32, or *Bacillus subtilis*. Interestingly, the three *Pseudoalteromonas* species did not express their pigments in colonies surrounding the inhibitory zone (Figure 8.2). The brominated compounds repressed pigment production at the inhibitory zone interface, not their growth. The inhibition of pigment formation was not observed for other pigmented species such as the *Cellulophaga lytica* strain ACEM 21.

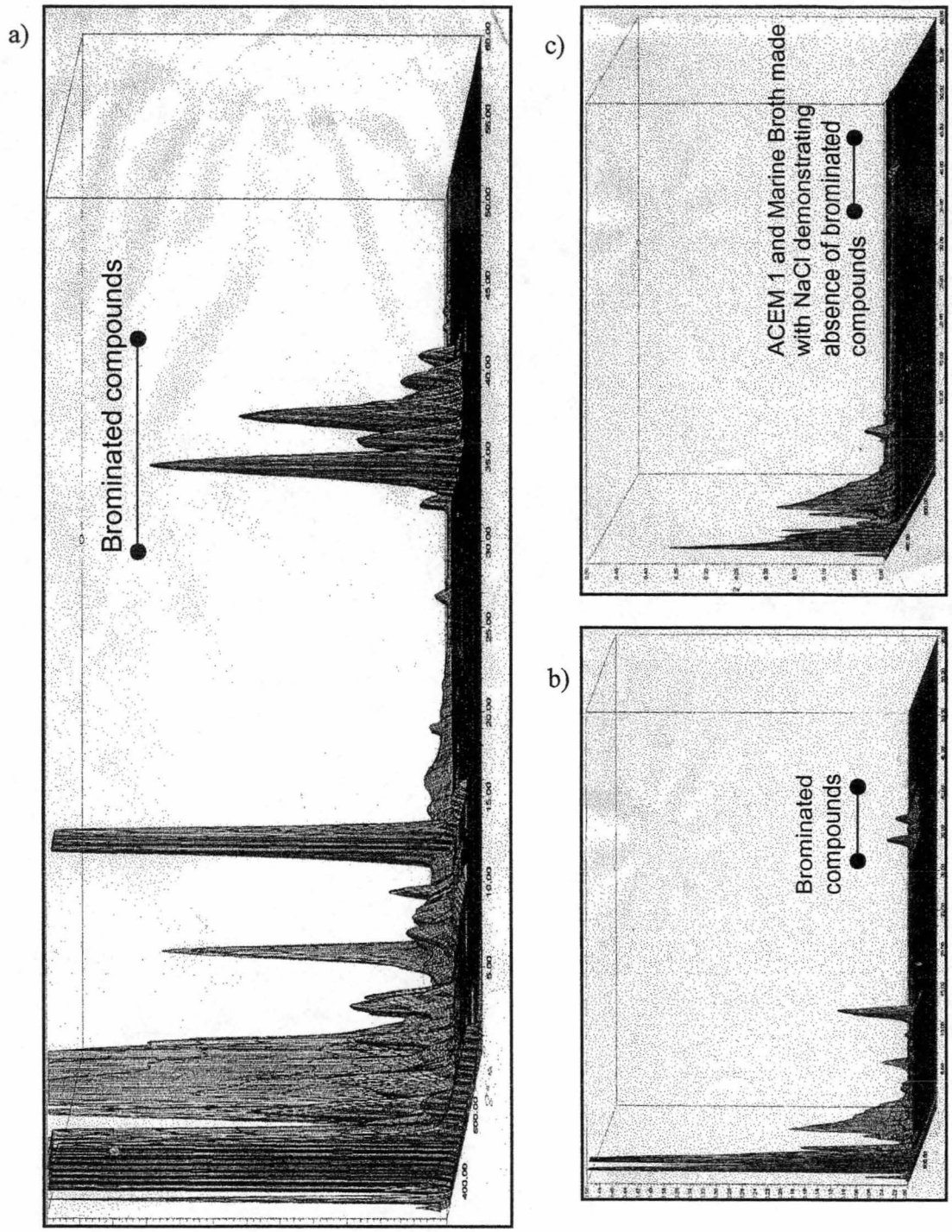


Figure 8.1 HPLC chromatogram with photodiode array detector showing absorption of the brominated compounds in the UV region. Figures b) represents the same trace as Figure a) but enables a comparison with Figure c) which demonstrates the effect of the addition of NaCl alone to the ACEM 1 culture instead of Sea Salts (Sigma) which contain bromine.

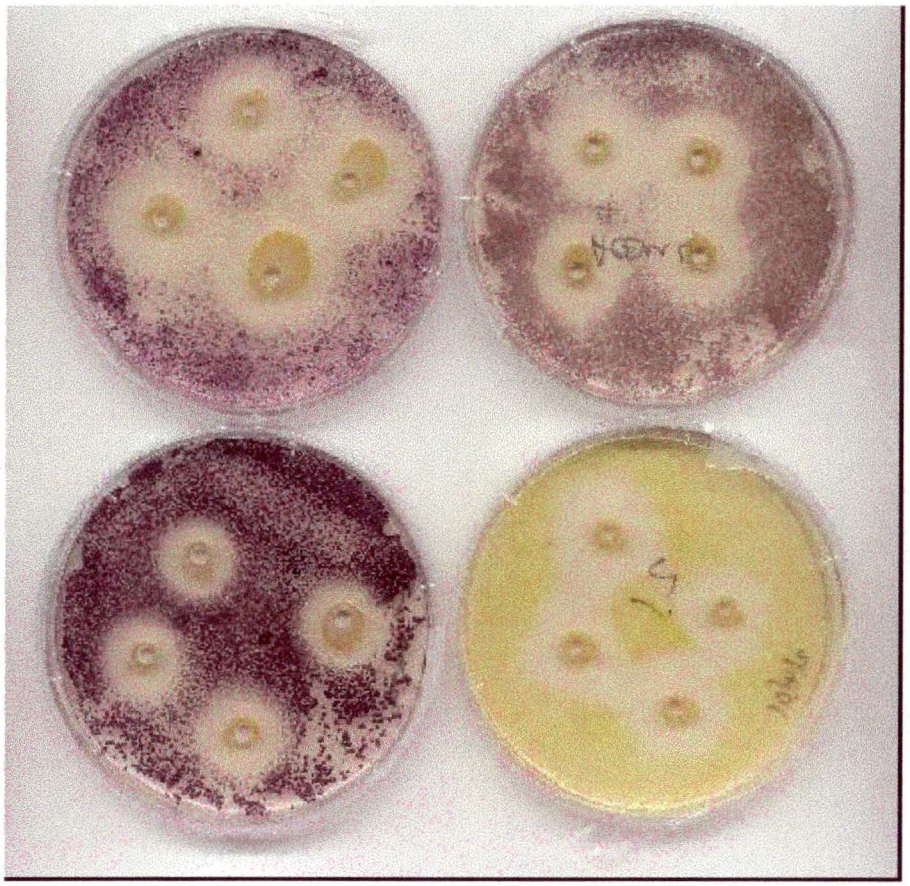


Figure 8.2 Zones of inhibition for ACEM 1 (50 µl bacterial cells plus supernatant) when applied to selected environmental bacteria (Clockwise from top left *P. ulva*, ACEM 4, ACEM 21 (*C. lytica*), *P. tunicata*.

