Algal-bacterial interactions:

a study of *Gymnodinium catenatum* and its associated bacteria

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By

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Declarations

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Abstract

Over the last decades, harmful algal blooms (HABs) have increased globally in both frequency and extent, leading to intensified efforts to determine the primary factors that are controlling the population and toxin dynamics of bloom-forming algal species. One poorly understood factor in HAB ecology, yet shown to be of great importance, is that of algal-bacterial interactions.

Efforts to investigate the relationships and mechanisms of interaction are often hindered by the complex composition of the algal-associated bacterial communities, wherefore this present study used simplified algal-bacterial experimental model systems. The model systems are based on the toxin producing dinoflagellate *Gymnodinium catenatum* and two of its associated bacteria, *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881, both known to support growth and survival of *G. catenatum* in culture. Using the experimental model systems, the work presented in this thesis examined the growth dynamics of *G. catenatum* and its associated bacteria, and studied the effect of bacteria on the toxicity of *G. catenatum.* The abundance of *Alcanivorax* and *Marinobacter* genotypes associated with natural populations of *G. catenatum* in the Derwent Estuary and D'Entrecasteaux Channel, south-east Tasmania was also investigated.

This thesis demonstrates that the bacterial community is important for growth of *G. catenatum* and showed that different bacteria, in particular *Marinobacter* sp. DG879, have a significant effect on the growth dynamics and toxicity of *G. catenatum* laboratory cultures. When grown with *Marinobacter* sp. DG879, *G. catenatum* was found to remain viable in culture for a period longer than ever recorded, suggesting that *Marinobacter* sp. DG879 produce a compound inducing a positive growth response in *G. catenatum,* or that it plays a role in carbon recycling or nitrogen fixation. Toxin analysis of the *G. catenatum* / *Marinobacter* sp. DG879 cultures revealed a toxin profile significantly different to other analysed treatments containing different bacteria. As the experimental design of the study sought to minimise genetic variation among treatments, and with biotransformation generally operating on extracellular toxins (this study focussed on intracellular toxins), the differing toxin profile seen in

the *G. catenatum* with *Marinobacter* sp. DG879 is most likely a response to *Marinobacter* sp. DG879 influencing toxin synthesis indirectly through its effect on dinoflagellate physiology. The field studies also suggest that *Marinobacter* sp. may have an important role in natural populations of *G. catenatum*, preceding *G. catenatum* blooms.

With the use of molecular detection methods becoming more widespread, and with more research on naturally occurring blooms, there is potential that specific elements of the bacterial population, such as *Marinobacter* sp. DG879 could be employed as indicator species of *G. catenatum* and other algal blooms, ultimately allowing for early detection and management of potentially harmful blooms.

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1.1 Introduction

The global increase in abundance and distribution of harmful algal blooms (HABs) over the past few decades (e.g. Hallegraeff, 1993; Anderson et al., 2002) has lead to intensified efforts to determine the primary causes controlling population and toxin dynamics of the bloom-forming algal species. The frequency, duration, and intensity of algal blooms are related to a number of chemical, physical, and biological factors. These factors include nutrient availability, light, mixing conditions, and temperature, as well as competition and predation between algal species (Granéli et al., 2008), and interactions between algae and organisms of different trophic levels (Granéli and Turner 2006).

Phytoplankton dynamics are influenced by abiotic (bottom-up) and biotic (top-down) factors, where top-down control refers to a top predator controlling the structure and population dynamics of the ecosystem, and bottom-up control refers to how nutrient supply, productivity and type of primary producers control the ecosystem structure (Hoekman et al., 2009). The traditional view of top-down/bottom-up control is limiting in terms of assessing phytoplankton population dynamics. There is currently a reasonably good understanding of the factors behind bottom-up control (e.g. Azam et al., 1987; Fasham et al., 1990; Anderson et al., 2002; Thompson et al., 2008). Due to the difficulty of studying interactions between microorganisms, and studying the microorganisms themselves quantitatively, the biological factors and their significance in top-down control are more poorly understood. Research over the last decades has uncovered a range of biological factors influencing phytoplankton growth dynamics, with virus-, parasite-, and bacterial interactions all shown to play important roles (Doucette et al., 1998; Thyrhaug et al., 2003; Park et al., 2004).

The interaction between algae and bacteria has emerged as an important factor influencing HABs (for reviews see Doucette, 1995 and Doucette et al., 1998), and it is

today a well-accepted fact that phytoplankton and bacteria, which are ubiquitous in the aquatic environment, form part of highly complex microbial food webs (Azam et al., 1983). The traditional view of marine microbes divided bacteria and microalgae into separate ecosystem compartments, with the interactions between the two either ignored or simplified to that of competition for nutrients. However, there is a considerable body of research over the past few decades that has established the existence of complex and sometimes highly specific relationships among the bacteria and phytoplankton (review Doucette et al. 1998).

1.2 Algal-Bacterial interactions

1.2.1 The Phycosphere

As early as 1972, the micro-environment surrounding the phytoplankton cell was recognized as a zone where bacteria and phytoplankton may interact. Bell and Mitchell (1972) coined the term “phycosphere” (Fig. 1.1), as analogous to the terrestrial “rhizosphere”; the area of soil immediately surrounding and influenced by plant roots. Hence, the “phycosphere” is the zone where any associated bacteria, free-living or attached, are influenced by the algal cell. Bell and Mitchell (1972) suggested that the phycosphere community would be formed by chemotaxis of the bacteria toward the algal cell, but did not consider more complex two-way interactions such as symbioses between the organisms. Despite this, the final words of their article anticipated the existence of such complex interactions of what might be found next: “It would appear that the phycosphere is a region of interactions that have only begun to be evaluated” (Bell and Mitchell, 1972, p. 276).

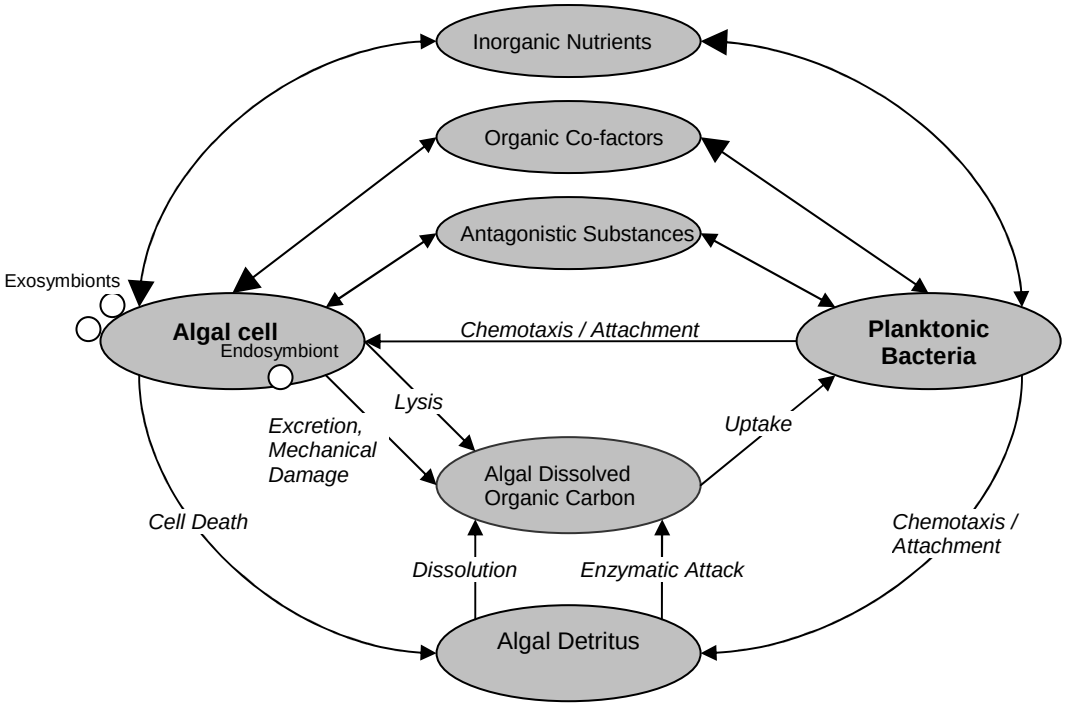


Figure 1.1 A schematic diagram of some of the interactions that occur within the Phycosphere. Arrows with a large head are showing the predominant direction of a process. Based on the review by Cole, 1982.

Ten years after Bell and Mitchell's (1972) description of the phycosphere, Cole (1982) gathered laboratory evidence which strongly suggested that a zone existed within which bacteria and algae closely interact in both stimulatory and inhibitory ways. The major driving force of the dynamics in the phycosphere was considered to be bacterial chemotaxis, with the size of the phycosphere dependent on the concentration of released matter, as well as on the degree of turbulence in the water (Cole, 1982). Following the work by Cole (1982), Azam et al. (1983) demonstrated that bacteria show kinesis (i.e. movement towards an increasing intensity of stimulus) in a field 10-100µm from algal cells, just close enough to take advantage of the DOC.

1.2.2 Algal-Bacterial associations and interactions

The physical relationship between algae and bacteria can range from intracellular (Silva, 1982, 1990; Seibold et al., 2001; Schweikert, 2003) to extracellular. The intracellular bacteria have been observed in several phytoplankton species, e.g. *Gonyaulax tamarensis* (Silva, 1990), *Noctiluca scintillans* (Seibold et al., 2001), and *Alexandrium tamarense* (Schweikert, 2003). The relationship between the bacteria and the phytoplankton appears mutually beneficial, where the intracellular bacteria syn-

thesize nutrients (for example, by photosynthesis, nitrogen fixation or an increase in enzyme activity) for the host, while the host supplies the bacteria with optimal habitats (Seibold et al., 2001). As for the extracellular bacteria, surface colonisation provides several advantages, for example enhanced access to released nutrients, or, in some cases, shelter from predation or protection from ultra-violet radiation (UV) (Dang and Lovell, 2000). Living, senescent and dead phytoplankton exudes soluble compounds (Wolter, 1982); for example, grazing, excretion, lysis, and autolysis are all responsible for release of algal dissolved organic carbon (Cole, 1982). Phytoplankton are known to release up to 25% of the total amount of organic carbon (fixed by photosynthesis) into the phycosphere (Doucette, 1995), and this dissolved organic matter (DOC) has been found to be rapidly remineralised by the bacterial community (Wolter, 1982; Rooney-Varga et al., 2005).

Phytoplankton and bacteria have been shown to influence each other on a range of different levels (for review see Doucette, 1995; Doucette et al., 1998). Algae produce organic compounds that can be used as a substrates for bacterial growth (Bell et al., 1972 & 1974) and also provide an increased surface area for bacterial attachment (Cole, 1982). Algal cells may also produce anti-bacterial compounds that select for particular bacterial groups (Romalde et al., 1990; Fukami et al., 1997). In turn, the bacterial community can also affect algal cells by the production of compounds that promote or inhibit algal growth, substances that are algicidal (Furuki and Kobayashi, 1991; Fukami et al., 1997; Stewart et al., 1997; Lovejoy et al., 1998; Croft et al., 2005), alter cell toxicity (Hold et al., 2001a; Bolch et al., 2001), or promote or interfere with sexual reproduction and cyst production (Adachi et al., 1999; 2001; Uribe and Espejo, 2003).

Algal-bacterial interactions are also influencing the microbial communities on a larger scale. There is a general trend for increasing bacterial abundance with increasing primary productivity (Azam et al., 1983; Linley and Newell, 1984), and, as composition of the microbial communities is dynamic over time, depth, and season, bacterial diversity and abundance is also affecting algal bloom development (Fukami et al., 1991; Rehnstam et al., 1993; Giovannoni et al., 1996). Shifts in bacterial communities are commonly seen, and research suggests that they occur when there is a change in the molecular organic compounds exuded by the phytoplankton: as the high mo-

lecular organic compounds (such as proteins or polysaccharides) are being decomposed to low molecular organic compounds, there also tends to be a shift in the bacterial communities (Fukami et al. 1981; Romalde et al., 1990; Riemann et al., 2000).

On the surface of fresh particulate organic matter (POM), where large amounts of high molecular organic compounds are present, the bacterial community with the ability to decompose such organic substrates becomes dominant, whereas, on the surface of old POM or in surrounding seawater where high molecular organic compounds are scarce, the community would instead be shifted towards that of low ability for organic compound decomposition (Fukami et al., 1981; van Hannen et al., 1999). A study on a bloom of *Lingulodinium polyedrum* from Californian waters by Fandino et al. (2001) found that the free living bacteria utilising low molecular organic compounds were numerically dominant, comprising more than 90 % of the total count and responsible for >70 % of the bacterial production. This group of bacteria is often dominated by *Roseobacter*, belonging to the α-Proteobacteria (DeLong et al., 1993; González and Moran, 1997).

1.2.3 Experimental model systems of algal and bacterial interactions

Naturally occurring phytoplankton communities commonly co-exist with 10s to 100s of different bacterial types (Groben et al., 2000; Alavi, et al., 2001; Green et al., 2004), leading to complex interactions between the algal and bacterial communities. This generates the potential for millions of phytoplankton-phytoplankton interactions, bacteria-phytoplankton interactions, and bacteria-bacteria interactions, making it close to impossible to control and study these complex relations.

Simplified experimental model systems of the algal-bacterial associations enable clarification of the mechanisms behind algal-bacterial interactions, and the significance of their importance in HAB dynamics. So far, experimental model systems have been developed for three dinoflagellate species, *Gymnodinium catenatum* (Graham), *Scrippsiella trochoidea* (Stein) and *Lingulodinium polyedra* (Stein) (Bolch et al. 2004, Green and Bolch, unpublished). The model systems use surface-sterilised resting cysts or resting cells of the dinoflagellates to which either a single or a mixture of bacterial strains are added before germination of the cysts (Fig. 1.2). The ef-

fects of bacteria on the dinoflagellate are then determined by the growth responses of the algal-bacterial cultures established from the cysts.

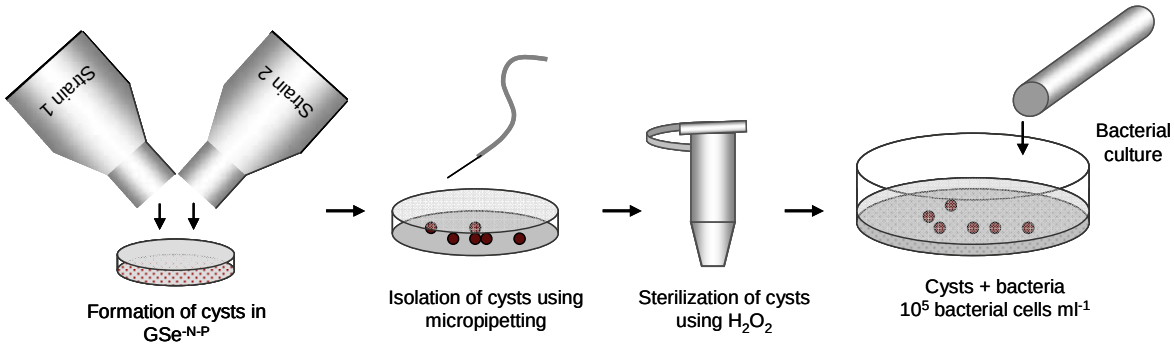


Figure 1.2 Diagram of the establishment of unibacterial *Gymnodinium catenatum* cultures. GSe-N-P is GSe medium (Blackburn et al., 2001) without nitrate and phosphate.

A library of culturable bacteria has also been isolated from cultures of these dinoflagellates that can be used in bacterial re-addition experiments with each species (Green and Bolch, unpubl.). Work with these model systems have indicated three main growth responses shown by the dinoflagellates after the addition of communities: 1) Algicidal response: found with members of the *Cytophaga* /*Flexibacter* group causing death of the alga at or just after germination; (2) Slow algal growth responses: typically caused by communities composed of *Roseobacter* sp. and *Rhodobacter* sp. (3) Enhanced algal growth response: *G. catenatum* showed growth similar to or greater than normal (i.e. grown with bacteria present at isolation of the alga into culture) when grown with communities composed of *Alcanivorax* sp. DG881 or *Marinobacter* sp. DG879 (Bolch et al., 2001; Vincent, 2003; Bolch et al., 2004; Green et al., 2004; Subramanian, 2008).

For the work conducted in this study, *Gymnodinium catenatum* was chosen as the model species for experimentation due to: (a) its well-known and easily manipulated life-cycle; (b) its relatively short resting cyst dormancy period (14-21 days) allowing comparatively rapid cycles of experimentation (Blackburn et al., 1989; 2001); (c) its relevance to increasing HAB events in the past decades (Hallegraeff 1993; Hallegraeff and Fraga 1998); and (d) because since its apparent introduction to Australian waters in the 1970s, the economic impacts on the aquaculture industry has classified

the dinoflagellate as a significant HAB species (Hallegraeff and Sumner, 1986; Hallegraeff et al., 1989, 1995).