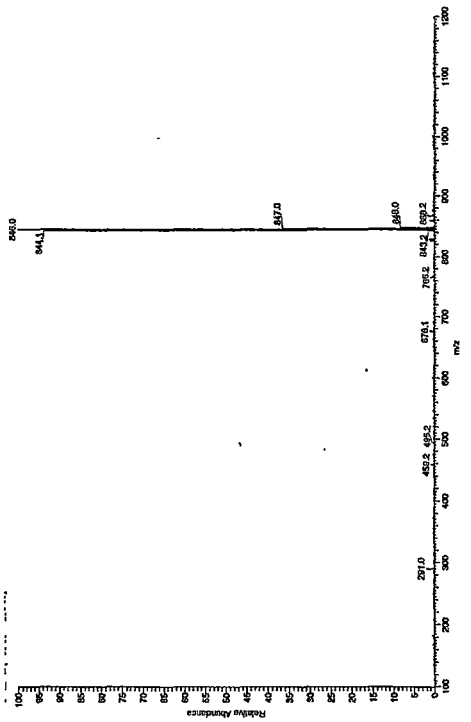
Wild type, non-pigmented ACEM 1 colonies were also used in the well diffusion assay to ascertain whether pigment production was related to antibiotic activity. Placing a non-pigmented-colony onto the culture lawn of the environmental cultures produced a zone of inhibition. The zones of inhibition were the same as those observed for the yellow-pigmented colonies.

When ACEM 1 supernatant was added at low concentrations (20 µl) to non-axenic algal cultures, growth of other bacterial species was hindered (see also chapter 4). It is possible that the autolysis mode of action observed for ACEM 1 demonstrated in chapter 4 is partly due to the production and activity of these brominated compounds.

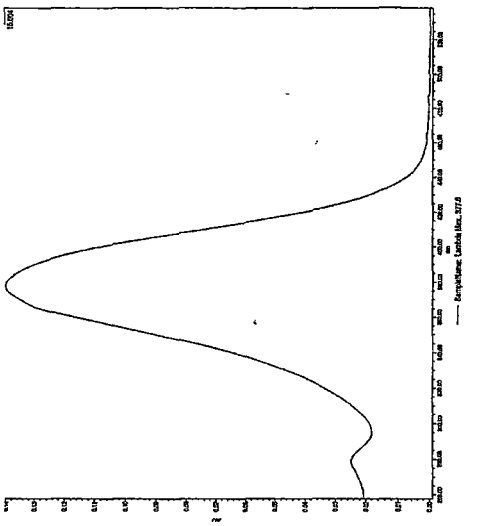
Previous studies demonstrated that the brominated compounds in *P. luteoviolacea* were small molecular weights and attached to the cells (Gauthier and Flatau 1976). In this study, the brominated compounds were extracellular as they were present in the filtered culture media. This does not preclude the presence of these compounds within the cells. Well inhibition assays using the type strain of *P. luteoviolacea* demonstrated no inhibitory activity. The cause may be the time in culture and the loss of these abilities. Other workers examining this genus have also found a lack of any bioactivity in many of the species, which supports this hypothesis (Holmström, C. pers. comm.). The addition of the HPLC fraction containing the brominated compounds to *G. catenatum* cultures extended the life of the *G. catenatum* culture (addition was equivalent to 10^{13} cells/l of ACEM 1 added to 100 ml of *G. catenatum* culture).

8.5 Structure: FAB-MS-MS

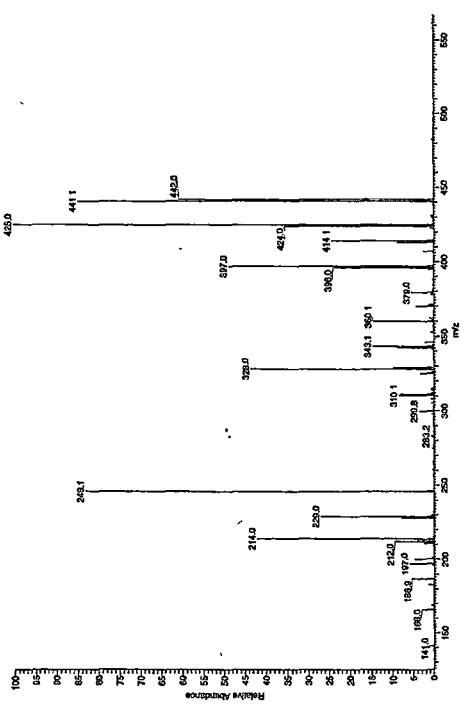
The brominated compounds were discovered as a result of their UV absorption when analysed with the photodiode array of the APCI on the LC-MS-MS. The UV absorption spectra of the most concentrated brominated peak is shown in Figure 8.3b. Several isomers of the brominated compounds were apparent (Figure 8.4b). Many had 2 to 4 isomers present. Compound separation on the HPLC was considered to be genuine isomerisation rather than an artefact of HPLC elution (peak splitting) as peaks eluting earlier and later had no indication of peak splitting.



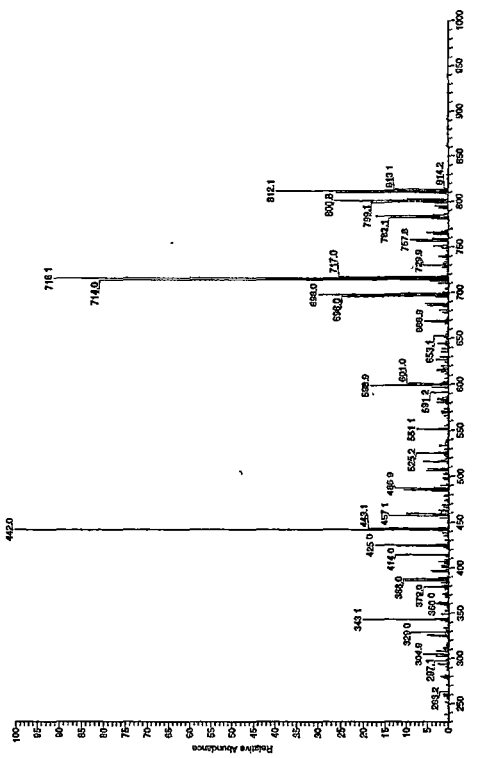
a) Mass spectral trace and molecular weight of major brominated compound



b) UV spectra of major brominated compound



c) MS – MS of 844/846 ion



d) MS – MS of 844/846 ion

Figure 8.3 Mass Spectral data, molecular weights, UV absorption and daughter ions (MS-MS) of the major brominated compound in ACEM 1.

Integration of the HPLC trace indicated that one particular brominated compound dominated (Figure 8.3), with up to 20 other similarly related compounds apparent at similar retention times to this peak (Figures 8.4, 8.5 and 8.6). All components contained bromine with one potentially containing a chlorine ion as well (Figure 5b, Figure 8.6b). The 18 compounds demonstrated isomeric and structural variation. The major structural differences were changes in the number of bromine and nitrogen atoms, and variation in the number of methyl groups. However, the overall structure of all the brominated compounds in ACEM 1 is likely to be very similar.

For mass spectral analysis, ions resulting from loss of C, H, N and O are usually small compared to those for bromine or other halogens. The presence of bromine ions in mass spectra is indicated by the fragmentation of the molecular weight ions. A compound that contains one chlorine atom will have an M+2 peak approximately 1/3rd the intensity of the molecular peak because of the presence of a molecular ion containing the ^{37}Cl isotope (Silverstein *et al.* 1991). A compound that contains one bromine atom will have an M+2 peak almost equal in intensity to the molecular ion because of the presence of a molecular ion containing the ^{81}Br isotope. A compound containing two bromine atoms (or one chlorine and one bromine) will therefore show a distinct M+4 peak in addition to the M+2 peak because of the presence of a molecular ion containing two atoms of the heavy bromine isotope. The ratio of these peaks for practical use of mass fragment graphs is shown in Table 1

Table 1: Intensities of isotope peaks relative to the molecular ion for combinations of bromine and chlorine; adapted from Silverstein *et al.* (1991).

Halogen present	Approximate % M+2	Approximate % M+4
Br	100	
Br_2	200	100
Br_3	300	290
Cl	33	
Cl_2	66	10
Cl_3	100	33
BrCl	130	33
Br_2Cl	230	160
$BrCl_2$	160	75

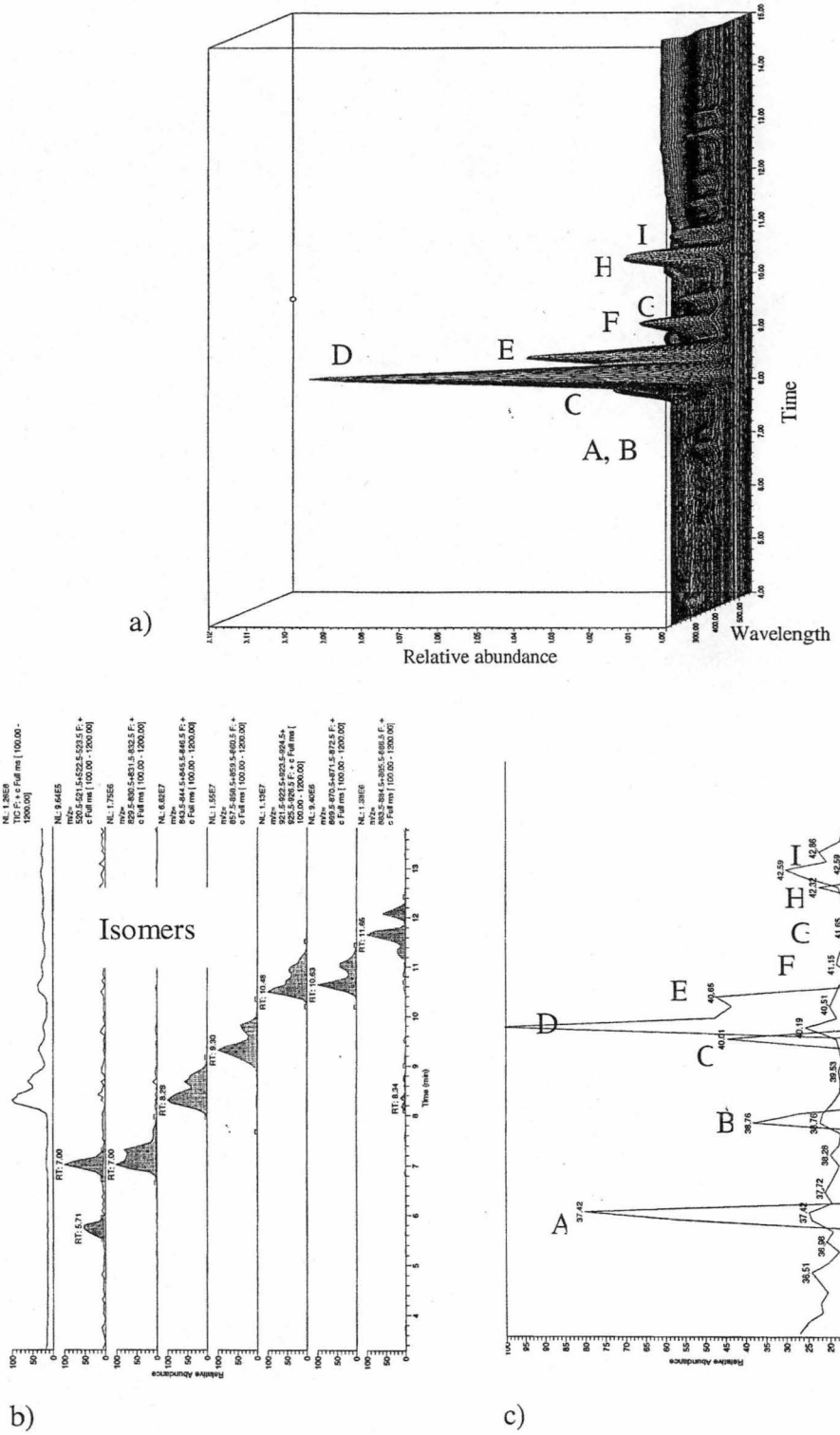


Figure 8.4 a) LC-UV photodiode array spectral data indicating absorption of the brominated compounds. b) Spectral data showing isomers of the brominated compounds. c) Retention time of brominated compounds and the relative abundance for the major ions present for the brominated compounds

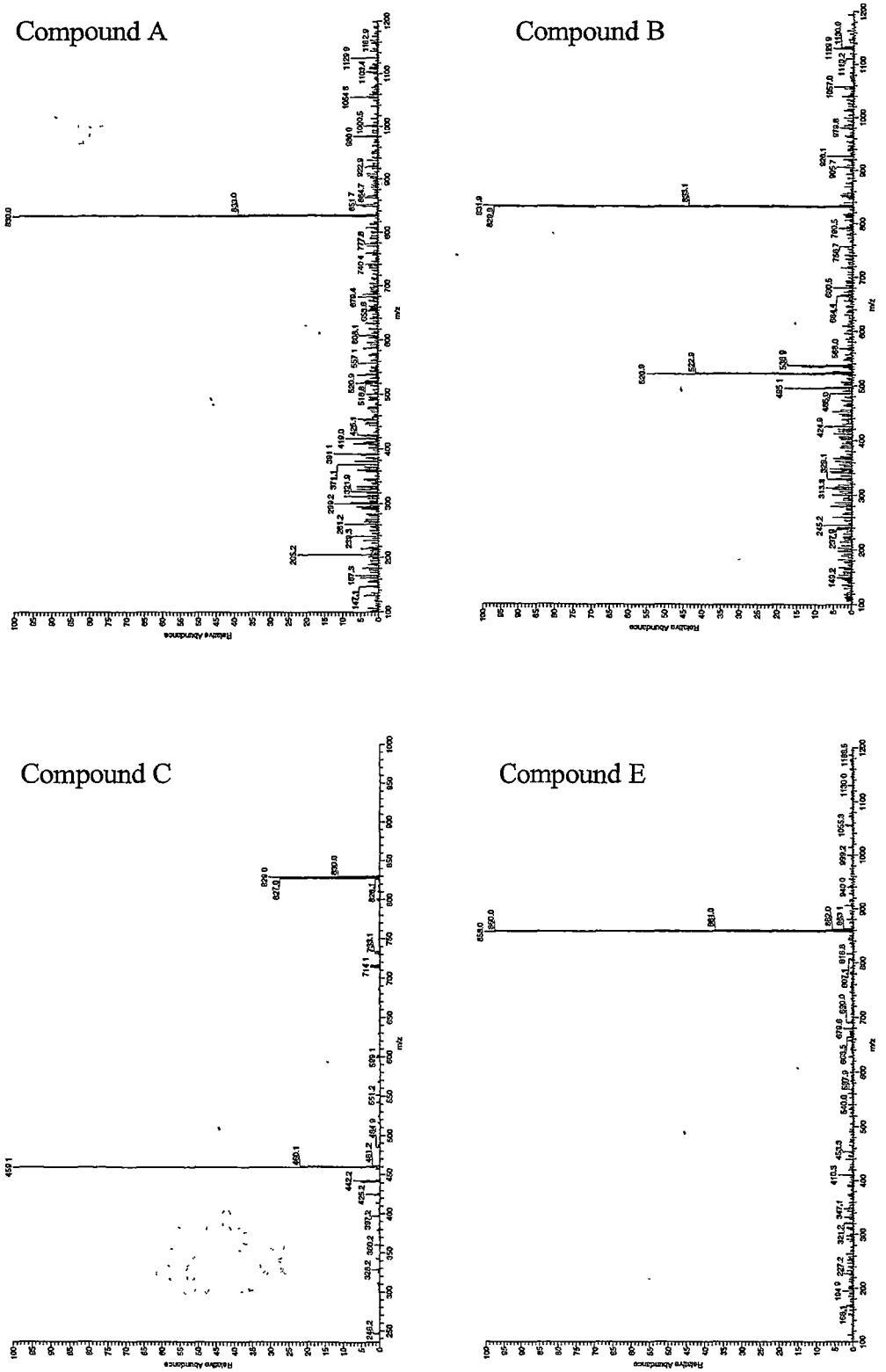


Figure 8.5 Spectral data and Molecular weights of some of the brominated compounds in ACEM 1