1.3 *Gymnodinium catenatum* biology and ecology

1.3.1 Species description

Gymnodinium catenatum is a marine, planktonic, dinoflagellate species associated with red tide paralytic shellfish poisoning (PSP) events all over the world (Hallegraeff et al., 1989). It is an athecate (i.e. without thecal plates), chain forming dinoflagellate (Fig. 1.3A and C) commonly seen in chain formations with up to 64 cells (Blackburn et al., 1989). The cells show differing morphology between single cells compared to when in chains, with single cells usually elongate-ovoid, 48-65 µm long and 30-43 µm wide (Fig. 1.3B), while chain formers tend to be smaller (31-40 µm long and 36-42 µm wide) and have a squarish-ovoid shape (Fig. 1.3C). Cell shape is shown to be strain depandant (Graham, 1943; Yuki and Yoshimatsu, 1987; Blackburn et al. 1989).

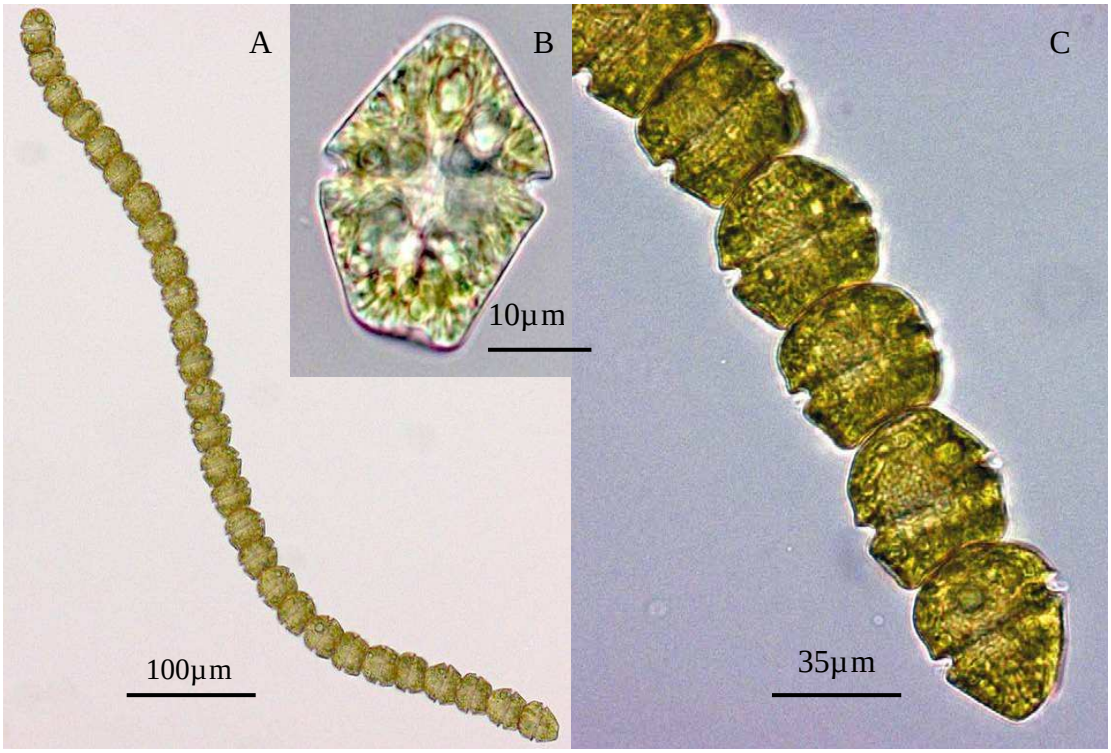


Figure 1.3 LM micrographs of *Gymnodinium catenatum*. (A) 28-cell chain, scale bar 100µm. (B) Single cell of *G. catenatum* showing slightly elongated shape in comparison to the chain-forming cells seen in (C).

The resting cyst of *G. catenatum*, which is produced by sexual reproduction (Blackburn et al., 1989), has a very distinct morphology. It is spherical, brown, and with the surface covered with microreticulate ornamentation, showing patterns reflecting the apical groove and cingulum of the vegetative cell (Anderson et al., 1988) (Fig. 1.4).

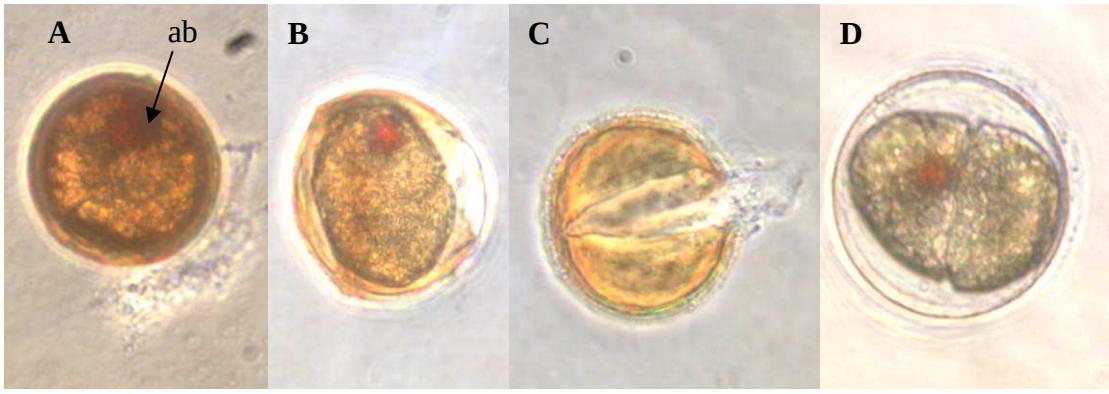


Figure 1.4 Light micrographs of a *Gymnodinium catenatum* resting cyst going through germination/excystment. (A) The characteristic brown cyst with the red accumulation body (ab); (B) the cyst starting to rupture, displaying a fully formed *G. catenatum* vegetative cell within the cyst; (C) The empty cyst; (D) The vegetative *G. catenatum* cell still within a protective membrane.

1.3.2 Life cycle of *Gymnodinium catenatum*

Gymnodinium catenatum displays both sexual (fusion of gametes) and asexual (vegetative division of cells) reproduction (Fig. 1.5). Blackburn et al. (1989) and Blackburn and Parker (2005) showed that *G. catenatum* gametes formed by asexual cell division are isogamous (morphologically identical to each other), and these gametes fuse to form a diploid planozygote. The planozygote is biflagellated, and much larger in size than both the vegetative cells and the gametes. The flagella are eventually lost, the planozygote loses its swimming ability and the cell attains a more rounded shape, with the sulcus and cingulum that were clearly visible before, fading away. The planozygote then transforms into a hypnozygote, the resting stage of the life cycle. After as little as two weeks of dormancy, the hypnozygote is able to undergo excystment, forming a biflagellated planomeiocyte (Fig. 1.4 a-d). This, in turn, divides to form haploid vegetative cells.

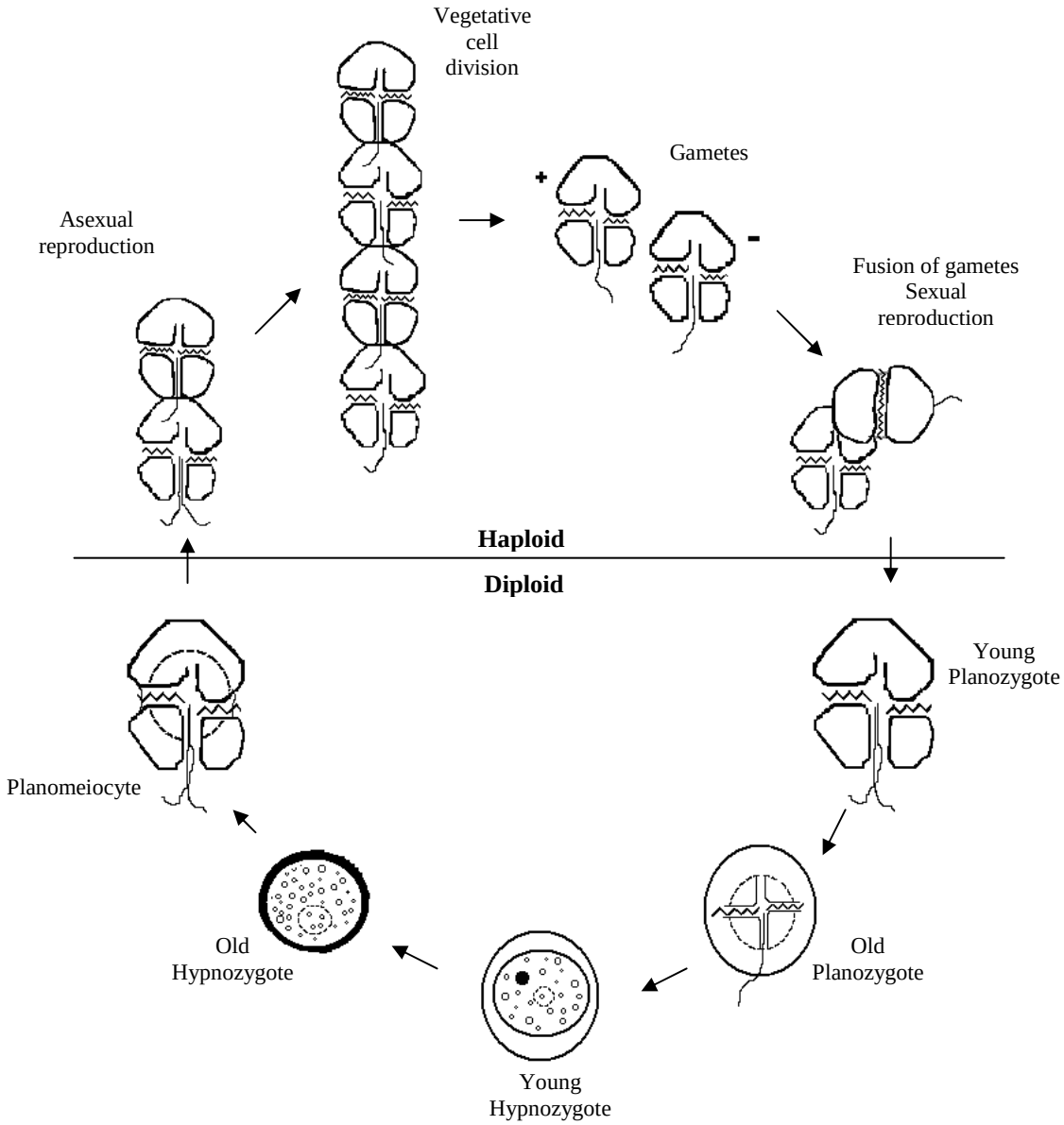


Figure 1.5 Life cycle of *Gymnodinium catenatum* as described by Blackburn et al. (1989) and Blackburn and Parker (2005).

1.3.3 Habitat, location and distribution

Gymnodinium catenatum was first discovered and described by Herbert W. Graham (Graham, 1943) following a bloom in Californian waters, and has since been reported from most continents, with an alarming global spread over the last two decades (Fig. 1.6). Vegetative cells are found in both tropical and temperate waters (Hallegraeff and Fraga 1989) and at least some of the increased global distribution is thought to have been a result of transfer in ships' ballast water (McMinn et al., 1997).

In Tasmania, Australia, analyses of historic plankton samples and cysts in sediment depth cores strongly support that *G. catenatum* is not indigenous to Tasmania but was introduced in the region after 1973 (Hallegraeff and Bolch, 1992; McMinn et al., 1997). Blooms have since then been recurring events from January to June (late summer to early winter) in the Huon and Derwent estuaries of south-eastern Tasmania. However, spatial distribution, duration and magnitude exhibit significant interannual variability (Hallegraeff et al., 1995; Volkman et al., 2009).

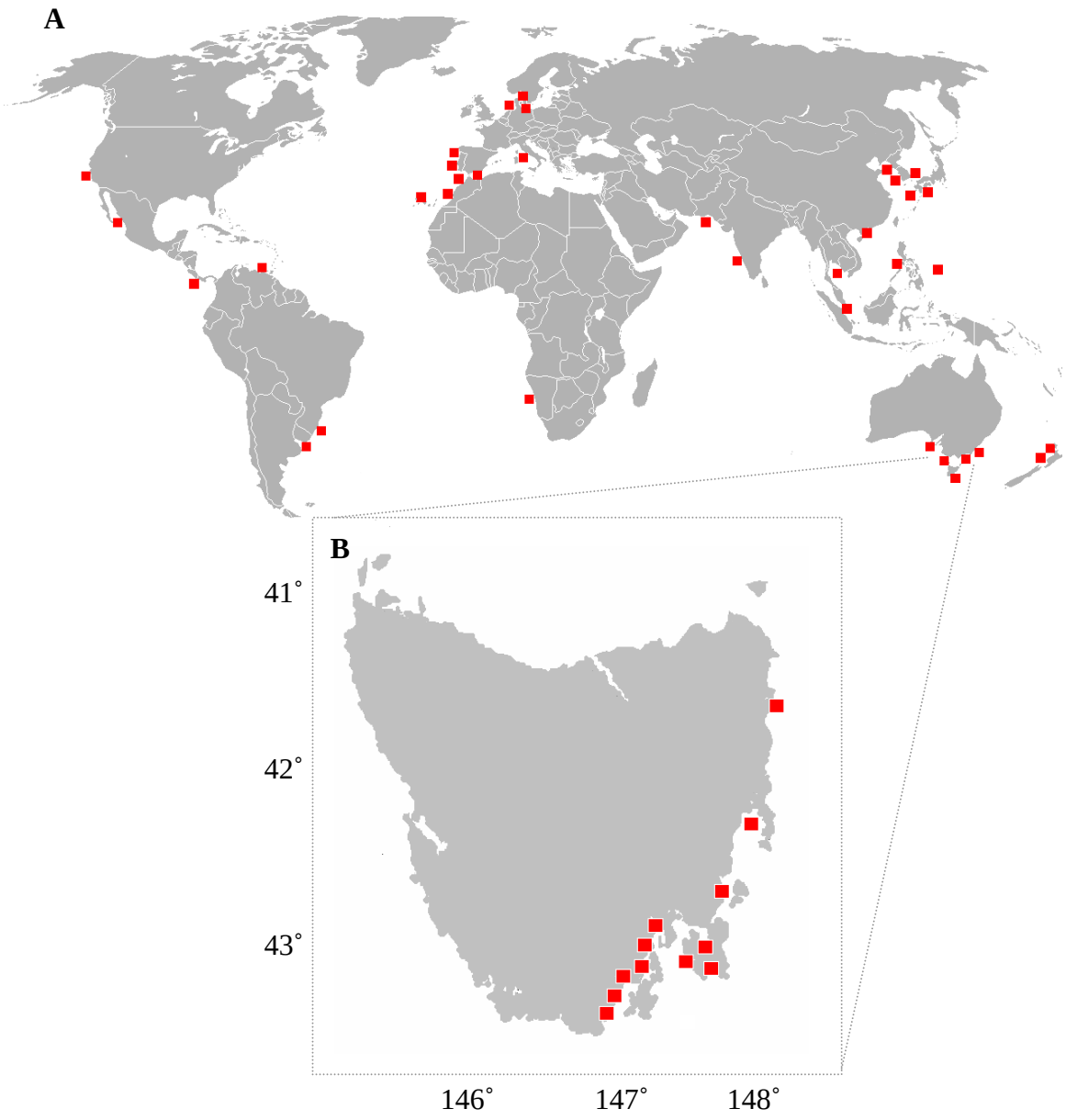


Figure 1.6 Known global (A) and Tasmanian (B) distribution of *Gymnodinium catenatum*, after Bolch and Reynolds (2002).

1.3.4 Toxicology of *Gymnodinium catenatum*

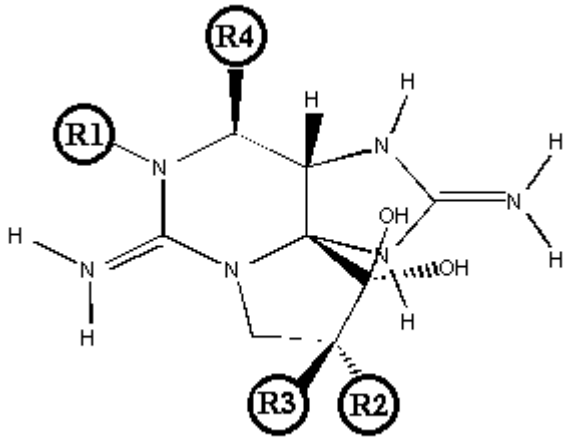
Gymnodinium catenatum is a well known paralytic shellfish toxin (PST) producer (Table 1.1), and is the only unarmoured dinoflagellate known to produce these toxins. PSTs are the most acutely neurotoxic compounds produced by marine and freshwater microalgae, and there are currently over 20 different PSTs known, all based on the same basic alkaloid, tetrahydropurine compound, saxitoxin (STX)

(Negri et al., 2003a) STX blocks sodium channels in nerve cells, thus causing their neurotoxic effects (Negri et al., 2003a). The structures of the PSTs are formed from substitutions at four distinct positions around the basic PST structure (Fig. 1.7). The first chemical confirmation of the toxin production by *G. catenatum* was achieved using samples from Tasmania by Oshima et al. (1987) who detected saxitoxin (STX), decarbamoyl saxitoxin (dcSTX), the N-sulfocarbamoyl gonyautoxins (GTX1-GTX5) and the less toxic N-sulfocarbamoyl-11-hydroxysulfate C toxins (C1-C4). Later, during a study by Oshima et al. (1993), Tasmanian strains revealed the presence of deoxy-decarbamoyl saxitoxin (doSTX) and deoxy-decarbamoyl gonyautoxin 3 (doGTX3), and recently during a survey of the paralytic shellfish poisoning (PSP) toxins, Negri et al. (2003b) found three novel saxitoxin analogues GC1, GC2, and GC3 in the *G. catenatum* strain GCDE09 (see summary in Table 1.1).