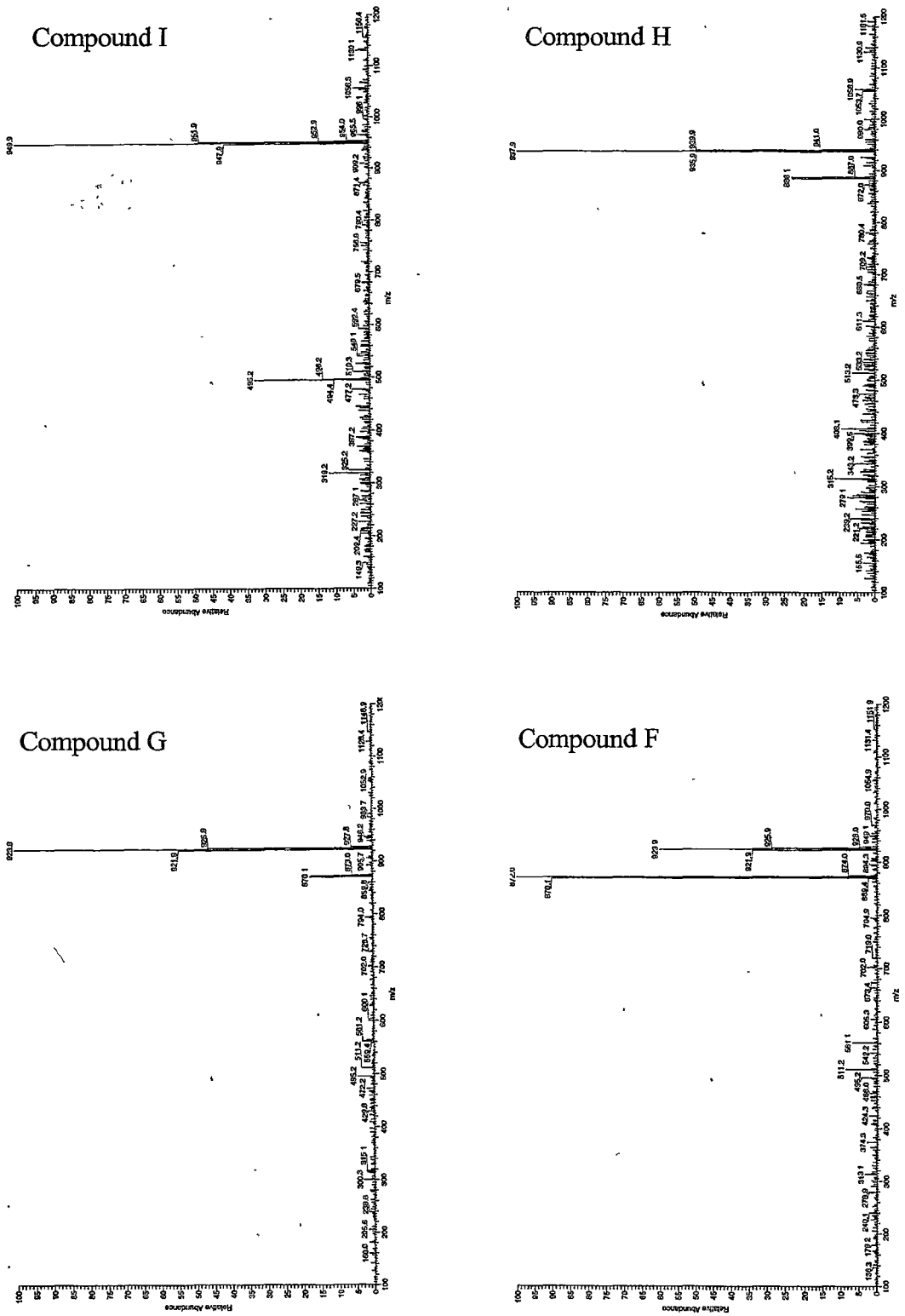


Figure 8.6 Spectral data and Molecular weights of some of the brominated compounds in ACEM 1

Using the information from the above ratio calculations some of the compounds in ACEM 1 contain two bromine atoms (Figure 8.5a) while others contain one atom (Figure 8.6b).

The dominant brominated compound contained one bromine (Figure 8.3). The odd-numbered molecular weight ion indicated that nitrogen was also present in the compound and signifies the occurrence of odd numbers of nitrogen ions. The molecular weights of the other brominated compounds not indicated in the figures are as follows: 860, 600, 522, 520. It is also possible that because of the presence of the M+2 ion, the major compound contains one sulphur atom (M+2 is over 10% abundance which may indicate the presence of sulphur or high oxygen numbers).

8.6 Structure: NMR

The proton and Carbon NMR of the main brominated compound again demonstrates the presence of halogens (identified as bromine from mass spectral data) aromatic rings, double bonds and oxygen (Figures 8.7 and 8.8).

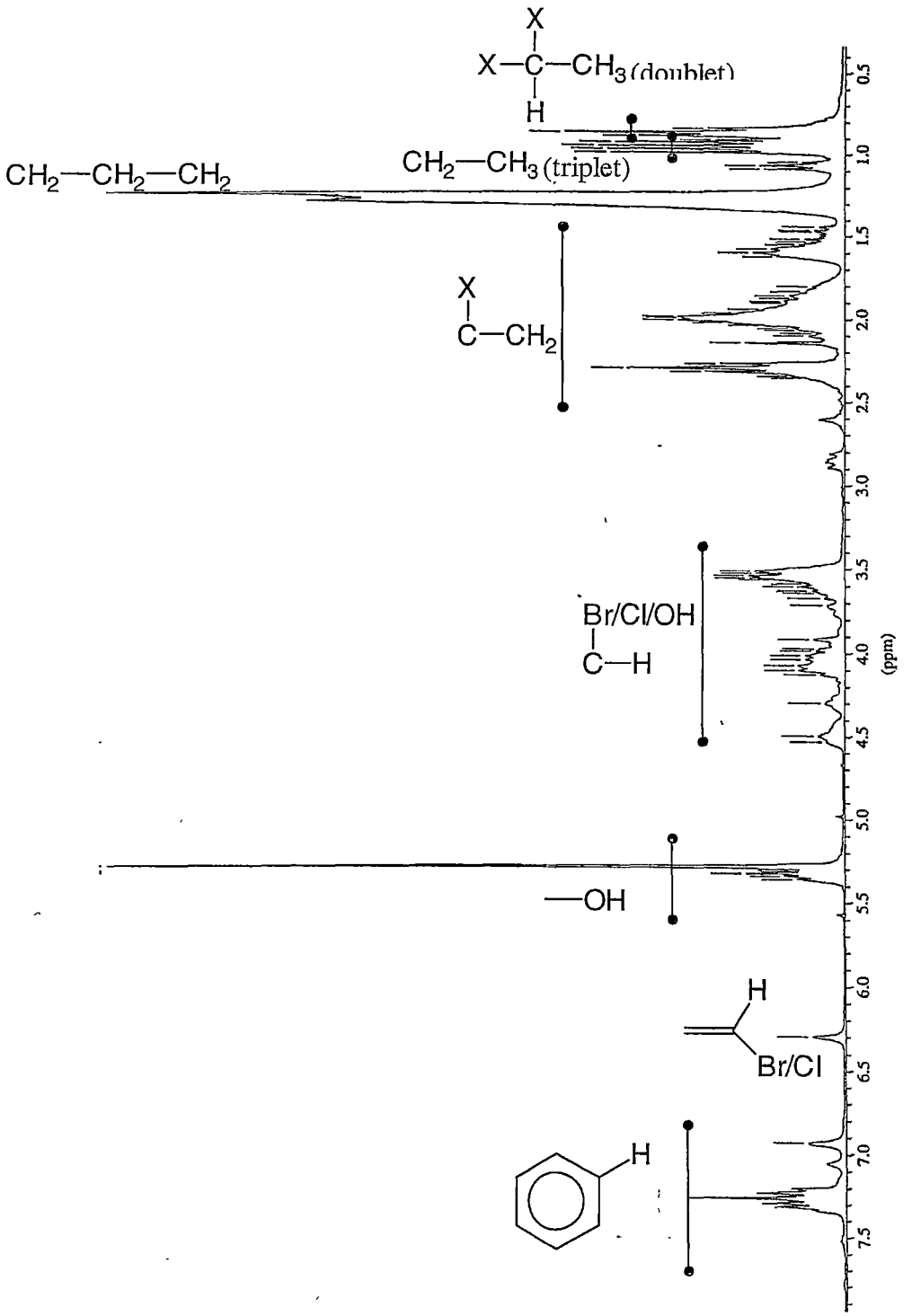


Figure 8.7 Proton magnetic resonance spectrum of major brominated compounds from ACEM 1. Solvent used was DCM. Chemical shifts of functional groups are labelled on the chart.