Table 1.1 Toxins produced by *Gymnodinium catenatum*

Toxin	**Common name / abbreviation**	**Ref.**
N-sulfocarbamoyl-11-hydroxy-sulfate toxins	C1 - C4	1, 2
Gonyautoxin / 11-hydroxysulfate toxins	GTX1 - GTX6	1, 2
Decarbamoyl-11-hydroxysulfate toxins	dcGTX1 - dcGTX4	3
Decarbamoylgonyautoxin 2	dcGTX2	2
Decarbamoylgonyautoxin 3	dcGTX3	2
Decarbamoylsaxitoxins	dcSTX	1, 2, 3
Neosaxitoxin	NEO / neoSTX	1, 2, 3
13-Deoxydecarbamoylgonyautoxin 2	doGTX2	1, 2
13-Deoxydecarbamoylgonyautoxin 3	doGTX3	1, 2
13-Deoxydecarbamoyl saxitoxins	doSTX	1, 3
G. catenatum toxins		
Epimeric 11-hydroxysulphate derivative of GC3	GC1	3, 4
Epimeric 11-hydroxysulphate derivative of GC3	GC2	3, 4
4-hydroxybenzoate ester derivative of dcSTX	GC3	3, 4

1. Oshima et al., 1993; 2. Negri et al., 2001; 3. Negri et al., 2003b; 4. Negri et al., 2007



Paralytic Shellfish Toxin	R1	R2	R3	R4
STX	H	H	H	$CONH_2$
NeoSTX	OH	H	H	$CONH_2$
C1	H	OSO_3^-	H	$CONHSO_3^-$
C2	H	H	OSO_3^-	$CONHSO_3^-$
C3	OH	OSO_3^-	H	$CONHSO_3^-$
C4	OH	H	OSO_3^-	$CONHSO_3^-$
GTX1	OH	OSO_3^-	H	$CONH_2$
GTX2	H	OSO_3^-	H	$CONH_2$
GTX3	H	H	OSO_3^-	$CONH_2$
GTX4	OH	H	OSO_3^-	$CONH_2$
GTX5	H	H	H	$CONHSO_3^-$
GTX6	OH	H	H	$CONHSO_3^-$
dcGTX1	OH	OSO_3^-	H	CH_2OH
dcGTX2	H	OSO_3^-	H	CH_2OH
dcGTX3	H	H	OSO_3^-	CH_2OH
dcGTX4	OH	H	OSO_3^-	CH_2OH
dcSTX	H	H	H	CH_2OH
NEO / neoSTX	OH	H	H	$CONH_2$
doGTX2	H	OSO_3^-	H	CH_3
doGTX3	H	H	OSO_3^-	CH_3
doSTX	H	H	H	CH_3
GC1	H	OSO_3^-	H	$C_8H_8O_3$
GC2	H	H	OSO_3^-	$C_8H_8O_3$
GC3	H	H	H	$C_8H_8O_3$

Figure 1.7 General structure of saxitoxin with the four positions for substitutions of compounds that determine the particular paralytic shellfish toxin.

1.3.5 *Gymnodinium catenatum*-associated bacteria

In a recent study by Green et al. (2004) the cultivable bacterial community of laboratory grown *Gymnodinium catenatum* was investigated, identifying the bacteria as belonging to three main phyla, the Proteobacteria (70%), Bacteroidetes (26%) and Actinobacteria (3%). Of the Proteobacteria, 21% were indentified as γ-proteobacteria while 49% as α-proteobacteria, of which the Rhodobacteria group made up of more than 30 %, always being the most numerically abundant bacterium present in each culture (Table 1.2).

Table 1.2 The cultivable bacteria associated to laboratory-grown cultures of *Gymnodinium catenatum*, compiled from Green et al. (2004)

Phyla	Class	Family	No of isolates
Proteobacteria	Alpha (α) proteobacteria	Rhodobacteraceae	21
		Rhodospirillaceae	3
		Sphingomonadaceae	3
		Phyllobacteriaceae	2
		Rhodobiaceae	1
	Gamma (γ) proteobacteria	Alteromonadaceae	8
		Alcanivoraxaceae	3
		Oceanospirillaceae	1
		Moraxellaceae	1
Bacteroidetes	Flavobacteriales	Flavobacteriaceae	10
	Sphingobacteriales	Flexibacteraceae	6
Actinobacteria	Actinomycetales	Micrococcaceae	1
		Nocardioidaceae	1

Green et al. (2004) isolated two members of the Gamma (γ) proteobacteria with close phylogenetic relationships to either *Alcanivorax borkumensis* (SK2) or *Marinobacter hydrocarbonoclasticus* (ATCC 27132), from 6 out of 7 cultures of *G. catenatum.* Two bacterial strains, *Alcanivorax* sp. DG881 and *Marinobacter* sp. DG879 were isolated from the Tasmanian *G. catenatum* strains GCDE08 and GCHU11 (Australian National Algae Culture Collection), respectively (Green et al., 2004). Both bacteria are gram-negative, aerobic, and hydrocarbon degrading, and have been shown to be essential for germination and cell growth of *G. catenatum*. Without the bacteria, the host cell dies within days of germination (Bolch et al., 2001; Vincent, 2003; Bolch et al., 2004; Green et al., 2004).

This work laid the foundation for subsequent studies aimed at understanding the influence of bacteria on toxicity, growth and physiology. The influence of these hydrocarbon-degrading bacteria on the growth of *G. catenatum* was further investigated by Vincent (2003) who studied host-range specificity, and Subramanian (2008) who showed that the bacterial community has a significant effect on the growth rate and dynamics of *G. catenatum,* and that the bacterial community appear to be a more important factor than any variation associated with the individual's genotype.

Although algal-bacterial interactions have received an increasing amount of attention over the last decade (e.g. Hold et al., 2001a; Uribe and Espejo, 2003; Croft et al., 2005) the underlying mechanisms of the complex interactions between the organisms are far from determined. The interactions are highly specific and stimulation or inhibition of algal growth or survival by bacteria may vary greatly depending on the associated bacterial taxa (Doucette et al., 1998; 1995). In terms of *G. catenatum* and its close association to the two γ-proteobacteria *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 there are still a number of unanswered questions. The absolute requirement for the bacteria by *G. catenatum* has so far only been demonstrated in laboratory-grown *G. catenatum* cultures. The underlying mechanisms behind the close relationships are also unknown. By studying the growth dynamics of the bacteria and algae while in association with one another, it may be possible to determine whether growth fluctuations depend on the culture life stage or whether one of the organisms is driving growth of the other. Furthermore it is not known whether the relationship between these bacteria may be important for *G. catenatum* blooms in the

natural environment, and whether *Marinobacter* sp. and/or *Alcanivorax* sp. play a role in *G. catenatum* bloom formation.

1.4 Thesis aims and Outline

The overall aim of this thesis is to investigate the influence of the growth-stimulating marine bacteria *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 on the growth dynamics and toxicity of the toxic, HAB forming dinoflagellate *Gymnodinium catenatum* in bacteria-dinoflagellate model culture systems and natural Tasmanian *G. catenatum* populations. Specifically this thesis aims to:

1. Establish the *G. catenatum* algal-bacterial model with different *G. catenatum* mating strains to test for consistent behaviour of the model system with different *G. catenatum* strains.

2. Develop a quantitative molecular detection method for the growth-promoting bacteria *Alcanivorax* sp. DG881 and *Marinobacter* sp. DG879.

3. Examine the growth dynamics of *Alcanivorax* sp. DG881 and *Marinobacter* sp. DG879 in single- and mixed-bacterial *G. catenatum* model cultures.

4. Examine the abundance and dynamics of *Alcanivorax* and *Marinobacter* associated with naturally occurring *G. catenatum* populations.

5. Investigate the influence of bacterial community composition on saxitoxin production by *G. catenatum.*

The information that will emerge from the studies of this thesis will shed light on aspects of the complex interactions of the microbial loop, and particularly increase the current knowledge of the interactions between algae and bacteria.

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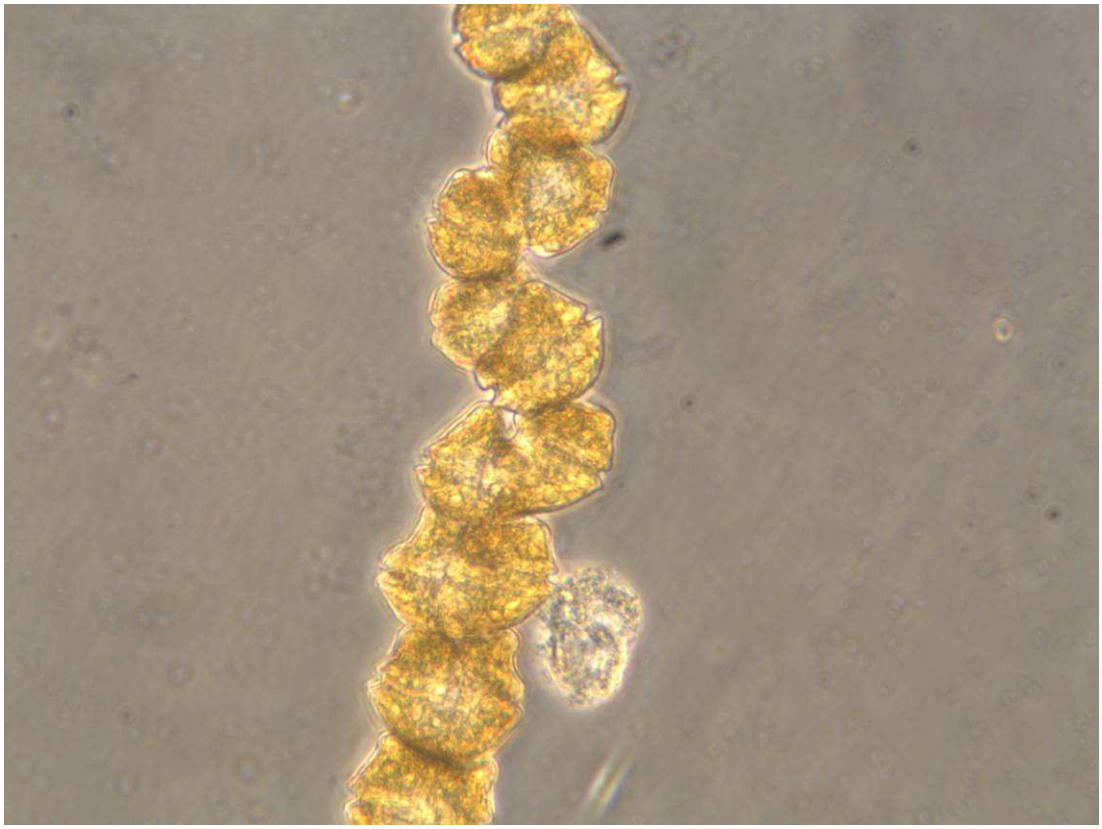
Chapter 2:

Establishment of uni-bacterial *Gymnodinium catenatum* model systems: reproductive compatibility of *G. catenatum* strains, resting cyst production and germination



Chapter 2: Establishment of uni-bacterial *Gymnodinium catenatum* model systems: reproductive compatibility of *G. catenatum* strains, resting cyst production and germination

2.1 Introduction

It is well-known that bacteria play an important role in oceanic processes. Bacteria is involved in nutrient regeneration where the bacteria decompose organic material and returns nutrients such as phosphates and nitrates for reuse by primary producers; as part of the carbon cycle, where the bacteria produce CO2 by fermentation of organic matter, and in the microbial loop, where the bacteria utilize DOC and are then in turn taken up by ciliates and heterotrophic flagellates (Wolter, 1982; Azam et al., 1983 and Doucette et al., 1998). The interactions that occur between the trophic levels in the natural environment are however highly complex, and in terms of algae and bacteria, algal communities have been known to interact with sometimes tens to hundreds of bacterial types (Groben et al., 2000; Alavi et al., 2001). As a result, studying the interactions between the two organisms in nature is very difficult, in turn having lead to algal-bacterial interactions being poorly understood, and with research of the interactions mostly confined to laboratory cultures.

Simplified experimental model systems (Chapter 1), which use surface-sterilised resting cysts of the algae to which either a single or a mixture of bacterial strains are added before cyst germination, can enable clarification of the mechanisms behind algal-bacterial interactions (Bolch et al. 2004, Green and Bolch, unpublished). The systems can allow for studies on, for example, algal toxicology, algal physiology, and algal growth dynamics, thereby demonstrating the potential significance of the algal-bacterial interactions. To date, experimental model systems have been developed for three dinoflagellate species, *Scrippsiella trochoidea* (Stein), *Lingulodinium polyedra* (Stein), and *Gymnodinium catenatum* (Graham) (Green and Bolch, unpublished).

Gymnodinium catenatum is a cosmopolitan dinoflagellate species that produces paralytic shellfish toxins (PSTs) potentially lethal to humans (Hallegraeff et al., 1989). There is evidence suggesting that *G. catenatum* has been introduced into Tasmanian waters by ships' ballast water (Hallegraeff and Bolch, 1992; McMinn et al., 1997), likely assisted by the resistant resting-cyst stage of its life cycle (see Chapter 1). Resting cysts are produced as part of the sexual life cycle, and aid in both long-term and short-term survival of some dinoflagellates, including *G. catenatum* (Blackburn et al., 1989; Blackburn and Parker, 2005). By producing a resistant cyst when conditions are less then favourable, *G. catenatum* has the ability to survive periods of darkness or low nutrient levels, undergoing excystment to form new vegetative cells when surrounding environmental conditions are more suitable for growth (Blackburn et al., 1989).

Resting cysts of *G. catenatum* can be created in laboratory cultures, and after manipulation of the cyst-associated bacteria, the effects of bacterial community on the algal growth dynamics can be studied. Typically *G. catenatum* cultures contain a range of bacterial species (Green et al., 2004). However, by manipulating the bacterial community of resting cysts of *G. catenatum*, cultures with specific bacterial assemblages can be generated, producing uni-bacterial or mixed-bacterial *G. catenatum* experimental model systems (Bolch et al., 2001; 2004). Using the experimental model systems, the underlying mechanisms and interactions between algae and bacteria can be investigated alongside each other (Bolch et al., 2001; 2004). A similar approach was also used by Alavi et al. (2001) on *Pfiesteria* sp. In this earlier study, axenic *Pfiesteria* sp. had bacteria, which was originally isolated from a bacterized *Pfiesteria* culture, added back to the dinoflagellate cells in order to study how and if a bacterial association could benefit dinoflagellate growth and physiology.

The experimental model systems with *G. catenatum* were initially developed by Drs David Green and Christopher Bolch, at the Scottish Association of Marine Science (Dunstaffnage Marine Laboratory, Scotland) (Bolch et al., 2001) by germinating surface-sterilised resting cysts and adding uni-bacterial strains to form a dinoflagellate-bacterium co-culture. The first step in this process is successful production of *G. catenatum* resting cysts, while the second step is to add known bacteria (cultivable unibacterial strains) to surface-sterilised cysts.

In this study, two bacterial species, *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 that both have been shown to be growth-promoting to *G. catenatum* (Bolch et al., 2001; Bolch et al., 2004) were used as the bacterial species in the *G. catenatum* unibacterial model systems. These *G. catenatum* model systems were then used for testing and validation of quantitative PCR detection methods for bacteria, prior to use in later chapters of this thesis.

This chapter details investigations of the reproductive compatibility of *G. catenatum* strains and their capacity to produce resting cysts as an essential first step in establishing the *G. catenatum* experimental model systems. Model systems developed to date have used resting cysts generated by crosses of *G. catenatum* strains GCDE08 and GCHU11 (see Appendix 3). However, as strain GCDE08 has been shown to be homothallic (self-crossing) and generally of low viability (see section 2.3.1) this chapter aims to establish model cultures using different crossing strains, and assess their germination, growth and survival. Studies by Blackburn et al. (1989; 2001), Parker (2002) and Vincent (2003) show that reproductive compatibility of *G. catenatum* can vary over time in long-term culturing. As a result, this study repeated the reproductive compatibility experiments, using the same strains as these four previous studies (to the extent possible as some strains are no longer held in culture) to asses the ability to sexually reproduce and form resting cysts. To determine the most successful cyst-producing strain crosses, ten different *G. catenatum* strains were crossed in pair-wise combinations. The successful crosses were then used for large-scale production of cysts, which in turn were used to establish the *G. catenatum* experimental model systems.

2.2 Materials and Methods

2.2.1 Algal strains

All *Gymnodinium catenatum* strains were obtained from the Australian National Algae Culture Collection (ANACC, Formerly CSIRO Collection of Living Microalgae; www.marine.csiro.au/microalgae). The *G. catenatum* strains (Table 2.1; Appendix 3) were cultured in 50 mL Erlenmeyer flasks in GSe medium (Blackburn et al., 2001; Appendix 1) at 18ºC, 65 µmol PAR m^{-2} sec^{-1}, with an 18:6 h light: dark light regime

from cool-white fluorescent lighting. All cultures were handled aseptically to prevent algal cross-contamination and bacterial contamination of the cultures.

Table 2.1 Culture strains of *Gymnodinium catenatum* used for the reproductive compatibility trial. The CS-code is a unique CSIRO numerical code for each strain held in the collection (further details available on www.marine.csiro.au/microalgae and Appendix 3)

Culture strain	CS-code	Tasmanian Origin	Date of isolation
GCDE05	CS-301/05	Derwent River	8/1 1987
GCDE06	CS-301/06	Derwent River	8/1 1987
GCDE08	CS-301/08	Derwent River	15/6 1987
GCDE09	CS-301/09	Derwent River	n/a
GCHU02	CS-302/02	Huon River	6/6 1986
GCHU11	CS-302/11	Huon River	20/6 1988
GCHU20	CS-302/20	Huon River	4/4 1990
GCHA01	CS-304/01	Hastings Bay	29/6 1990
GCVS04	CS-799	Verona Sands	1/1 2002
GCLV01	CS-800	Louisville	1/1 2002

2.2.2 Cyst production

To establish cyst production of compatible strains, 1 mL of each of two strains of *G. catenatum* (pair-wise combinations of all strains) as well as 1 mL of each strain on its own (self-crossing) in late logarithmic phase was transferred to a 35 mm diameter plastic Petri dish containing 10 mL of GSe medium without added nitrate or phosphate ($GSe^{-N-P)}$; a method used to enhance resting cyst formation (Blackburn et al., 1989). Petri dishes were incubated at 21 ºC ± 2 ºC, 80 µmol PAR m^{-2} sec^{-1}, on an 18:6 h light/dark regime. Cyst counts were conducted using a Sedgwick-Rafter counting chamber every 4 days for 5 weeks, and simultaneously, presence of different sexual life-stages of the *G. catenatum* cells (i.e. gamete swarming, gamete fusion, planozygotes and empty shells from hatched planozygotes) were noted.

2.2.3 Measurement of reproductive success

Cyst production and reproductive success by *G. catenatum* crosses was scored in all crosses according to the criteria of Blackburn et al. (2001) (Table 2.2).

Table 2.2 Scoring criteria and equivalent cyst concentrations for cyst production in *Gymnodinium catenatum* crosses. (From Blackburn et al. 2001)

Score	Cysts per cross	Cyst concentration (cysts L^{-1})
0	<4	$0 - 3.0 \times 10^2$
1	4-24	$>3.0 \times 10^2 - 2.0 \times 10^3$
2	25-124	$>2.0 \times 10^3 - 1.0 \times 10^4$
3	125-1200	$>1.0 \times 10^4 - 1.0 \times 10^5$
4	>1200	$> 1.0 \times 10^5$

In order to assess the variation in mating among strains in all crosses, three measures of reproductive success (Blackburn et al., 2001) were calculated from the scores in the crossing matrix.

- Compatibility index (CI_S): which is the number of compatible pairings resulting in a score of ≥ 1, divided by the total number of possible crosses (other than self crosses), it is regarded as a measurement of 'willingness to cross' with other strains. CI_S (max) = 1
- Average vigour (AV_S): The average of the scores (0-4) for maximum cyst production for all successful crosses involving a particular strain, i.e. an average measure of the number of cysts produced per cross in successful crosses. AV_S (max) = 4
- Strain reproductive compatibility (RC_S) was calculated as the product of the strain's CI and AV in all crosses (i.e. $RC_S = CI_S \times AV_S$). RC_S (max) = 4.

- A total compatibility index (TCI) was calculated among all strains as the percentage of all pair-wise crosses (including self-crosses) that produced viable resting cysts. TCI (max) = 100%

2.2.4 Bacterial strain maintenance and preparation

Both bacterial species, *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 were initially isolated from the Tasmanian *Gymnodinium catenatum* strain CGDE08/CS-301/08 (Green et al., 2004; Appendix 3) and maintained on Zobell's Marine (ZM1) agar (Zobell, 1941; Appendix 2) at 20 ºC in total darkness. Bacterial strains were prepared for use in model systems as follows: strains were aseptically transferred into ZM1 broth and incubated with shaking (70 rpm) at 20 ºC in total darkness for 3 days. After incubation, 1 mL of dense suspension was transferred to 1.5 mL centrifuge tubes and centrifuged for 10 minutes at 1500 rpm. In order to remove the ZM1 broth, the supernatant was replaced with GSe medium and the centrifugation/wash step repeated three times. The 'cleaned' bacterial cells in 1 mL GSe medium were transferred into sterile McCartney bottles containing 20 mL sterile GSe medium. The bottles were incubated at 20 ºC for 24 hours for acclimation of bacteria to algal growth medium.

2.2.5 Surface sterilization of cysts and cyst germination

From the studies of strain compatibility the cross GCHU11 × GCHA01 was chosen for large-scale cyst production as this was the cross producing the largest amount of cysts in the shortest amount of time. Cysts from crosses were harvested approximately 2 weeks after crossing using a manual micropipette, washed several times in fresh GSe medium, and placed in sterile 1.5 mL centrifuge tubes containing 0.5 mL GSe medium. Ten to fifteen cysts were added to each centrifuge tube, and 50 µL 6 % H_2O_2 (final conc. 0.5 %) was added to the tubes (Bolch and Hallegraeff, 1993). Tubes were wrapped in aluminium foil to limit photodegradation of H_2O_2 and left for 45 minutes at room temperature. After H_2O_2 incubation the tubes were centrifuged and 500 µL of the supernatant replaced with fresh GSe medium. This process was repeated 3 times in order to remove residual H_2O_2. Successful surface-sterilisation was checked by spread-plating 10 µL of the sterilised sample onto ZM1 agar which

was incubated at 24 °C for 4 days. If bacterial growth was seen after incubation, the media and cysts were discarded.

The sterilised cysts were then placed in 35 mm diameter polystyrene Petri dishes containing 10 mL GSe medium. Ten to fifteen cysts were placed in each Petri dish, and cleaned bacterial cultures were added at a concentration of 10^5 cells mL^{-1}. The different treatments were: (1) sterile cysts and *Alcanivorax* sp. DG881 in sterile GSe; (2) sterile cysts and *Marinobacter* sp. DG879 in sterile GSe; (3) a positive control containing non-sterilised cysts with 1 mL of a 5 µm filtrate from the GCHU11 x GCHA01 culture and sterile GSe, and (4) a negative control containing sterile cysts and sterile filtered (0.2 µm) seawater. All treatments were carried out in triplicate. Petri dishes were incubated at 21 ºC ± 2 ºC, 80 µmol PAR m^{-2} sec^{-1}, on an 18:6 h light/dark regime, and studied every 4 days over a 35 day period to determine germination of the cysts. Germination success was determined by counts of empty cysts left after cyst germination by surveying the bottom of the petridish by microscopy.

2.2.6 Establishment of *Gymnodinium catenatum*-bacterial model systems

To establish clonal cultures, *G. catenatum* cells were isolated from the Petri dishes containing either *G. catenatum* and *Alcanivorax* sp. DG881 or *G. catenatum* and *Marinobacter* sp. DG879 using a dilution isolation method. Cultures were diluted with sterile GSe medium to a concentration of approximately 1 cell or a single chain (2-8 cells long) of cells mL^{-1} and 1 mL^{-l} transferred to each of the wells of a 24-multiwell plate containing 2 mL GSe medium. Wells containing only one cell or cell chain (cell counts determined by microscopy) were labelled and the plate incubated under the culture conditions previously described for 1 week. Clonal cultures were established from the labelled wells, transferred into 50 mL Erlenmeyer flasks containing 25 mL fresh GSe medium and when sufficient cell concentration was achieved these were transferred to 50 mL flasks containing 40 mL GSe medium

2.2.7 Statistical analysis

Differences between treatments (i.e. sterile cysts with *Alcanivorax* sp. or sterile cysts with *Marinobacter* sp. DG879) and controls (positive control containing non-sterilised cysts and negative control containing sterile cysts and sterile seawater) in

terms of the cyst germination success and cells per germinated cyst (at day 36) were compared using one-way ANOVA, $p \leq 0.05$ (Microsoft Excel).

2.3 Results

2.3.1 Reproductive compatibility of *Gymnodinium catenatum*

Nine of the ten strains crossed were heterothallic (i.e. out-crossing; can only undergo sexual reproduction with a genetically different strain; Blackburn et al., 1989), and one, GCDE08 was also homothallic (able to produce resting cysts in self-crosses) (Blackburn and Parker, 2001) (Table 2.3). All strains produced cysts with at least one other strain, although GCDE05, GCHU20 and GCHU02 were only compatible with one other strain. The remaining strains were compatible with 2 or more strains.

Table 2.3 Compatibility matrix of Gymnodinium catenatum strains. Scores (0-4) indicating the number of cysts produced per cross (cyst concentration), (scoring criteria as Blackburn et al., 2001, Table 2.2). * = crosses producing < 4 cysts and scored as unsuccessful. Original strain names have been shortened from GCDE05 to DE05 etc.

Strain	DE05	DE06	DE08	DE09	HU02	HU11	HU20	HA01	VS04	LV01
DE05	0	0	0*	0	0	1	0*	0	0	0
DE06		0	0*	0	0*	2	1	0	0	1
DE08			1	3	0	2	0*	3	2	2
DE09				0	0*	1	0*	0	0	0
HU02					0	1	0	0	0	0
HU11						0	0	2	0	0*
HU20							0	0	0	0
HA01								0	0	0
VS04									0	1
LV01										0*

The total compatibility index (TCI) for all strains was 31%, indicating that almost one third of all crosses were capable of producing viable resting cysts. The mean RC_S was low (0.52, Table 2.4), and highly variable between strains. GCDE08 and GCHU11 showed the highest reproductive compatibility (1.44 and 1.22 respectively), but considering the ability of GCDE08 to self-cross it is not possible to de-

termine whether the cysts formed are from homothallic or heterothallic reproduction. The time to appearance of the first cysts ranged from 12 to 52 days (Table 2.5).

Table 2.4 Summary of the overall reproductive compatibility of each *Gymnodinium catenatum* strain measured by compatibility index (CIS), average vigour (AVS), and reproductive compatibility (RCS)

Strain	CI_S	AV_S	RC_S
DE 05	0.11	1.00	0.11
DE 06	0.33	1.33	0.44
DE 08	0.67	2.17	1.44
DE 09	0.22	2.00	0.44
HU 02	0.11	1.00	0.11
HU 11	0.67	1.83	1.22
HU 20	0.11	1.00	0.11
HA 01	0.22	2.50	0.55
VS 04	0.22	1.50	0.33
LV 01	0.33	1.33	0.44
Mean	0.30	1.57	0.52

Table 2.5 Summary of the time (days) for the first resting cyst to appear in the cross. Original strain names have been shortened from GCDE05 to DE05 etc.

Strain	DE05	DE06	DE08	DE09	HU02	HU11	HU20	HA01	VS04	LV01
DE05	--	--	--	--	--	52	--	--	--	--
DE06		--	--	--	--	28	24	--	--	32
DE08			36	40	--	20	--	20	52	40
DE09				--	--	32	--	--	--	--
HU02					--	12	--	--	--	--
HU11						--	--	12	--	--
HU20							--	--	--	--
HA01								--	--	--
VS04									--	12
LV01										--

As GCDE08 was shown to be homothallic, and produce non-viable offspring, the strain cross chosen for further use in large-scale cyst production was GCHU11 × GCHA01. The strain cross produced the highest amount of cysts over the shortest period of time, on average 4.0×10^3 cysts l^{-1} in a 4 week period, and with the first cysts appearing after 12 days. After GCDE08, GCHU11 showed the highest compatibility index of 0.67 and the highest reproductive compatibility of 1.22, while GCHA01 showed the highest average vigour of 2.50.

2.3.2 *Gymnodinium catenatum* resting cyst germination success

Statistical analysis (one-way ANOVA) showed no statistical difference in germination success between the treatments ($p = 0.17$, $df = 15$, $F = 1.99$) (Fig. 2.1). The first germination of cysts was observed in the two controls at Day 4, and after 8 days cysts had germinated in all treatments.

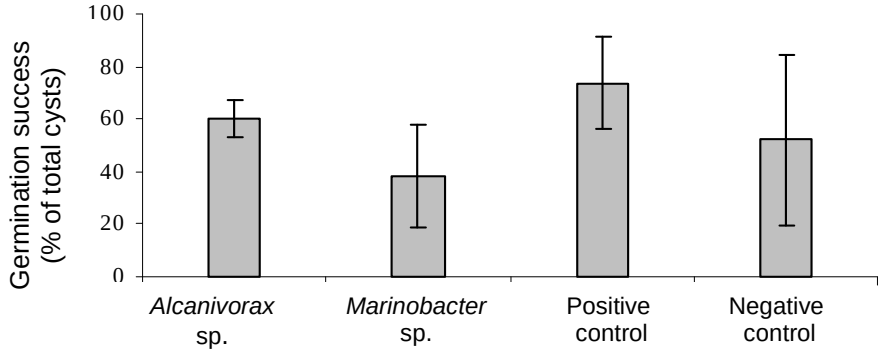


Figure 2.1 The percentage of germination success of *Gymnodinium catenatum* cysts with differing bacterial treatment. Error bars ± SD, n=4

The mean number of vegetative *G. catenatum* cells produced per resting cyst by Day 36 (i.e. the number of vegetative cells produced from cell division of the planomeiocyte; see Figure 1.5), is shown in Figure 2.2. The *Alcanivorax* sp. DG881 treated *G. catenatum* cysts produced an average of 5 cells cyst^{-1}, the *Marinobacter* sp. DG879 treated cysts produced on average 1.5 cells cyst^{-1}, the positive control reached the highest number of 8.5 cells cyst^{-1} while the negative control, in which the cells were placed in sterile seawater, never reached above one cell cyst^{-1} during the entire time period. This is a presumed planomeiocyte (Blackburn et al., 1989). Statistical analysis (one-way ANOVA) showed no statistical difference between the treatments at Day 36 ($p = 0.16$, $df = 11$, $F = 2.25$). The analysis is however limited due to the small sample size. Vegetative cells produced in the *Alcanivorax* sp. DG881, *Marinobacter* sp. DG879, and positive control treatments demonstrated viable progeny (as per Blackburn et al., 2001: germinated cells viable beyond the 8-cell stage), while the germling cell (planomeiocyte) germinated from the negative control were unable to reproduce following germination.

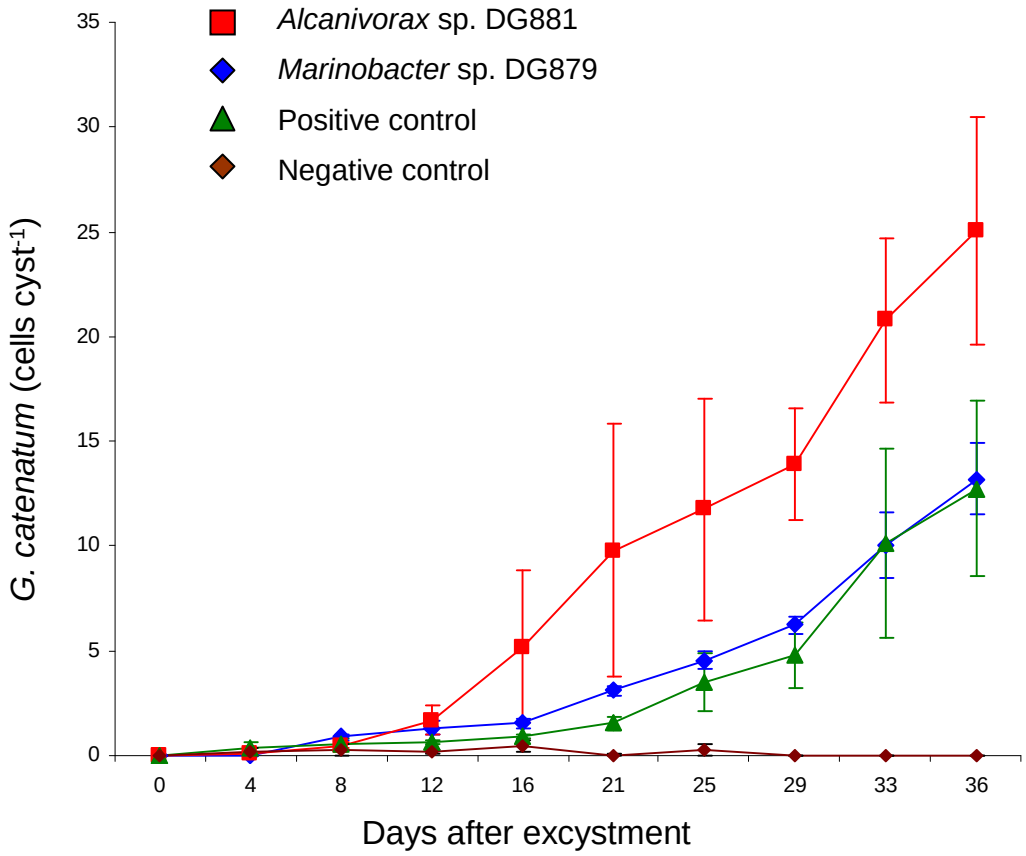


Figure 2.2 *Gymnodinium catenatum* vegetative reproduction following germination: mean *G. catenatum* vegetative cells produced per resting cyst. Error bars ±SE, n=3

Cysts in the negative control (sterilised cysts without added bacteria) germinated but did not produce viable progeny. Planomeiocytes produced in the negative control were non-motile, and had low levels of pigmentation in comparison to normal vegetative cells.