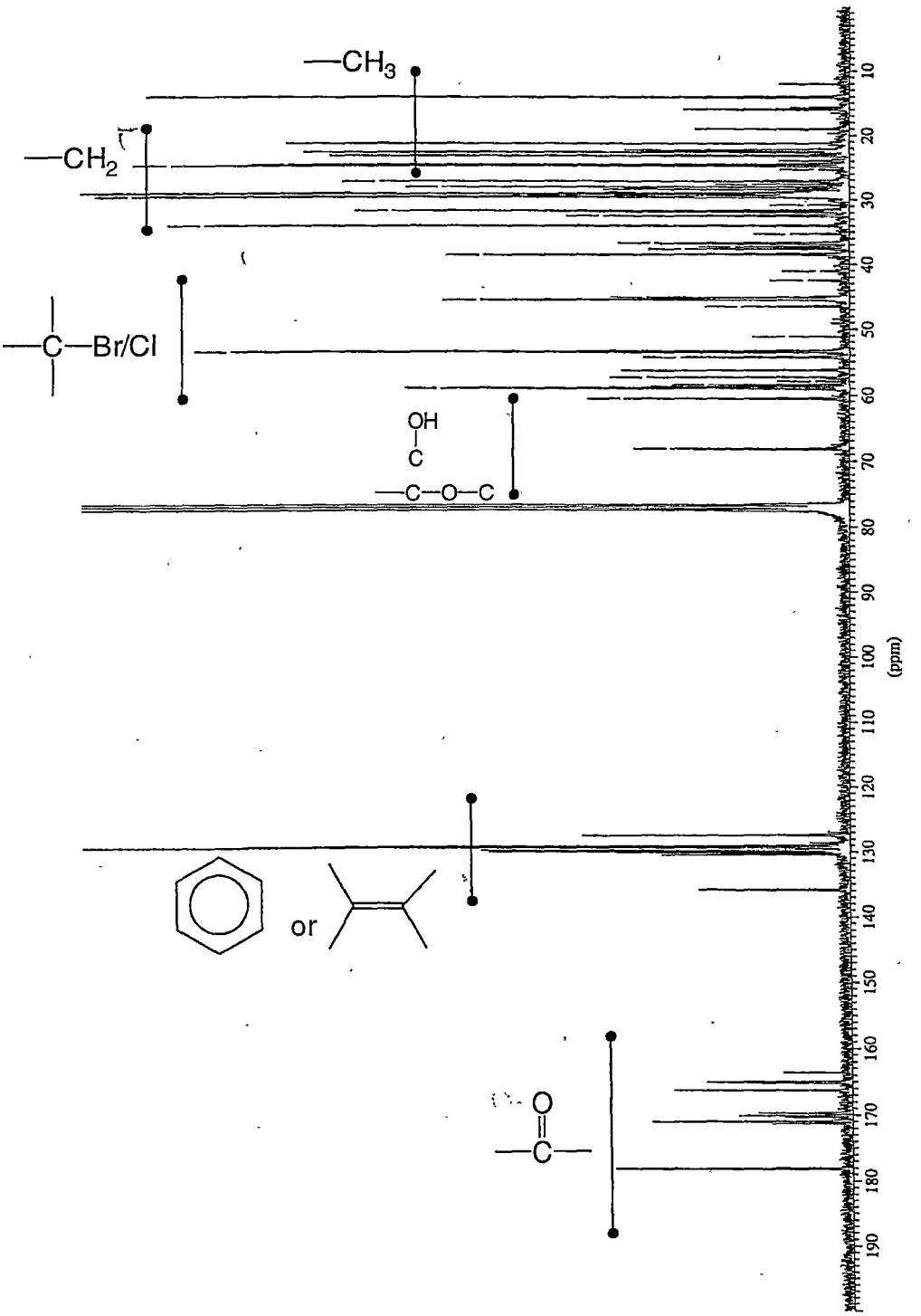


Figure 8.8 Decoupled ^{13}C-spectrum of the dominant brominated compound isolated from ACEM 1. Solvent used was $CDCl_3$. Chemical shifts of functional groups are labelled on the chart.

Discussion

The first antibiotic isolated and characterised from a marine bacteria was a 2,3,4-tribromo-5(1'-hydroxy-2',4'-dibromophenyl) pyrrole isolated from a species of seagrass *Thalassia* (Lowell 1966). Another compound, 2-(3',5'-dibromo 2'hydroxyphenyl)-3,4,5-tribromopyrrole, was isolated from the same species in the late 1970s (Faulkner 1978). Interestingly, although these bacteria were originally placed in the respective genera *Pseudomonas* and *Chromobacterium,* their taxonomy suggests they are probably *Pseudoalteromonas* spp..

Other antibiotic producing *Pseudoalteromonas* species have also been closely associated with macrophytes suggesting a commensal relationship between the two (Lemos *et al.* 1985). The relationship of macrophytes with *Pseudoalteromonas* also extends to the production of anti-algal and anti-biofouling compounds demonstrated by the species *P. ulva and P. tunicata* (Egan *et al.* 2001b, Holmström and Kjelleberg 1999). Elucidation of the anti-algal and anti-biofouling compounds produced by *Pseudoalteromonas* species has proved elusive.

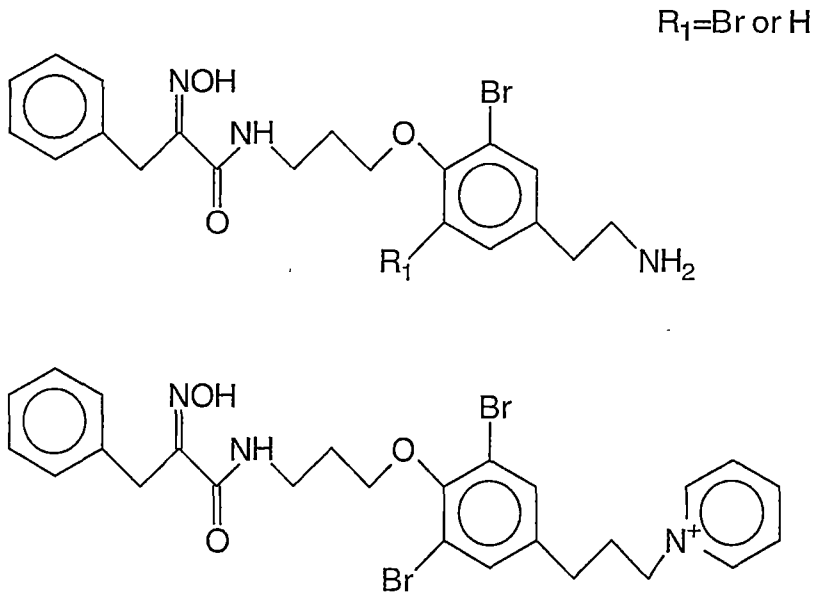
Four other *Pseudoalteromonas* species demonstrate antibiotic activity; *P. luteoviolacea, P. rubra, P. citrea* and *P. aurantia.* A study of *P. luteoviolacea* indicated that some of the antibiotic compounds, although not elucidated, were small-brominated secondary metabolites (MW<300) (Anderson *et al.* 1974). *P. luteoviolacea* (then identified as *Chromobacterium marinum*) was found to produce the same antibiotic determined by Faulkner (1978) as well as tetrabromopyrrole, hexabromo-2,2'-bipyrrole, 4-hydroxybenzladehdye and n-propyl-4-hydroxybenzoate. Gauthier *et al.* (1982) observed that the small molecular weight compounds produced by *P. luteoviolacea* were similar to the compounds found in three other *Pseudoalteromonas* species; *P. rubra, P. citrea* and *P. aurantia.* All of these compounds are a much lower molecular weight than those found in ACEM 1.

Larger antibiotic compounds produced by *P. luteoviolacea* were also discovered (Gauthier and Flatau 1976). These high molecular weight compounds (~100,000 MW) were found to be associated with a protein produced by the bacteria and only

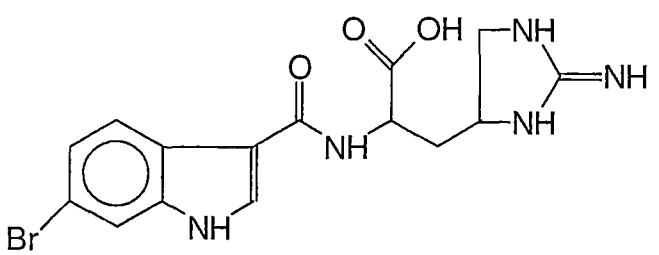
occurred in late log to stationary phase (McCarthy *et al.* 1994). Consequently, the dominant heterocyclic antibiotics produced by *Pseudoalteromonas* are pyrroles. Pyrroles are based on a five membered nitrogen-containing compound and have an aromatic nature. Pyrroles are usually found in association with sponges and many have bioactive abilities (Rao *et al.* 2000, Gribble 1999).

There are many naturally occurring brominated pyrroles in the marine environment as they are 10^{18} times more reactive than benzene to electrophilic aromatic bromination (Gribble 1999). Naturally occurring pyrrole derivatives are haem and chlorophyll. Interestingly, the structures of the marine harmful algal bloom toxins anatoxin (produced by freshwater cyanobacteria) and domoic acid (produced by marine diatoms and red algae) (Falconer 1993) are also pyrrole derivatives. It is therefore worthy to note that ACEM 1 was closely associated with the toxic algae *G. catenatum*. It has been suggested that bacteria either produce the compounds that cause paralytic shellfish toxins or their initial derivatives for species such as *G. catenatum*. This hypothesis has created much discussion (Doucette *et al.* 1998). Species from the genus *Pseudoalteromonas* have demonstrated the ability to produce tetrodotoxins (chapter 1) and paralytic shellfish toxins (chapter 1) as well as secondary metabolites, but there are still questions in relation to the link between bacterially produced bioactive compounds and algal or sponge metabolites.

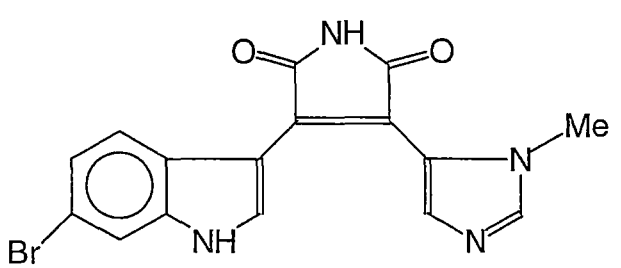
Brominated compounds isolated from marine animals and sponges with similar molecular weights as those in this study are discussed further below. The examples demonstrate the bioactive nature of these brominated metabolites. Many of the brominated compounds come from the sponge species *Psammaplysilla purea* and *P. purpurea* of the family *Aplysinellidae* which produces 95% of their secondary metabolites as brominated structures (Rao *et al.* 2000).



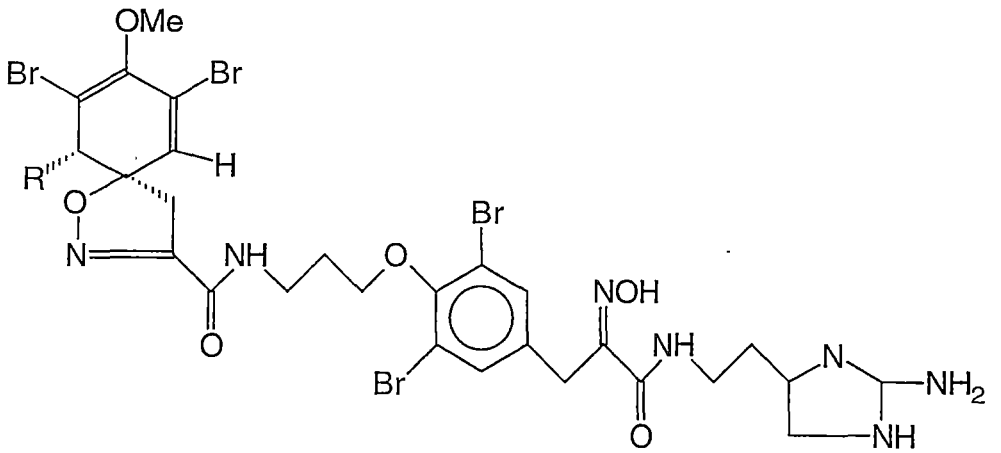
Two of nine bromotyrosine derived metabolites and purpuramines from *P. purpea* (Yagi *et al.* 1993. Tetrahedron, 49, 3749). All show antibacterial activity against *Staphylococcus aureus*, MW 520-636.



One of three indoles from the ascidian *Leptoclinides dubuis* which contains the rare amino acid enduracididine (Garcia *et al.* 1996. Journal of Natural Products, 59, 782), MW 393.



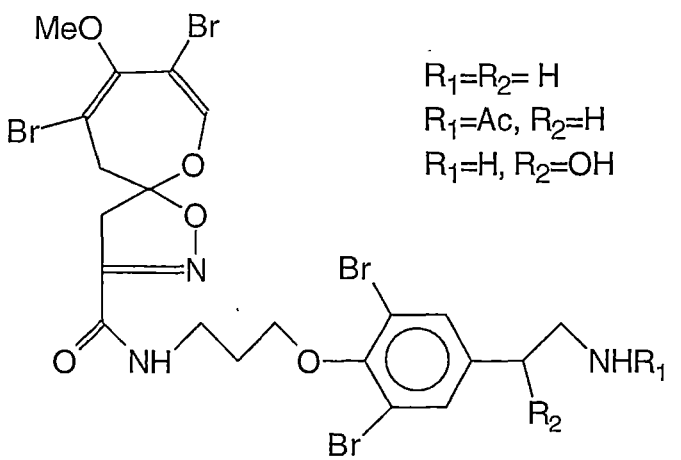
One of four didemnimides from the ascidian (sea squirt) *Didemnum conchyliatum* that are potent feeding deterrents (Vervoort *et al.* 1997. Journal of Organic Chemistry, 62, 1486), MW 367.