2.3.3 Establishment of *Gymnodinium catenatum*-bacterial model systems

All replicates of the clonal cultures established from the *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 treatments survived although they displayed slow growth for the first three weeks. During this period the cells showed limited motility (direct observation by microscopy) and were mostly confined to the bottom of the flasks. However, the established cultures grew into dense highly motile cultures over a period of approximately six weeks (two transfers). Three cultures of *G. catenatum* with *Marinobacter* sp. DG879 and five cultures of *G. catenatum* with *Alcanivorax*

sp. DG881 have currently been maintained for approximately 3 years. All replicates of the cultures established from the positive control treatment (non-sterilised cysts with added 5 µm filtrate from the GCHU11 x GCHA01 culture in sterile GSe) also survived, though they were only kept alive for a period of 4 weeks.

2.4 Discussion

2.4.1 Reproductive compatibility of *Gymnodinium catenatum*

In the present study, nine of the ten strains tested were heterothallic, with the exception of strain GCDE08 which was homothallic as well as heterothallic (also shown by Parker, 2002 and Vincent, 2003). Blackburn et al. (2001) provided evidence of a multiple group mating system in *G. catenatum*, with varying levels of compatibility between the different mating groups. Although Blackburn et al. (2001) did not find GCDE08 to be homothallic, another Tasmanian strain, GCHU05, was shown to be. It produced viable offspring, and thereby proving that mating is possible between sibling clones of some *G. catenatum* strains. Sibling clones are clones from the same parental cross, they are 'brothers and sisters'; this in comparison to a clone, which is a genetically identical individual derived from a single cell by repeated sexual division.

In regards to the GCDE08 self-cross, it appears that the homothallism has evolved over time in culture. Neither of the studies by Blackburn et al. (1989 and 2001) reported that strain GCDE08 was homothallic, whereas this study found a cysts production of $3.0 \times 10^2 - 2.0 \times 10^3$ cysts l^{-1}. Parker (2002) also found a production of $2.0 \times 10^3 - 1.0 \times 10^4$ cysts l^{-1}, and Vincent (2003), showed a cyst yield between $4.2 \times 10^3 - 8.3 \times 10^3$ l^{-1}. Note that the crosses published by Blackburn et al. (2001) were conducted in the early 1990s (S. Blackburn, Pers. Comm.). Progeny produced by GCDE08 self-crosses in this study were non-viable (i.e. progeny not surviving beyond the 8-cell stage; Blackburn et al., 2001). This was also demonstrated by Vincent (2003). One suggested reason for homothallism has been that it is derived from heterothallism in response to the problems caused when a compatible mating type is unavailable (Goodenough, 1985), or as a result of limited parthenogenesis (reproduc-

tion from a female gamete without fertilization by a male gamete), as was suggested by Pfiester and Anderson (1987). While studying *Alexandrium tamarense*, Destombe and Cembella (1990) suggested that long-term culturing of clonal strains could result in mating type mutations, which could cause strains that were originally unable to form resting cysts without the presence of another stain, to be able to do so. It is possible that this is the case for GCDE08 which have been found to not be able to self-cross in older studies but is now known to do so. In terms of use in strain crosses for experimental model cultures, self-crossing strains as GCDE08 can still be used, however, one must take into account that the self-cross is generally of lower viability, and cysts can have been be produced from either homothallic or heterothallic reproduction depending on the gametes' origin.

An interesting observation was that inter-crosses between GCDE08 and any other strain (except GCHU02 and GCHU20) gave a cyst production up to two magnitudes higher than the GCDE08 self–cross. Theoretically, if the cell concentration of the inocula was the main factor controlling the mating response, then cyst production should have been relatively similar as an equal amount of vegetative cells from both original cultures initially were added together, which could suggest a combined hetero- and homothallism. Combined hetero- and homothallism within the same species has been shown in other dinoflagellates, e.g. *Alexandrium tamarense* (Anderson, 1980; Destombe and Cembella, 1990) and *Karenia brevis* (Walker, 1982). Some of the GCDE08 inter-crosses (with GCDE09, GCLV01, and GCVS04), as well as the self-cross) gave rise to cysts of an irregular appearance with a slightly oblong shape. This irregular resting cyst appearance has previously also been seen in Spanish strains of *G. catenatum* (Figueroa, 2005), although no remarks were made as to the significance of this appearance.

Overall, the results of *G. catenatum* reproductive compatibility found in this study show similarities to those found by Blackburn et al. (2001) (Table 2.6). Although Blackburn et al. (2001) did not find GCDE08 to be self-crossing, several of the other crosses are comparable with respect to their cyst-production capacity. Of the 14 crosses in this study that did not produce cysts, only one (GCDE05×GCDE08) produced cysts in Blackburn et al.'s (2001) study. The cyst production of GCDE08×GCHU11, GCDE08×GCDE08, GCDE05×GCHU11 and GCDE09×

GCHU11 are an order of magnitude higher than reported in Blackburn et al. (2001) and the cross between GCDE08 and GCDE09 show 3 orders of magnitude greater cyst production, this despite the fact that the studies used the same crossing methods and culture strains. Changes in reproductive compatibility over time has also been found by Destombe and Cembella (1990) and was also studied by Blackburn et al. (2001), suggesting that long-term culturing of a strain and undetected environmental factors may affect the ability to sexually reproduce.

Table 2.6 Comparison of cyst production between present study (first number) and Blackburn et al. (2001) (second number). Numbers indicating level of cyst production as described in Table 2.2. Original strain names have been shortened from GCDE05 to DE05 etc.

Strain	DE05	DE06	DE08	DE09	HU02	HU11
DE05	0 / 0	0 / 0	0 / 1	0 / 0	0 / 0	1 / 0
DE06		0 / 0	0 / 0	0 / 0	0 / 0	2 / 3
DE08			1 / 0	3 / 0	0 / 0	2 / 1
DE09				0 / 0	0 / 0	1 / 0
HU02					0 / 0	1 / 2
HU11						0 / 0

Studies have shown that long-term culturing appears to have an effect on the reproductive compatibility of both *Alexandrium minutum* and *G. catenatum* (Parker, 2002). While Parker (2002) repeated a strain-crossing study with four *A. minutum* strains, using the same strains that were originally used by Blackburn seven years earlier (unpublished data), Parker found only one of the strains to be reproductively compatible within the culture, in comparison to the results from Blackburn where three of the four cultures were reproductively compatible. Observations of sexual reproduction within cyst-derived strains suggest that the strains can contain multiple mating types, as observed for *Ceratium cornutum* and *Alexandrium catenella* (Beam and Himes, 1984; Yoshimatsu, 1984). The apparent loss of reproductive compatibility within the *A. minutum* germinated resting cyst strains (after 7 years in culture) seen by Parker (2002), may therefore suggest that a genetic selection is acting within the culture to favour one mating type over another. In terms of *G. catenatum*, loss of reproductive compatibility can however not be due to the selection of one mating

type over the other (as seen in *A. minutum* described above). In contrast to the *A. minutum* study, all the *G. catenatum* strains used in the studies were clonal cultures, thus suggests that the change in reproductive compatibility is instead a result of an actual shift in reproductive compatibility rather than the selection for one mating type over the other within the strain (Parker, 2002). Loss of sexual viability has also been observed in Chlorophytes (*Gonium pectorale*; Coleman et al., 1994), where it appears that mating types in complex mating systems are not particularly stable in long-term culture.

Some of the strains used in this study have been isolated for more than 20 years which theoretically can lead to a greater opportunity for selection of particular bacterial associations, and subsequently influence the results. However, despite being isolated as early as 1987, GCDE08 (from which *Marinobacter* sp. DG879 was originally isolated) have been found to have a bacterial composition remarkably similar to that of other cultured dinoflagellates (Green et al., 2004), suggesting that the established algal-bacterial arrangement may remain even after long-term culturing.

In a previous *G. catenatum* growth study, Vincent (2003) measured reproductive compatibility on some of the strains that were also used in the present study. As different scoring criteria were used it is only possible to compare presence/absence of resting cysts (Table 2.7). Despite using the same strains and the same culturing conditions, 9 of the 27 crosses (Vincent did not cross GCLV01) varied between the two studies, again indicating that compatibility and cyst production can vary between studies, and cannot be assured. Vincent (2003) found these 9 crosses to be cyst-producing while in this current study, they were not. This suggests that the crosses in this study are either false negatives (i.e. the crosses are able to produce cysts, although it could not be confirmed in this study) or alternatively, that there has been a consistent breakdown of compatibility over time.

Table 2.7 Comparison of cyst production between current study (first symbol) and Vincent (2003) (second symbol). (+) indicates cysts production, (-) indicates no cysts production. *n.a* = not attempted cross. Original strain names have been shortened from GCDE06 to DE06 etc.

Strain	DE06	DE08	DE09	HU11	H 01	VS04	LV01
DE06	- / -	- / +	- / -	+ / +	- / +	- / -	+ / *n.a*
DE08		+ / +	+ / +	+ / +	+ / +	+ / +	+ / +
DE09			- / -	- / +	- / -	- / -	- / +
HU11				- / -	+ / +	- / +	- / +
HA01					- / -	- / +	- / +
VS04						- / -	- / +
LV01							- / -

The variation in the period of time from crossing to cyst production (ranging between 12 to 52 days) could be due to a number of factors including prior culture history and also that in early stationary phase batch cultures, a number of different life-history stages may be present in different proportions (Cembella et al., 1990). This in turn could influence the rate at which cysts were being produced.

2.4.2 *Gymnodinium catenatum* resting cyst germination success

The short dormancy period demonstrated by *G. catenatum* in this study (approximately two weeks) is similar to that shown in other studies (Blackburn et al., 1989; 2001; Figueroa, 2005), and equally short dormancy periods have also been found for some other dinoflagellates, for example *Scrippsiella trochoidea* (Binder and Anderson, 1987) and *Alexandrium monilatum* (=*Gonyaulax monilata)* (Walker and Steidinger, 1979). The short dormancy period is a great advantage in establishment of unibacterial algal model systems.

The germination success of 74% in the positive control (non-sterilised cysts with a 5µm filtrate) found in this study is comparable to an earlier study showing the cross GCDE08×GCHU11 to have a germination success of 60-80% (Vincent, 2003). Considering the two bacterial treatments, *G. catenatum* cysts incubated with either *Alcanivorax* sp. DG881 or *Marinobacter* sp. DG879 cultures showed comparable germination success to that found by Vincent (2003), with 59 ± 7.1 % and 82 % respectively for the *Alcanivorax* sp. DG881 treatment, and 38 % ± 19.6 % in comparison to

50 % found by Vincent (2003) for the *G. catenatum* cells treated with *Marinobacter* sp. DG879.

Production of vegetative cells from germinated cysts at Day 36 (Day 40 in Vincent, 2003) was lower in the current study compared to results from Vincent (2003). In the current study the *Alcanivorax* sp. DG881 treatment gave rise to 6 times less cells than the same treatment in Vincent (2003), the *Marinobacter* sp. DG879 treatment yielded less cells while the positive controls were comparatively similar. It is likely that the lower cell per cyst production could be a demonstration of the overall health of the GCHU11 and GCHA01 strains used in the cross, or related to different environmental factors between the two studies. The complex mating system that is found in *G. catenatum* may also be an explanation of the slow growth of the cells post germination, as there is considerable variation between cyst production and viability of progeny depending on the strain cross (Blackburn et al., 2001). Although the resting cysts germinate readily, they appear to not be able to vegetatively reproduce to the same extent as the GCDE08 × GCHU11 cross used by Vincent (2003). However, this slow initial growth did not affect long-term maintenance and vigour (see chapter 4) of the *G. catenatum* / bacterial experimental model systems.

2.4.3 Establishment of *Gymnodinium catenatum*-bacterial model systems

The initial slow growth of the isolated clonal uni-bacterial *G. catenatum* cultures was not unexpected as *G. catenatum* is known to grow slowly when densities are below 10^5 cells L^{-1} (S. Blackburn, pers. comm.). A similar pattern has been found in *Pyrodinium bahamense* where the inoculum size was found to be critical in culture maintenance (Blackburn and Oshima, 1989). Initially, Oshima et al. (1985) showed that when using an inoculum of 1000 cells mL^{-1}, *P. bahamense* var. *compressum* could grow well even under suboptimal conditions, while later, Blackburn and Oshima (1989) found that an initial density of at least 700 cells mL^{-1} was necessary in order to establish a vigorous culture. This may possibly have to do with 'growth factors' (e.g. bacterial exudates or growth enhancing compounds produced by the algae itself) in the medium transferred from the parent culture. Seliger (in Blackburn and Oshima, 1989) also found that isolation success and promotion of cell division was algal density dependent, with the creation of a microenvironment (i.e. single

cells placed in glass capillary tubes immersed in Petri dishes with growth media) being more successful in promoting cell division than when using a Petri dish alone . These results suggest that there may be a production of growth enhancing compounds released by the algal cell, with cell division rate dependent on the concentration of the compound.

Despite the low level of cells produced by the cysts in the *Marinobacter* sp. DG879 treatment, the isolated clonal *G. catenatum* cells proved to be resilient and cultures subsequently grew well. Developed uni-bacterial *G. catenatum* cultures were used in studies examining algal-bacterial interactions using molecular probes (Chapters 3 and 4). In this study, the on-going growth of the uni-bacterial *G. catenatum* cultures, compared with the failure of the negative control (cysts without bacterial addition), demonstrate an essential interaction between the bacteria and *G. catenatum*, with growth of *G. catenatum* only achievable in the presence of the bacteria *Marinobacter* sp. DG879 or *Alcanivorax* sp. DG881. The mechanisms of interaction between *G. catenatum* and the bacteria have not been fully elucidated; however a recent study by Amin et al. (2009) demonstrates that a group of *Marinobacter* (including *Marinobacter* sp. DG879) produce an unusual dicitrate siderophore called Vibrioferrin. Vibrioferrin is a small organic molecule that binds iron and thereby increases the iron solubility, which in turn allows the bacteria to take up the iron-siderophore complex. Their results suggest that Vibrioferrin facilitates photochemical redox-cycling of iron, promoting algal absorption of iron by >20-fold. Additionally, these culture experiments suggested that algal cells release organic molecules utilised by bacteria for growth by *Marinobacter* sp. (Amin et al., 2009).

Conclusion

This study investigated reproductive compatibility of Tasmanian *G. catenatum* strains, identified the best strain inter-cross for resting cyst production, established uni-bacterial *G. catenatum* cultures with either *Marinobacter* sp. DG879 or *Alcanivorax* sp. DG881, and demonstrated the obligate requirement for the specific bacteria for survival and growth of *G. catenatum*. The established uni-bacterial cultures formed the basis of further investigations of algal-bacterial interactions in *G. catenatum*, including development of bacterial quantitative PCR detection methods.