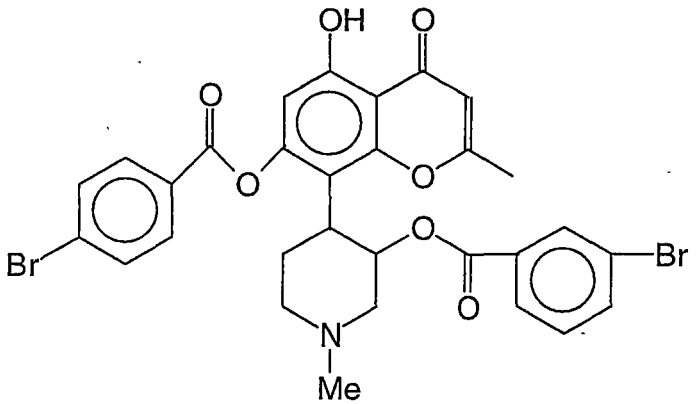
R=OH: Activates myosin EDTA-ATPase at 0.3-30 μm and inhibits myosin Ca^{+}-ATPase and Na^{+}, K^{+}-ATPase (Nakamura *et al.* 1987. European Journal of Biochemistry 167, 1), MW 934

R= MeO: stabilizer of smooth muscle myosin filaments that modulate ATPase activity of dephosphorilated myosin (Takito *et al.* 1986. Journal of Biological Chemistry, 13, 861), MW 948.

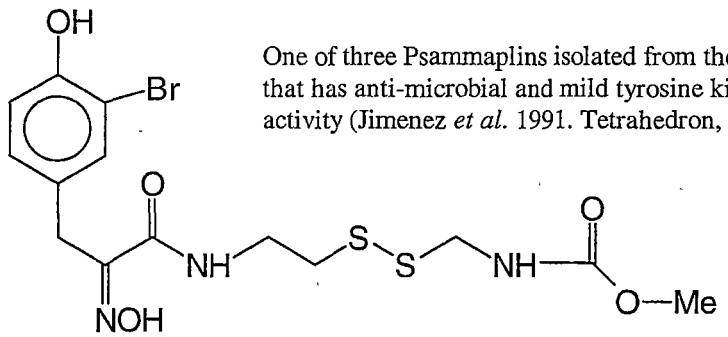
Both compounds were isolated from the sponge *P. purpea*



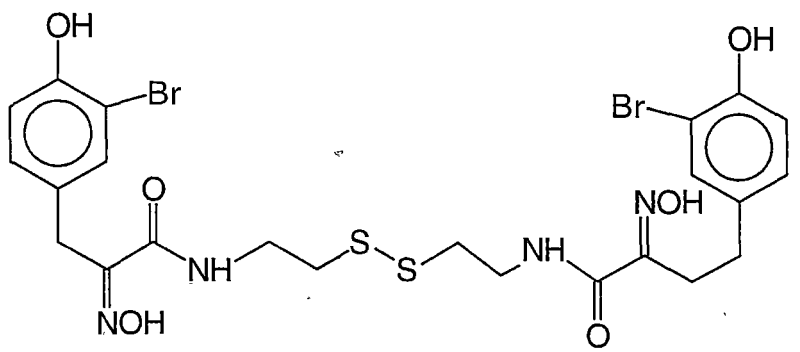
These three compounds are anti-microbial Psammaplysins isolated from the sponge *P. purea* (Roll *et al.* 1986. Journal of the American Chemical Society, 107, 2916). Psammaplysins are derived from dibromotyrosine via benzene oxideoxepin intermediates, MW 710-726.



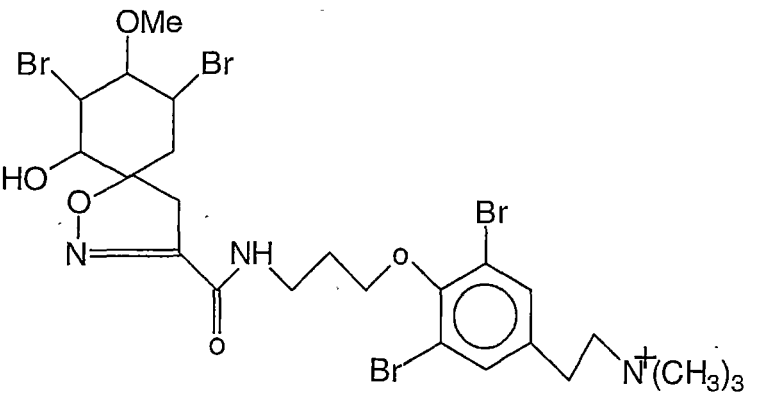
Tubastraine produced by stony coral *Tubastraea micrantha* which is avoided by the Crown of Thorns sea star (reviewed in Gribble *et al.* 1997. Accounts of Chemical Research, 31,141), MW 712.



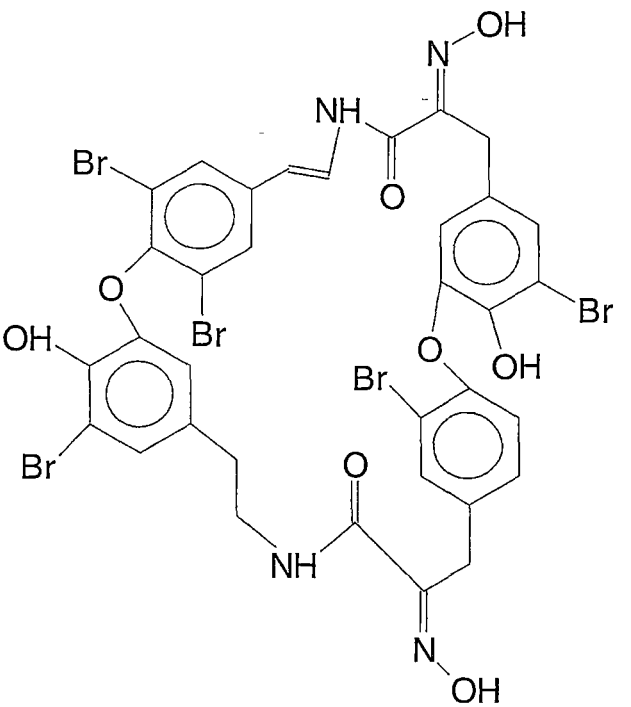
One of three Psammaplins isolated from the sponge *P. purpurea* that has anti-microbial and mild tyrosine kinase inhibitory activity (Jimenez *et al.* 1991. Tetrahedron, 47, 2097), MW 451



Psammaplysin isolated from the sponge *Psammaplysilla* (Quinoa *et al.* 1987. Tetrahedron letters, 28, 3229) MW 665.



A purealidin from the sponge *P. purea* that demonstrates antibacterial activity (Kobayashi *et al.* 1991. Tetrahedron, 47, 6617), MW 747.



A macro-cyclic peptide identified as a bastadin that demonstrates anti-microbial activity, isolated from the sponge *P. purea.* (Carney *et al.* 1993. Journal of Natural products. 56. 1531). MW 992.

Sponges of the genus *Agelas* are also chemically defended from fish predation by brominated pyrrole alkaloids (Assmann *et al.* 2000). Another group of sponges (*Aplysina* spp.) have demonstrated a high degree of antimicrobial activity which was thought to be enhanced by the associated bacteria (Hentschel *et al.* 2001). These bacteria were from the genus *Pseudoalteromonas* and the α proteobacteria class.

The structures identified from sponge extracts demonstrate similarities to each other and it would be realistic to compare the MS and NMR traces of the brominated compounds produced by ACEM 1 to these compounds. Most of the compounds reported in the literature that are produced from sponges demonstrate anti-microbial activity. Some show anti-tumour activity but many are larger than the small metabolites produced so far by other *Pseudoalteromonas* species, or are smaller than the bioactive polysaccharides or protein associated metabolites. To my knowledge antimicrobial compounds of a similar size to those found in ACEM 1 have not been isolated from any bacteria or sponge species. Many of the larger molecules with a molecular weight similar to the compounds isolated in this study, contain 4 to 8 bromines atoms or are polysaccharides. However, it would be realistic to hypothesize that the structure of the brominated compounds in ACEM 1 may have a similar structure to some of the bacteria or sponge brominated metabolites previously described (e.g. aerothionin, aeroplysinin, dibromoverongiaquinol, bromochloroverongiaquinol and heptylquinolone).