2.5 References

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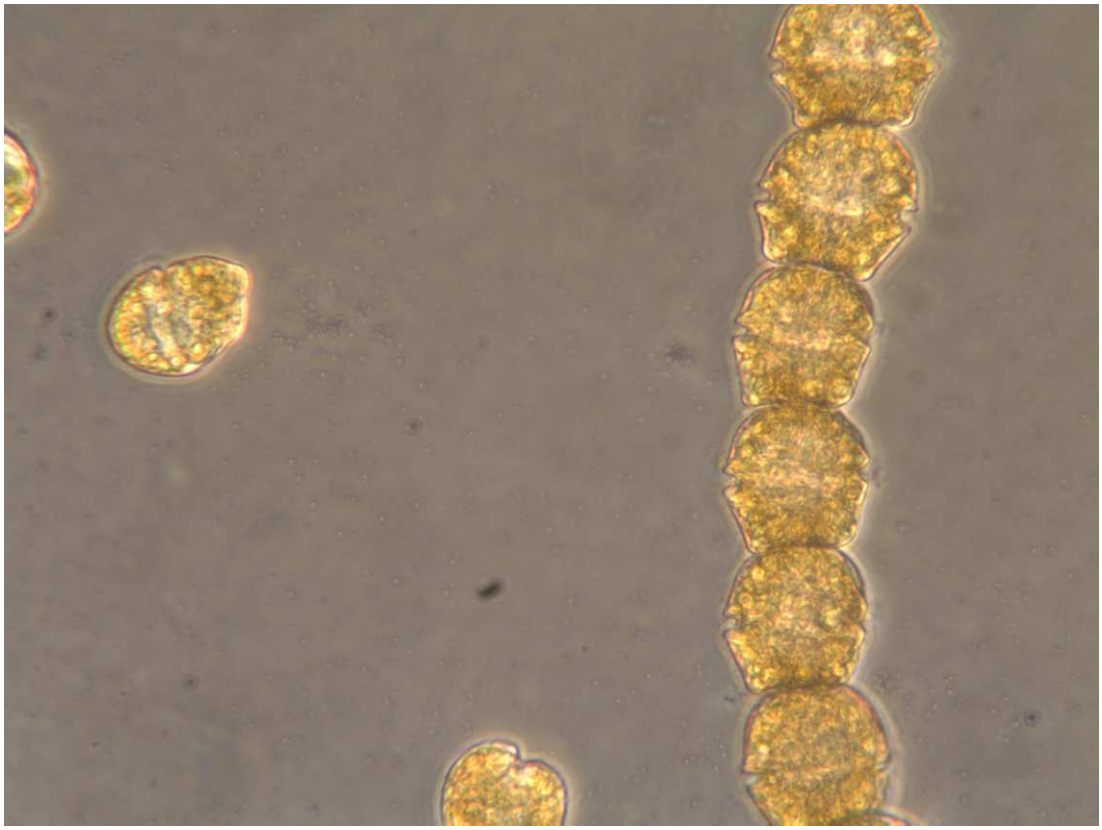
Chapter 3:

Development of and validation of a quantitative Real-time PCR assay for *Marinobacter* sp. and *Alcanivorax* sp. associated with *Gymnodinium catenatum*



Chapter 3: Development of and validation of a quantitative Real-time PCR assay for *Marinobacter* sp. and *Alcanivorax* sp. associated with *Gymnodinium catenatum*

3.1 Introduction

Algal-bacterial interactions are considered to be of greater importance to the ecosystem than previously believed (Doucette et al., 1998). Complex algal-bacterial interactions have been identified at several stages of algal life cycles, affecting sexual reproduction and cysts formation (Adachi et al., 2001), inhibiting or promoting growth (e.g. Fukami et al., 1997; Ferrier et al., 2002), or involved in toxin production (e.g. Bates et al., 2004). Algal-bacterial interactions can potentially also act as a major factor influencing algal bloom formation for example by bacterial production of growth factors such as cytokinins (plant hormones) that promote proliferation of algae (e.g Fukami et al. 1991; Furuki and Kobayashi, 1991; Maruyama and Simidu, 1986; Iwasaki, 1979), by bacterial selection for particular algal species (Ishio, et al., 1989), or by bacterial production of Vitamin B_{12} for algal utilisation (Nishijima and Hata, 1989).

Since its introduction via ballast water in the mid 1970s (Hallegraeff and Bolch, 1992; McMinn et al., 1997), the toxic dinoflagellate *Gymnodinium catenatum* has formed re-occurring blooms in southeast Tasmanian waters, affecting the local aquaculture industry. A shellfish quality assurance program has been in place since the 1980s (Brown and Turnbull, 2006) and from a whole of ecosystem perspective, monitoring of *G. catenatum* has been identified as a critical part of ecosystem management (Volkman et al., 2009).

Two bacterial strains, *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881, both initially isolated from a cultured Tasmanian *G. catenatum* strain (GCDE08 / CS-301/08), have individually been shown to be essential for growth and survival of *G. catenatum* in laboratory-grown cultures (Vincent, 2003; Bolch et al., 2004; Green et

al., 2004). Subsequent research also showed that the bacterial community composition has a significant effect on the growth rate and the dynamics of *G. catenatum* in laboratory cultures (Subramanian, 2008). Considering the impact these bacteria have in laboratory cultures, it is possible that the same bacteria may also play a significant role in regulating populations in the natural environment. Several other examples of the stimulative effect of bacteria have been demonstrated. For example Fukami et al. (1997) found that some diatoms showed biomass increase after addition of specific bacteria, while Ferrier et al. (2002) found growth of *Alexandrium fundyense* to be 'dramatically' stimulated by a bacterial assemblage dominated by a member of the *Alteromonadaceae* family.

The classic way to enumerate bacteria in water samples is to do counts of colony forming units on solid media or direct counts (Fuchsluger et al., 2010). However, plate counts are based on the ability of the bacteria to grow on a defined nutrient medium, and underestimates of bacterial numbers can be caused by clumps of bacteria forming only one colony, while overestimates can be caused by bacteria originally being in a starved state and therefore unable to grow on rich nutrient media. Also, only 1% of all bacteria are culturable (Sharma et al., 2005). Plate counts are also time-consuming as it may take several days to yield results. Direct count techniques have shown to be more reliable (including non-viable and viable but non-culturable), but the differences in numbers of bacteria observed depend on the staining technique and physicochemical characteristics of the samples, as well as individual investigator bias (Kepner and Pratt, 1994). Molecular detection and quantification by Real-time PCR has been shown to be a fast and reliable method to identify and enumerate bacterial species without having to culture, and has been used as a tool for quantifying bacteria in environmental samples (Huijsdens *et al*., 2002; Harms *et al*., 2003) and various other bacteria (Luo et al., 2004; Rousselon et al., 2004).

Real-time PCR uses two general approaches to obtain a fluorescent signal from the synthesis of PCR product. The first depends on the property of fluorescent dyes such as SYBR Green to bind to double stranded DNA, while the second approach use fluorescent resonance energy transfer (FRET). This second approach use a variety of means to alter the relative spatial arrangement of photon donor and acceptor molecules (the donor molecule is the dye or chromophore that initially absorbs the energy

and the acceptor is the chromophore to which the energy is subsequently transferred); these molecules can be attached to probes, primers or the PCR product and are usually selected so that amplification of a specific DNA sequence brings about an increase in fluorescence at a particular wavelength (Edwards et al., 2004) One of these approaches is the TaqMan™ probe with the 5'-3' exonuclease activity of the *Taq* polymerase, which was developed by Holland et al. (1991). It is a technique that uses not only two primers (like conventional PCR), but a third fluorogenic oligonucleotide probe designed to hybridize within the sequence that is targeted by the primers, making the assay more specific and reliable than conventional PCR, and allows for real-time detection of the amplified DNA.

The use of real-time PCR detection based on 16S rDNA or 16-23S rDNA-ITS sequences has become an increasingly routine technique over the last years (e.g. Syutsubo et al., 2001; Nadkarni et al., 2002; Park et al., 2007). As mentioned, this type of analysis circumvents the restrictions of traditional microscopy and culturing techniques in the quantification of microbial communities, allowing for rapid detection of species-specific growth dynamics. This chapter describes the development and validation of a quantitative PCR approach to detect and quantify *Alcanivorax* sp. DG881 and *Marinobacter* sp. DG879 associated with the toxic dinoflagellate *Gymnodinium catenatum*. This approach will allow the investigation of the growth dynamics of the two bacterial species and *G. catenatum* in controlled uni-bacterial and mixed-bacterial *G. catenatum* cultures as well as complex natural marine microbial communities.

3.2 Materials and Methods

3.2.1 Bacterial cultures

The strains used in this study are listed in Table 3.1. Bacterial strains were generously provided by Dr. David H. Green of the Scottish Association Marine Science (SAMS). The *Alcanivorax* cultures were maintained on ZM1 agar with an addition of sodium acetate (NaAc, 10g L^{-1} media; Green et al., 2004; Appendix 2), while all other cultures were maintained on ZM1 agar (Zobell, 1941; Appendix 2). All cultures were held at 20°C in total darkness, with regular transfers every 7 days.

Table 3.1 Bacterial strains used in this study

Bacterial Species	Strain code	Microalgal strain originally isolated from	Bacterial Isolator	Origin of algal host
Marinobacter sp.	DG 879	*G. catenatum* GCDE08	D.H.Green	Derwent Estuary, Tasmania, 1987
Alcanivorax sp.	DG 881	*G. catenatum* GCDE08	D.H. Green	Derwent Estuary, Tasmania, 1987
Roseobacter sp.	DG 874	*G. catenatum* GCDE08	D.H. Green	Derwent Estuary, Tasmania, 1987
Brachybacterium sp.	N/A	*G. catenatum* GCHU11	E. Albinsson	Huon Estuary, Tasmania, 1988
Janibacter sp.	N/A	*G. catenatum* GCDE06	E. Albinsson	Huon Estuary, Tasmania, 1988

3.2.2 DNA extraction

Extraction of bacterial DNA was conducted using cetyltrimethylammonium bromide (CTAB) purification method (Wilson, 1987) on both pure bacterial cultures and on uni-bacterial *G. catenatum* cultures (see section 3.2.9) with the following minor alterations. From the pure bacterial cultures, 2-3 colonies were emulsified in 1 mL sterile demineralised water, pelleted by centrifugation at 13000 rpm for 2 min and supernatant replaced with 1 mL TE Buffer (10 mM Tris HCl pH 8.0, 1 mM EDTA). From the uni-bacterial *G. catenatum* cultures, 5 mL samples were filtered through 10µm Nucleopore® polycarbonate filters to remove algal cells and the bacteria then collected onto 0.2 µm cellulose acetate filters. Both bacterial pellets and filters containing bacteria were frozen at -20 °C until DNA extraction could be conducted.

At the time of extraction, bacterial pellets were thawed and centrifuged at 13 000 rpm for 2 min, and the supernatant was replaced with 507 µL fresh TE Buffer before following the protocol below. Filters containing bacteria were cut into strips, placed in microcentrifuge tubes and like the bacterial cell pellets, extracted using 507 µL TE Buffer and 30 µL Lysozyme (50 mg/ml; Amresco, USA), followed by incubation at

37 °C for 20 min. After incubation, 30 µL of 10 % sodium dodecyl sulphate (SDS), and 3 µL Proteinase K (20 mg/ml; Roche Applied Science, Germany) was added and incubated at 56 °C for 30 min. 100 µL of 5 M NaCl and 80 µL CTAB/NaCl (0.7 M NaCl, 10 % CTAB) were then added and incubated for 20 min at 65 °C. The resulting cell lysates were extracted by phenol/chloroform precipitation: An equal volume (750 µL) of chloroform: isoamyl alcohol (24:1) was added to the microcentrifuge tubes and centrifuged for 5 minutes at 13 000 rpm. The upper layer of the supernatant was removed to a new microcentrifuge tube and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, after which a centrifugation step (13 000 rpm, 5 minutes) followed. The supernatant containing the DNA was again transferred to a new 1.5 mL microcentrifuge tube and precipitated with an equal volume of 100 % Isopropanol. Tubes were centrifuged at 13 000 rpm for 15 minutes, supernatant carefully removed and the pelleted DNA washed with 200 µL 70 % ethanol, dried at room temperature for 10 minutes and re-suspended in 50 µL TE-Buffer. All extracted DNA was stored at -20 °C until analysis.

3.2.3 Design of TaqMan probe and primer sets

DNA sequences of the 16-23S rDNA-ITS region of six algal-associated *Alcanivorax* species (including *Alcanivorax* sp. DG881) and six *Marinobacter* species (not including *Marinobacter* sp. DG879) (Table 3.2), were provided by Mr. Steven Myers (National Centre for Marine Resources and Sustainability, NCMCRS, University of Tasmania).

Table 3.2 Detail of bacterial strains from which 16-23S rDNA ITS sequences were used for *Alcanivorax* and *Marinobacter* primers and probe design.

Bacterial species	Strain code	Origin	Micro-algal species of isolation
Alcanivorax	DG 881	Tasmania, Australia	*Gymnodinium catenatum*
	DG 813	Yellow Sea, Korea	*Gymnodinium catenatum*
	DG 1190	Plymouth, United Kingdom	*Gymnodinium simplex*
	DG 1404	Unknown	*Emiliania huxleyi*
	DG 1592	West coast of New Zealand	*Emiliania huxleyi*
	MBIC 4348	Noroshi, Japan	Seawater
	SK2	North Sea	Oil spill from sea water
Marinobacter	DG 879	Tasmania, Australia	*Gymnodinium catenatum*
	DG 1194	Tamar, United Kingdom	*Alexandrium tamarense*
	DG 1197	Portugal	*Gymnodinium microreticulatum*
	DG 1239	Plymouth, United Kingdom	*Scrippsiella trochoidea*
	DG 1402	unknown	*Emiliania huxleyi*
	MH125a	Millport, Scotland	*Achnanthes* sp.
	MCBI5591	Sendai Port, Japan	Free-living (Sediment)
Roseobacter	DG 874	Tasmania, Australia	*Gymnodinium catenatum*
Flexibacter	DG 1129	Tasmania, Australia	*Gymnodinium catenatum*

The 16-23S rDNA-ITS region for *Marinobacter* sp. DG879 was attained by PCR amplification of the region in both directions using the primer set G1 5'-GAAGTCGTAACAAGG-3' and L1 5'-CAAGGCATCCACCGT-3' (Jensen et al., 1993). Following amplification, the DNA was sequenced using ABI-Prism 'BigDye' terminator chemistry (Applied Biosystems, USA) according to manufacturer's standard protocols. The resulting 4 *Marinobacter* sp. DG879 sequences were aligned with each other and base-calls manually checked using BioEdit Sequence Alignment Editor V.7.0.5.3 (Hall, 1999).

As real time PCR assays can be made more specific with the use of TaqMan™ probes, TaqMan™ Minor Groove Binding (MGB) probes and primers were designed for the

Alcanivorax sp. DG881 and *Marinobacter* sp. DG879 strains using ABI PRISM Primer Express 2.0 software (Applied Biosystems, USA). The fluorescent dye FAM™ was used as the reporter dye at the 5'end of the probe, coupled with a non-fluorescent quencher at the 3' end of the probe.

For the *Alcanivorax* sp. DG881 assays (Fig. 3.1 shows a graphic diagram of the primer and probe location), the 16-23S rDNA-ITS sequence from DG881 was used as a reference sequence and aligned with sequences of the 6 other *Alcanivorax* strains (Table 3.2) together with *Marinobacter* sp. DG879, *Roseobacter* sp. DG874, and *Flexibacter* sp. DG1129 (Fig. 3.2), which are all known to be associated with laboratory-grown cultures of *G. catenatum*; using BioEdit Sequence Alignment Editor V.7.0.5.3 (Hall, 1999). The predicted total length of the product was 60 base pairs (Fig. 3.1).

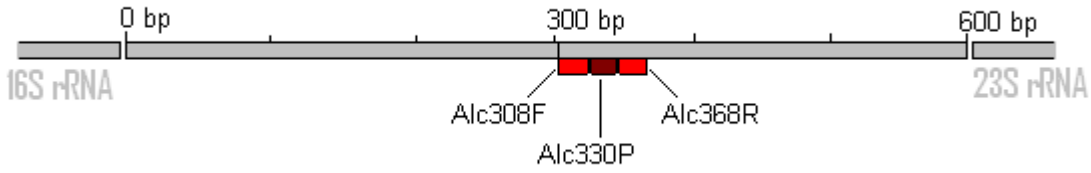


Figure 3.1 Graphic diagram of the *Alcanivorax* sp. DG881 primer and probe positions on the 16-23S rDNA ITS sequence.

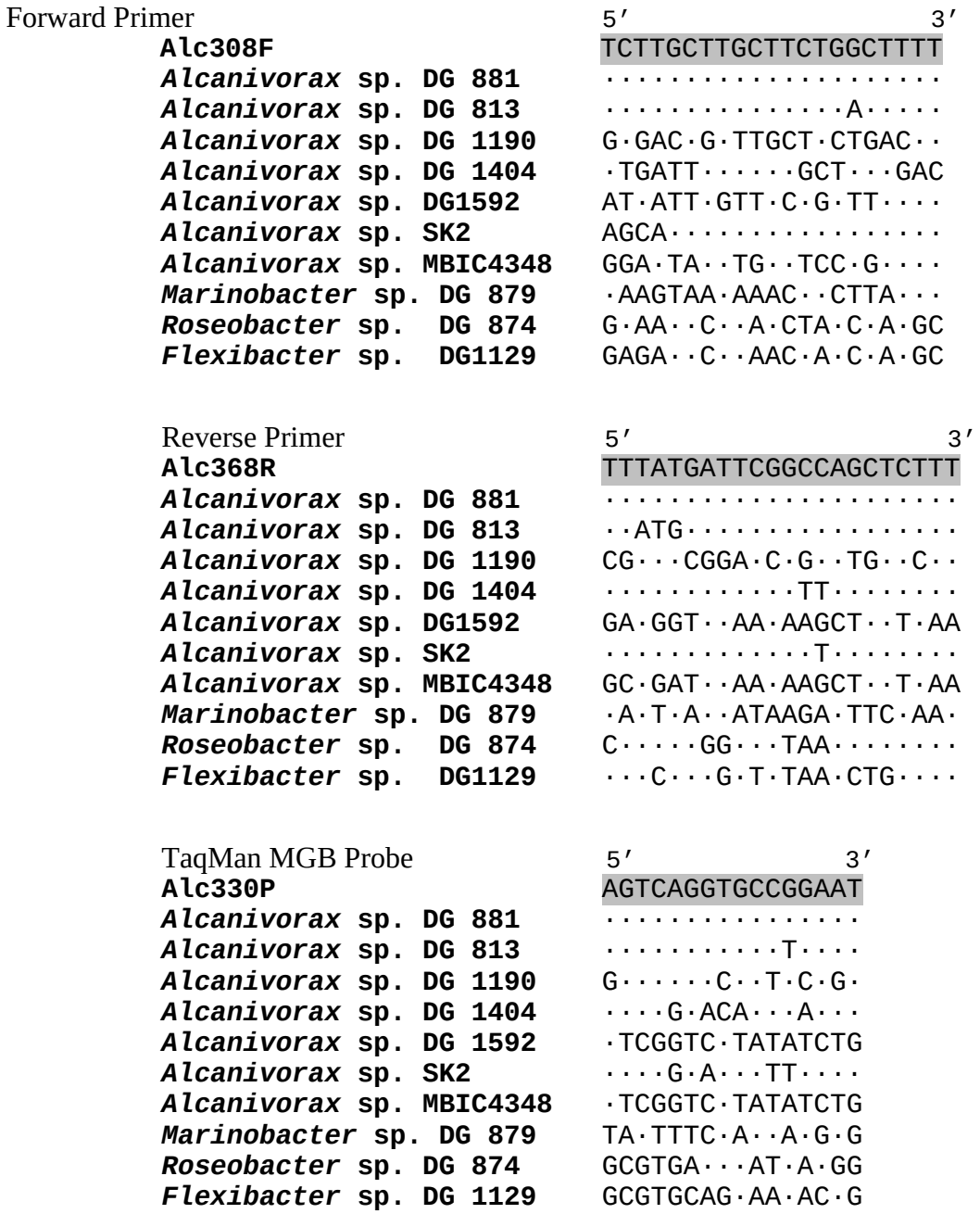


Figure 3.2 Sequence alignment of partial 16-23S rDNA with *Alcanivorax* sp. DG881-specific real-time PCR primers (Alc308F and Alc368R) and probe (Alc330P). Sequences of the primers and probe based on *Alcanivorax* sp. DG881 were aligned with 4 closely related strains, together with *Marinobacter* sp. DG879, *Roseobacter* sp. DG874 and *Flexibacter* sp. DG1129. GenBank accession numbers are: *Roseobacter* sp. DG 874 -AY258075 and *Flexibacter* sp. DG1129 –AY258133. Dots indicate similarities between the aligned sequences.

For development of the *Marinobacter* sp. DG879-specific primers and probe (Fig. 3.3), a consensus sequence attained from sequencing and alignment of 4 *Marinobacter* sp. DG879 cultures (the aligned region of the 4 sequences showed 100% similarity) was aligned with 6 other *Marinobacter* sequences (Table 3.2) together with *Alcanivorax* sp. DG881, *Roseobacter* sp. DG874, and *Flexibacter* sp. DG1129 (Fig. 3.4) using BioEdit Sequence Alignment Editor V.7.0.5.3 (Hall, 1999). The total length of product is 63 base pairs.

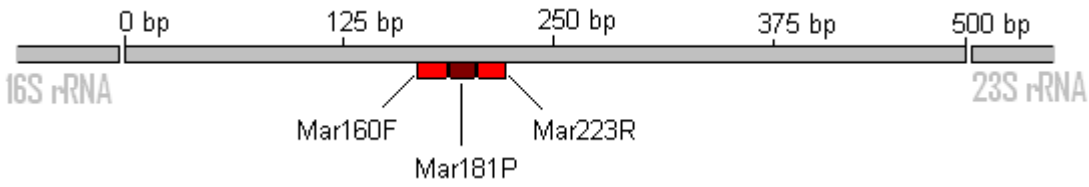


Figure 3.3 Graphic diagrams of the *Marinobacter* sp. DG879 primer and probe positions on the 16-23S rDNA ITS sequence.

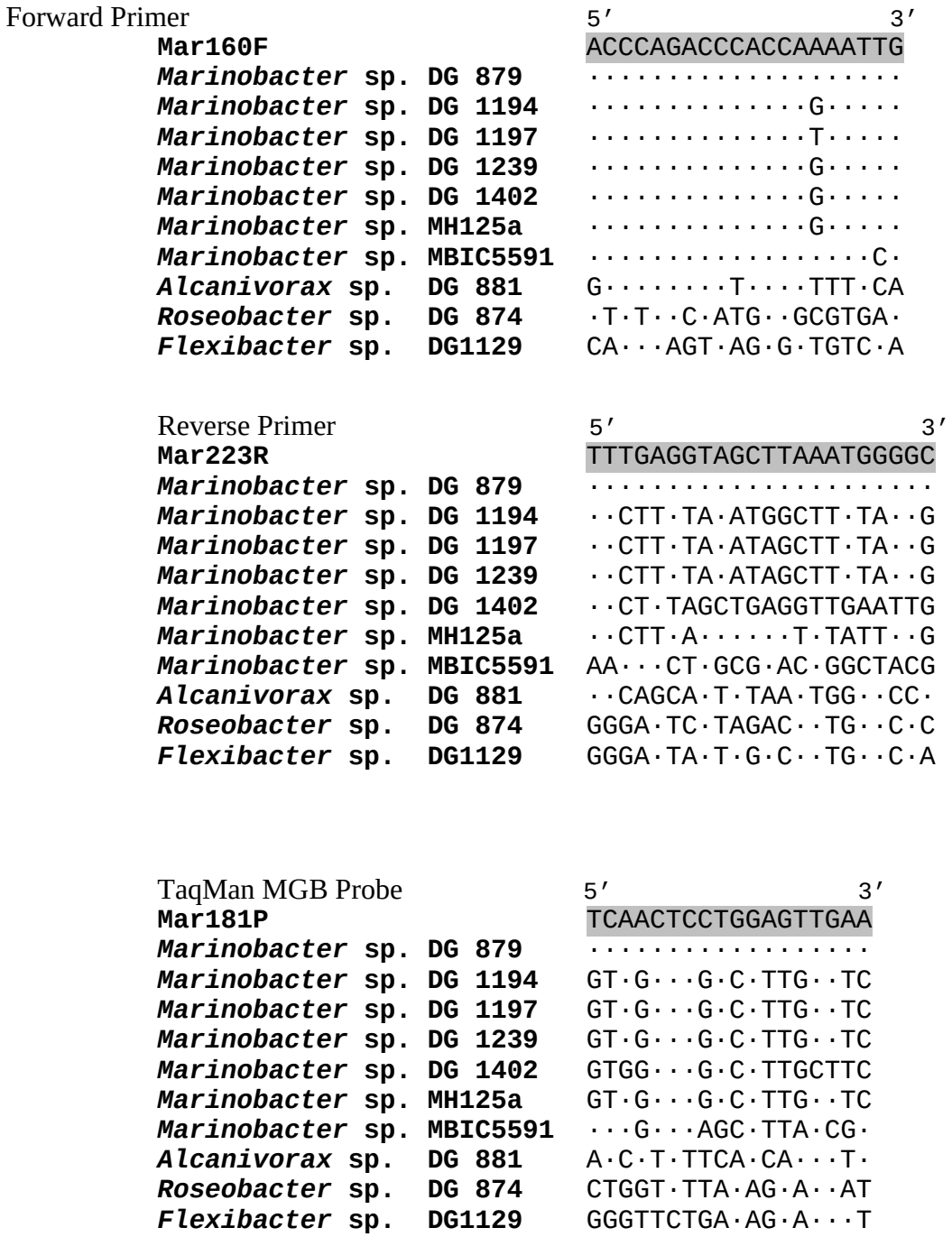


Figure 3.4 Sequence alignment of partial 16-23S rDNA with *Marinobacter* sp. DG879-specific real-time PCR primers (Mar160F and Mar223R) and probe (Mar181P). Sequences of the primers and probe based on *Marinobacter* sp. DG879 were aligned with 5 closely related species, together with *Alcanivorax* sp. DG 881, *Roseobacter* sp. DG 874 and *Flexibacter* sp. DG1129 who are all known to be present in laboratory cultures of *Gymnodinium catenatum*. GenBank accession numbers are: *Roseobacter* sp. DG 874 -AY258075 and *Flexibacter* sp. DG1129 –AY258133. Dots indicate similarities between aligned sequenses.

The primers and probes developed in this study are shown in Table 3.3.

Table 3.3 Primers and probes developed for *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 detection and quantification in this study. FP = Forward primer, RP = Reverse primer, and P = Probe

Bacterial strains	DNA Origin	Primer or Probe	Sequence (5'- 3')
Alcanivorax sp. DG 881	16-23 rDNA ITS	FP Alc308F	TCTTGCTTGCTTCTGGCTTTT
		RP Alc368R	AAAGAGCTGGCCGAATCATAAA
		P Alc330P	FAM-AGTCAGGTGCCGGAAT-MGBNFQ
Marinobacter sp. DG 879	16-23 rDNA ITS	FP Mar160F	ACCCAGACCCACCAAAATTG
		RP Mar 223R	GCCCCATTTAAGCTACCTCAAA
		P Mar181P	FAM-TCAACTCCTGGAGTTGAA-MGBNFQ

3.2.4 Specificity of TaqMan® probes and primers

The specificity of the designed primers and probes in Table 3.3 was tested as described below.

1. Cross-reactivity with other bacterial sequences

Potential cross-reactivity with other bacterial sequences was examined by comparing the sequences of the probes and primers to bacterial sequences available in NCBI's (National Center for Biotechnology Information) GenBank using the Basic Local Alignment Search Tool (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2. Real-time PCR using SYBR Green

A real-time PCR was developed using SYBR Green, without the dual-labeled MGB TaqMan probe to test the specificity of the primers alone. The qPCR was performed using template DNA from *Roseobacter* sp. DG874, *Janibacter* sp., *Brachybacterium* sp., and the target strains *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 (Figs

3.5a, b; 3.7 a, b). When using SYBR Green in a real-time PCR system, any double stranded (ds) DNA, including non-specific real-time PCR products such as primer-dimers, or amplification of non-target DNA will be detected, thereby giving inaccurate fluorescence readings, and in turn inaccurate results. However, when using SYBR Green, the produced PCR products can be melted as a final step in the PCR reaction, in a so called 'melt curve analysis'. As each specific PCR product produces a specific melt curve, non-specific PCR products such as primer-dimers or amplification of non-target DNA can be detected. The SYBR Green qPCR was performed on a 7500 Real time PCR systems (Applied Biosystems) using a Quantifast SYBR Green PCR Kit (QIAGEN, www.qiagen.com). Reactions used 12.5 µL Quantifast SYBR Green PCR Master Mix (containing HotStarTaq®*Plus* DNA Polymerase, QuantiFast SYBR Green PCR Buffer, dNTP mix, and ROX passive reference dye), 0.2 µL of both forward and reverse primers [100 µM], DNA template to a concentration of ≤100 ng per reaction, and RNase-free water to 25 µL. Thermal cycling was performed on a ABI7500 Real time PCR systems (Applied Biosystems) using two-step thermal cycling as follows: 1 cycle of 95 ºC for 5 min (HotStarTaq®*Plus* DNA polymerase activation), 40 cycles of combined 95 ºC for 10 s (denaturation) followed by 32 s at 60 ºC (annealing/extension). A dissociation step of 1 cycle of 95 ºC - 15 s, 60 ºC-1 min, and 95 ºC - 15 s was added to the run to generate a melting curve analysis of the amplified fragments.

3. Screening of specific bacterial species

Specificity of the developed TaqMan primer/probes were tested by screening 4 bacterial strains: *Roseobacter* sp. DG874, *Janibacter* sp., *Brachybacterium* sp., and either *Marinobacter* sp. DG879 or *Alcanivorax* sp. DG881 depending on probe being tested.

4. Screening of bacteria isolated from *G. catenatum*

A total of 31 bacterial strains isolated from *G. catenatum* culture CGDE08 (Appendix 3) were screened using the developed TaqMan assays. The bacterial strains were isolated using a dilution series ranging from 10^0 to 10^{-6} spread plated onto ZM1 agar (Zobell, 1941) incubated at 28 ºC for 7 days in total darkness. Isolated colonies were re-streaked onto ZM1 agar and grown for 7 days and DNA extracted and screened with the TaqMan primer/probe combinations as described above (section 3.2.2).

DNA from the bacterial isolates that gave a positive result using the real-time qPCR was sequenced (as explained in 3.2.3) using universal bacterial 16s rDNA primers (Weisburg et al., 1991) and identified by comparison with bacterial rDNA sequences available on the NCBI GenBank database.

5. Screening of bacteria isolated from whole water

A total of 62 bacterial strains were isolated from a surface (1 m depth) whole water sampled from the CSIRO wharf, Derwent Estuary, Hobart, Tasmania (42º53'12 S: 147º20'21 E). The bacterial strains were isolated and maintained as described above in number 4, and DNA was extracted and screened with the TaqMan primer/probe combinations as described in section 3.2.2. As for the bacterial isolates from cultured *G. catenatum*, DNA from the whole water isolates that gave a positive result using the real-time qPCR was sequenced using universal bacterial 16s rDNA primers (Weisburg et al., 1991) and identified by comparison with bacterial rDNA sequences available on the NCBI GenBank database.

6. Trial on uni-bacterial and multi-bacterial cultures of *G. catenatum*

Finally, the assays were trialed with uni-bacterial cultures of *G. catenatum* containing either *Marinobacter* sp. DG879 or *Alcanivorax* sp. DG881 in order to determine whether the presence of *G. catenatum* would affect the specificity of the PCR (see section 3.2.9 for uni-bacterial culture development). For each primer/probe set, a total of 6 cultures (three uni-bacterial *G. catenatum* cultures and three *G. catenatum* cultures with mixed bacterial flora) were screened. Bacterial DNA from the uni-bacterial cultures was extracted as described in section 3.2.2, while DNA from the *G. catenatum* cultures was extracted first after the *G. catenatum* cells had been collected on 10µm Nucleopore® polycarbonate filters (1 mL of culture onto each filter) and rinsed three times with sterile GSe medium to remove the majority of the *G. catenatum* associated bacteria.

3.2.5 Real time PCR assay conditions

Quantitative PCR (qPCR) assays were performed on a 7500 Real time PCR systems (Applied Biosystems) and reactions for the Taqman qPCR assays were set up according to manufacturer's instructions (Table 3.4). Conditions for SYBR Green assays were set up as described in section 3.2.4.

Table 3.4 Reagents and concentration for use in Real-time PCR assays

Reagent	Concentration of stock	Concentration in thermal cycling reaction	Volume (µL) to be used in reaction
TaqMan® Universal PCR Master Mix	2x	1x	25
Primer (Forward)	9mM	0.9 mM	5
Primer (Reverse)	9mM	0.9mM	5
TaqMan® Probe	2.5mM	0.25mM	5
DNA template	100ng/ul	10ng	5
Demineralised H_2O	------	------	5
Total			**50**

Thermal cycling conditions consisted of 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min (AmpliTaq Gold® Polymerase activation), then 40 cycles of 15 s each at 95 °C followed by 1 min at 60 °C for annealing and extension. Fluorescence data were collected at the end of each cycle and determination of the cycle threshold line was calculated automatically by the instrument.

3.2.6 Construction of standard curves

Standard curves were constructed to examine the relationship between cell concentration of *Marinobacter* sp. DG879 or *Alcanivorax* sp. DG881 and concentrations estimated by the qPCR method. The concentration of bacterial cells was estimated by triplicate haemocytometer counts (Neubauer) using a light-microscope before harvesting of cells and subsequent genomic DNA extraction (as explained in section 3.2.2). Ten-fold serial dilutions of the extracted bacterial DNA were then quantified by real-time PCR. C_T values were determined by the threshold line, which intersec-

tion with the amplification plot determines the value of C_T (Figs 3.10, 3.12). I.e. the C_T value is the number of PCR cycles that elapse before the threshold is reached, and is a measure of the amount of input nucleic acid. Target bacterial cell concentration in experimental samples was estimated by comparison with the standard curve.