The findings for ACEM 1 support the increasing recognition that bacteria can produce complex secondary metabolites or their derivatives. According to a leading natural product chemists the study of bacterial metabolites is yet to be fully realised (Faulkner 2001, 2000). It has been demonstrated that bacteria or cyanobacteria can play a role in the production secondary metabolites of sponges which are often the most concentrated sources of these compounds. Research into the bryozoan *Amathia wilsoni* isolated from Tasmanian waters indicated that it produced several brominated secondary metabolites. These metabolites were smaller than those produced by ACEM 1 and were concentrated on the surface of the bryozoan. The metabolites correlated with a rod shaped bacterial species that only occurred on the tip of the bryozoan (Walls *et al.* 1995). Other bryozoans

from the area had a less patchy distribution of bacteria and lower bacterial numbers. However, an *Oventricosa* species had the lowest bacterial numbers, but was still chemically defended (Walls *et al.* 1993), indicating the complexity of these interactions. Another example is a brominated compound produced by a symbiotic cyanobacteria rather than the resident marine sponge (Unson *et al.* 1994). A further study of the sponge demonstrated that metabolites were localised to both the cyano- and the symbiotic bacteria of the sponge (Bewley *et al.* 1996).

Variations in the antibacterial activity of the sponge *Ircinia ramosa* demonstrated that the associated bacteria were capable of producing antibacterial metabolites (smaller than ACEM 1). Interestingly, the antibacterial activity originated from polar solvent fractions in cooler months and appeared in non-polar fractions in warmer months (Thakur and Anil 2000). The authors concluded that the chemical nature and production of antibacterial compounds produced by the sponge or its associated bacteria appeared to be governed by the environment. This is relevant for the bactericidal and algicidal features of ACEM 1. Both types of bioactive compounds produced by ACEM 1 have the potential to vary in the marine environment. The capability of switching one or other of these abilities on or off would make energetic sense in a natural environment. This remains an area for further research.

Future analyses using FISH may be helpful in the discovery of more of these animal/plant/bacterial relationships and to further understand whether or not bacteria are responsible for the production of secondary metabolites or their precursors. Studies so far have demonstrated that a diversity of species exists on sponges (Webster *et al.* 2001). Identification of these genera using FISH may help to rectify whether these relationships are symbiotic or merely coincidental for a particular species. Specific FISH probes such as those for the genus *Pseudoalteromonas* would be valuable, as this genus has already demonstrated the ability to produce brominated secondary metabolites.

9. Thesis Conclusions

Algicidal bacteria were present in the estuary and members of their respective classes were often associated with algal blooms. The discovery of five algicidal isolates from the Huon Estuary adds to the present knowledge of this type of bacterial action. For the first time, two gram-positive species demonstrated algicidal activity. The other novel algicidal species were from the more traditional algicidal genera, *Cellulophaga* and *Pseudoalteromonas*. All isolates demonstrated similar algicidal activity against *G. catenatum* vegetative cells. Some isolates, such as the genus *Cellulophaga,* have the potential to be far more physically active in the water column which may provide a mechanism for nutrient sequestering.

Isolation of such diverse genera indicates the discovery of five algicidal species is a positive outcome for the search for new bacterial isolates that have bioactive potential in the marine environment. In addition to the algicidal compounds, ACEM 4 produced inhibitory biofouling compounds and ACEM 1 produced antibacterial compounds. Discovery of these additional activities further demonstrates the biotechnological potential for the genus *Pseudoalteromonas*. The ability of this genus to produce a spectrum of bioactive compounds via a number of unrelated mechanisms demonstrates the adaptability of the genus to the variable conditions that occur in the marine environment. Diverse ranges of bioactive activities are not new to the genus *Pseudoalteromonas*. They are however interesting, when examining the ecological context of when these compounds are produced in their natural environment. Future work on understanding whether algicidal and antibiotic mechanisms switch on and off with different environmental triggers, would be a valuable contribution to knowledge of the ecology of microbial systems. The potential for systems such as quorum sensing shows great promise in this area with respect to activation or inactivation of these bioactive mechanisms. Recent advances have demonstrated that certain quorum sensing mechanisms can be effective at cell concentrations found in the marine environment.

The relationship of estuarine bacteria both to each other and within their environment can vary. The use of morphological techniques, FISH and fatty acid analysis identified bacterial and algal community composition changes in the water column over the sampling season. The bacterial community included many genera with seasonal transitions resulting in the dominance of a few genera at any one time. Changes in bacterial species were related to shifts in algal community composition. This indicates that any one bacterial group does not consistently dominate the composition of a marine bacterial community. These microbial groups, made up of a combination of different genera, have the capability to vary in much the same way as algal species vary over the season.

For most of the year, apart from during the *Pseudonitzschia* spp. bloom, CFB and α proteobacteria were dominant classes in the surface and pycnocline samples. Identification of γ proteobacteria was not possible using BCFA analysis but the absence of BCFA in the integrated samples despite the higher bacterial numbers indicate that γ proteobacteria or species without BCFA were a major part of the community. FISH analyses also demonstrated high levels of γ proteobacteria during the decay of the *G. catenatum* bloom and the commencement of the *Pseudonitzschia* spp. bloom. The photic depths where the diatom bloom occurred demonstrated a decrease in BCFA concentration at this time indicating a community shift from species with BCFA to species with less BCFA. The integrated water samples reflected a microbial community with a remarkable capacity for microbial degradation and/or heterotrophic feeding. Many of the fatty acids detected below the pycnocline were breakdown products of those fatty acids present in the upper photic layer. This finding may be explained by the presence of heterotrophic or bacterial sources in the deeper waters. The genus *Shewanella* may have played a part in nutrient recycling deeper in the water column, as they were often present in the integrated water samples in winter. The ability of novel temperate *Shewanella* isolates to produce the essential fatty acid EPA at similar levels to those previously found only in barophilic and psychrophilic niches is a unique finding. This feature may be of biotechnological interest. Future use of the FISH probe for the psychrotrophic section of the genus *Shewanella* may help determine if this group is present in ecologically significant proportions, allowing nutritional benefits to accrue to higher trophic levels.

The field study reported in this thesis demonstrated that it is useful to apply a variety of techniques to determine the microbial composition of a community. The research represents the first combined use in the marine environment of traditional morphology, fatty acid analysis, and a molecular method (FISH). It is also one of the first Australian studies to use FISH in an estuarine or marine system. There are complex variations within an estuarine system such as flushing time, tidal flow, estuary shape and human or agricultural inputs. The large amount of humic material in the Huon Estuary distinguishes it from many river systems and is a major factor in the biological processes that occur within the estuary. In addition, this aspect also has implications for research methodologies, as some techniques, particularly FISH, are compromised by the presence of these substances.

Algicidal activity in the marine environment could be effective at bacterial concentrations that occur during algal blooms. Alternatively, algicidal activity may be artificially enhanced due to cell density on particulate matter. Potentially, *G. catenatum* vegetative cells could be affected in the natural environment by high numbers of algicidal bacteria either by natural means or via human intervention. Physical factors may mitigate this effect, such as the rapid flushing time of the Huon Estuary or biological factors such as the production of *G. catenatum* resting cysts which occur throughout the alga's lifecycle. Algicidal bacteria appear to be ineffective at harming the cyst stage so using them as a control method may be ineffective. The control of other toxic algal species by the use of algicidal bacteria, might be possible if the toxic species have no resting cyst or occur in an enclosed, low flow environment. In practice, many toxic algal blooms occur in open ocean areas and estuaries which are not easily confined. Fresh water systems, by contrast, are enclosed and have been successfully treated for algal and cyanobacterial blooms using chemical methods. Identification of bacterial biocides antagonistic to toxic fresh water algal species might be a practical research direction for the investigation of algicidal bacteria.

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