3.2.7 Detection limits

Serial 10-fold dilutions of extracted bacterial DNA from cultures of known cell concentration were used. Detection of bacterial DNA was tested over a wide range of dilutions, ranging between 600 ng μL^{-1} and 6.0×10^{-8} ng μL^{-1} for the *Alcanivorax* assay, and 150 ng μL^{-1} to 1.5×10^{-8} ng μL^{-1} for the *Marinobacter* assay.

3.2.8 Algal cultures

The *G. catenatum* cultures used in the study (GCDE06 and GCHU11; Appendix 3) were obtained from the Australian National Algae Culture Collection, ANACC (formerly CSIRO Collection of Living Microalgae (www.marine.csiro.au/microalgae). Cultures were maintained at 20 °C (± 2 °C) in 50 mL Erlenmeyer flasks containing 40 mL GSe medium (Blackburn et al., 2001, Appendix 1), with a light regime of 12:12 h light/dark using cool white fluorescent light at a light intensity of 65 µmol PAR m^{-2} sec^{-1}. Establishment of the uni-bacterial *G. catenatum* cultures used was conducted as described in Chapter 2, sections 2.2.2, and 2.2.4 to 2.2.6.

3.3 Results

3.3.1 Specificity of TaqMan® probes and primers

1. Cross-reactivity with other bacterial sequences

The result from the comparison of the primer and probe sequences to the NCBI GenBank is shown in Table 3.5. The *Marinobacter* assay (Mar160F/ Mar223R/ Mar181P) showed the three closest matches to the primers and probe to be of a *Marinobacter* clone (100 % match) while the probe sequence showed to have a 100% match with sequences from chimpanzee and human, and a 94 % sequence identity to

a deep-sea bacterium. The *Alcanivorax* sp. DG881 assay (Alc308F/ Alc368R/ Alc330P) showed a more varied result, with both primers and the probe showing sequence identity to an uncultured bacterium (forward primer 100 % sequence identity, reverse primer 100 % and probe 97 % sequence identity respectively) and the *Alcanivorax borkumensis* SK2 (90 %, 100 % and 93 % respectively). Forward primer and probe also resembled strains of *Synechococcus* (90 %) and *Rhodococcus* (93 %) respectively.

Table 3.5 Comparison of probe and primer sequences with GenBank sequences, showing the 3 closest matches identified using BLAST.

	Primer / Probe	**Nearest sequence identified by BLAST search**	**% Sequence identity**
Marinobacter sp. DG 879	FP Mar160F	Uncult. *Marinobacter* sp. clone I3K-289ITS8 16S-23S rRNA	100 %
		Uncult. *Marinobacter* sp. clone I3K-289ITS7 16S-23S rRNA	100 %
		Uncult. *Marinobacter* sp. clone I3K-289ITS4 16S-23S rRNA	100 %
	RP Mar 223R	Uncult. *Marinobacter* sp. clone I3K-289ITS8 16S-23S rRNA	100 %
		Uncult. *Marinobacter* sp. clone I3K-289ITS7 16S-23S rRNA	100 %
		Uncult. *Marinobacter* sp. clone I3K-289ITS4 16S-23S rRNA	100 %
	P Mar181P	*Pan troglodytes* (chimpanzee)	100 %
		Homo sapiens (human)	100 %
		Oceanobacillus iheyensis (deep-sea bacterium)	94 %
Alcanivorax sp. DG 881	FP Alc308F	Uncult. Bacterium AD203-H6 genomic sequence	100 %
		Alcanivorax borkumensis SK2, complete genome	90 %
		Synechococcus sp. RCC307 genomic DNA sequence	90 %
	RP Alc368R	*Alcanivorax borkumensis* SK2	100 %
		Alcanivorax borkumensis SK2	100 %
		Uncult. Bacterium AD203-H6 genomic sequence	100 %
	P Alc330P	Uncult. Bacterium AD203-H6 genomic sequence	97 %
		Rhodococcus erythropolis PR4 DNA	93 %
		Alcanivorax borkumensis SK2	93 %

2. Real-time PCR using SYBR Green

Primer specificity testing using SYBRGreen qPCR assays showed that primer pairs for each bacterial species were not species-specific unless used in conjunction with the species specific respective probe. The primer pair Mar160F / Mar223R amplified the *Marinobacter* target DNA, but was also able to amplify *Roseobacter* sp. DG874, *Brachybacterium* sp., and *Alcanivorax* sp. DG881 (Fig. 3.5), this despite a total of 24 mismatches between the DNA sequences of the three bacteria (Fig. 3.4)

However, amplification of *Marinobacter* sp. DG879 started after 11 cycles, whereas amplification of non-target species started only after 31 cycles (Fig. 3.6a). The melting curve showed high similarities between most bacterial strains (Fig 3.6b), and although *Marinobacter* sp. DG879 showed the highest melting temperature of 78.5 ºC there was no distinct difference between *Marinobacter* sp. DG879 and the other bacteria which melting curves followed on temperatures shortly below.

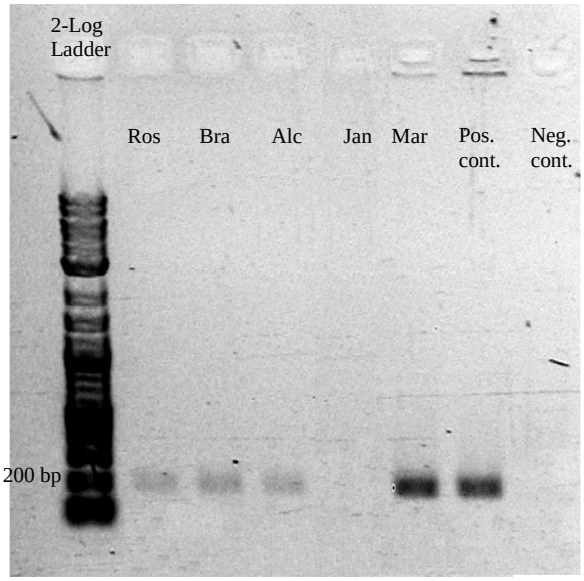


Figure 3.5 Specificity of *Marinobacter* SYBR green qPCR with primer pairs Mar160F / Mar 223R resolved by agarose gel electrophoresis. Primers tested against extracted DNA from pure cultures of Ros = *Roseobacter* sp. DG874, Bra= *Brachybacterium* sp., Alc= *Alcanivorax* sp. DG881, Jan= *Janibacter* sp., and Mar= *Marinobacter* sp. DG879. Positive control = *Marinobacter* sp. DG879

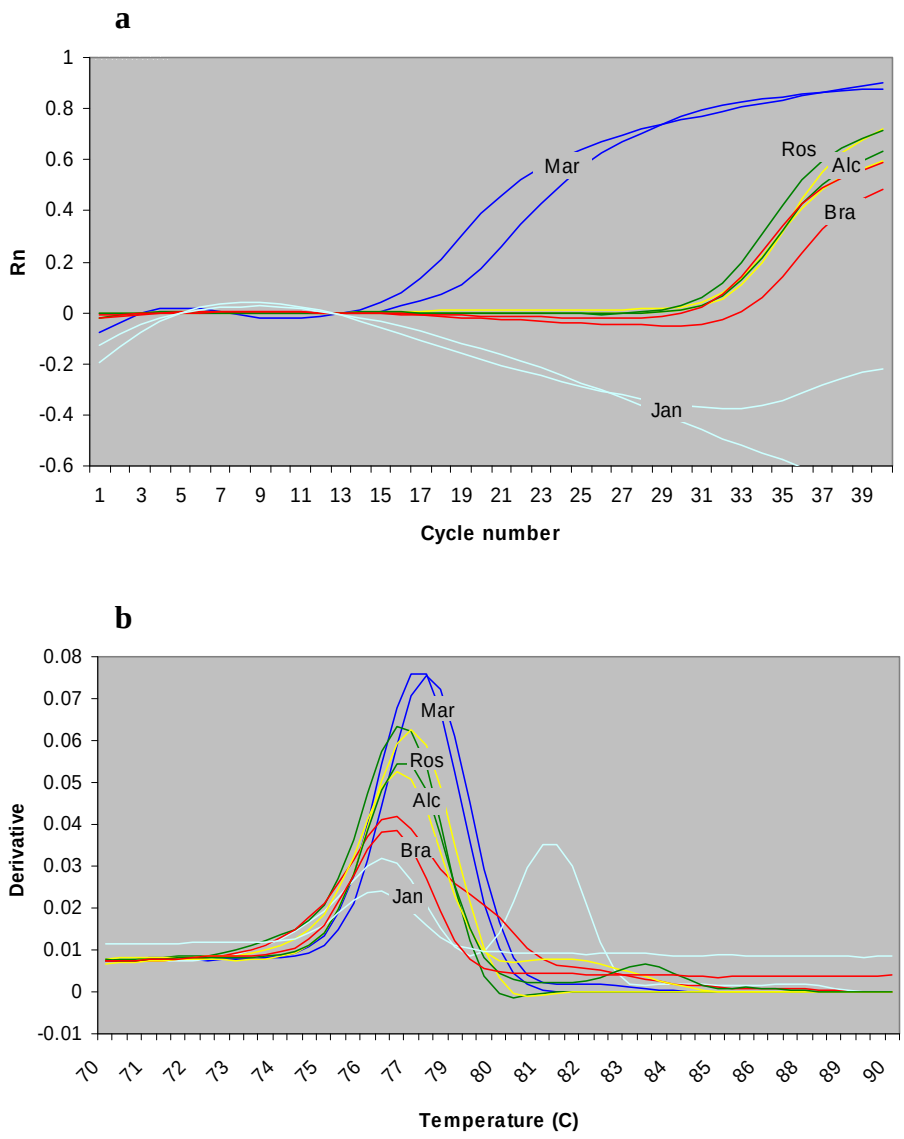


Figure 3.6 Specificity of the SYBR Green-based *Marinobacter* qPCR using primers Mar160F and Mar223R. (a) Primers tested against extracted DNA from pure cultures of *Marinobacter* sp. DG879 (Mar), *Alcanivorax* sp. DG881 (Alc), *Roseobacter* sp. DG874 (Ros), *Brachybacterium* sp. (Bra) and *Janibacter* sp. (Jan) (b) melting curve analysis of the PCR products.

The primer-pair Alc308F / Alc368R showed to amplify the target DNA from *Alcanivorax* sp. DG881, however, non-target species amplification was also found displayed as bands of DNA in the electrophoresis of all the other tested species (Fig. 3.7). However, amplification of *Alcanivorax* sp. DG881 started after 12 cycles and amplification of the other tested bacteria started only after approximately 30 cycles (Fig. 3.8a). Melting temperatures for the different amplicons were different, with *Al-*

canivorax sp. DG881 having a melting temperature of 78°C compared to 73-74°C for the other tested species (Fig. 3.8b).

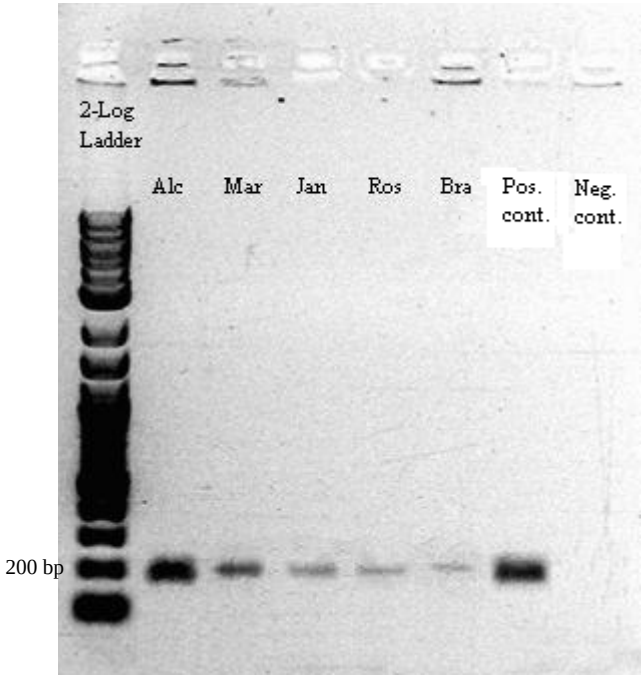


Figure 3.7 Specificity of the primer pair Alc308F / Alc368R resolved by agarose gel electrophoresis. Primers tested against extracted DNA from pure cultures of Alc= *Alcanivorax* sp. DG881, Mar= *Marinobacter* sp. DG879, Jan= *Janibacter* sp., Ros = *Roseobacter* sp. DG874, Bra= *Brachybacterium* sp., and. Positive control = *Alcanivorax* sp. DG881

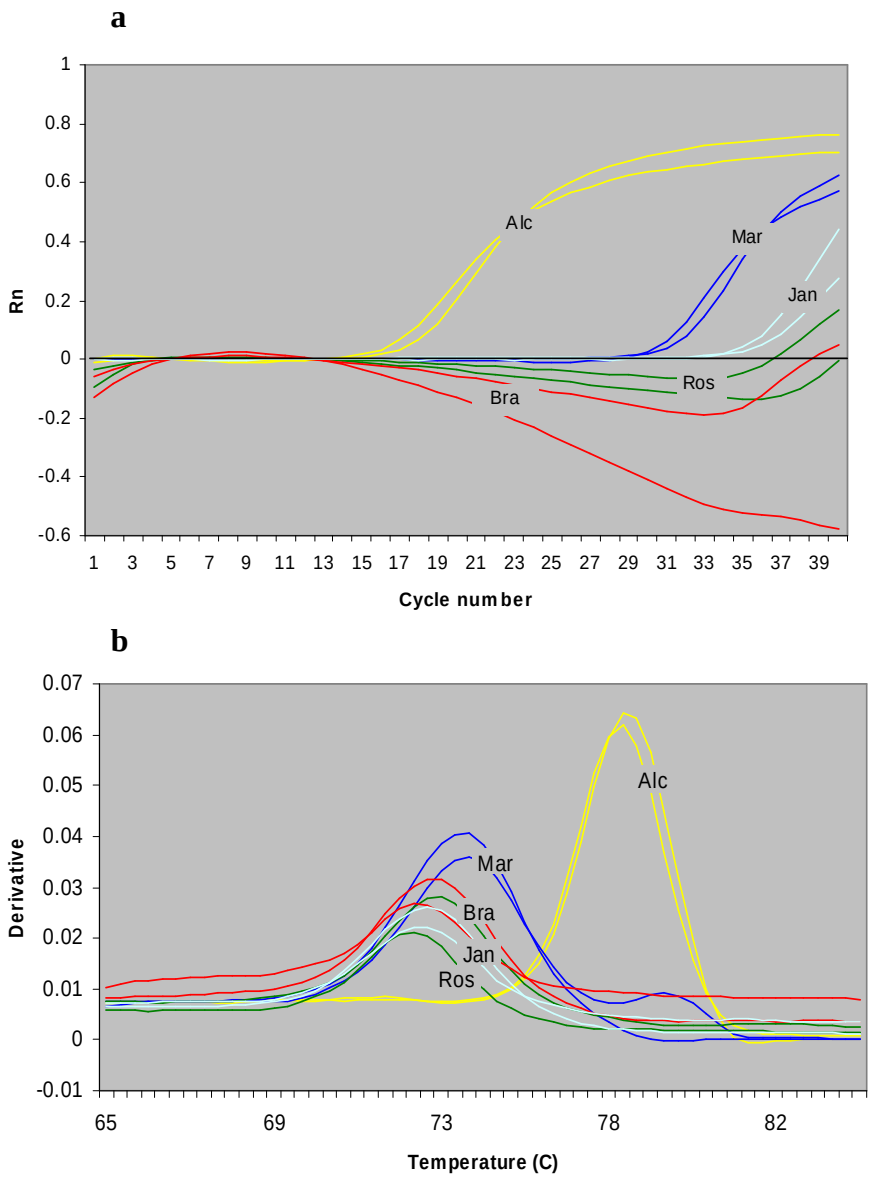


Figure 3.8 Specificity of the primers Mar160F and Mar223R using SYBR Green based system. (a) Primers tested against extracted DNA from pure cultures of *Marinobacter* sp. DG879, *Alcanivorax* sp. DG881, *Roseobacter* sp. DG874, *Brachybacterium* sp. and *Janibacter* sp. (b) Melting curve analysis of the PCR products

3. Screening of specific bacterial species

Both TaqMan™ probe assays were able to detect and amplify the bacterial species for which they were designed and did not amplify product from DNA from pure cultures of the other non-target bacteria tested. The primer/probe set Mar160F/Mar223R/ Mar181P was shown to be species-specific to *Marinobacter* sp. DG879, and did not amplify any of the other tested species (Fig. 3.9a), as was the case for the primer-probe set developed for *Alcanivorax* sp. DG881, Alc308F/ Alc368R/ Alc330P (Fig. 3.9b).

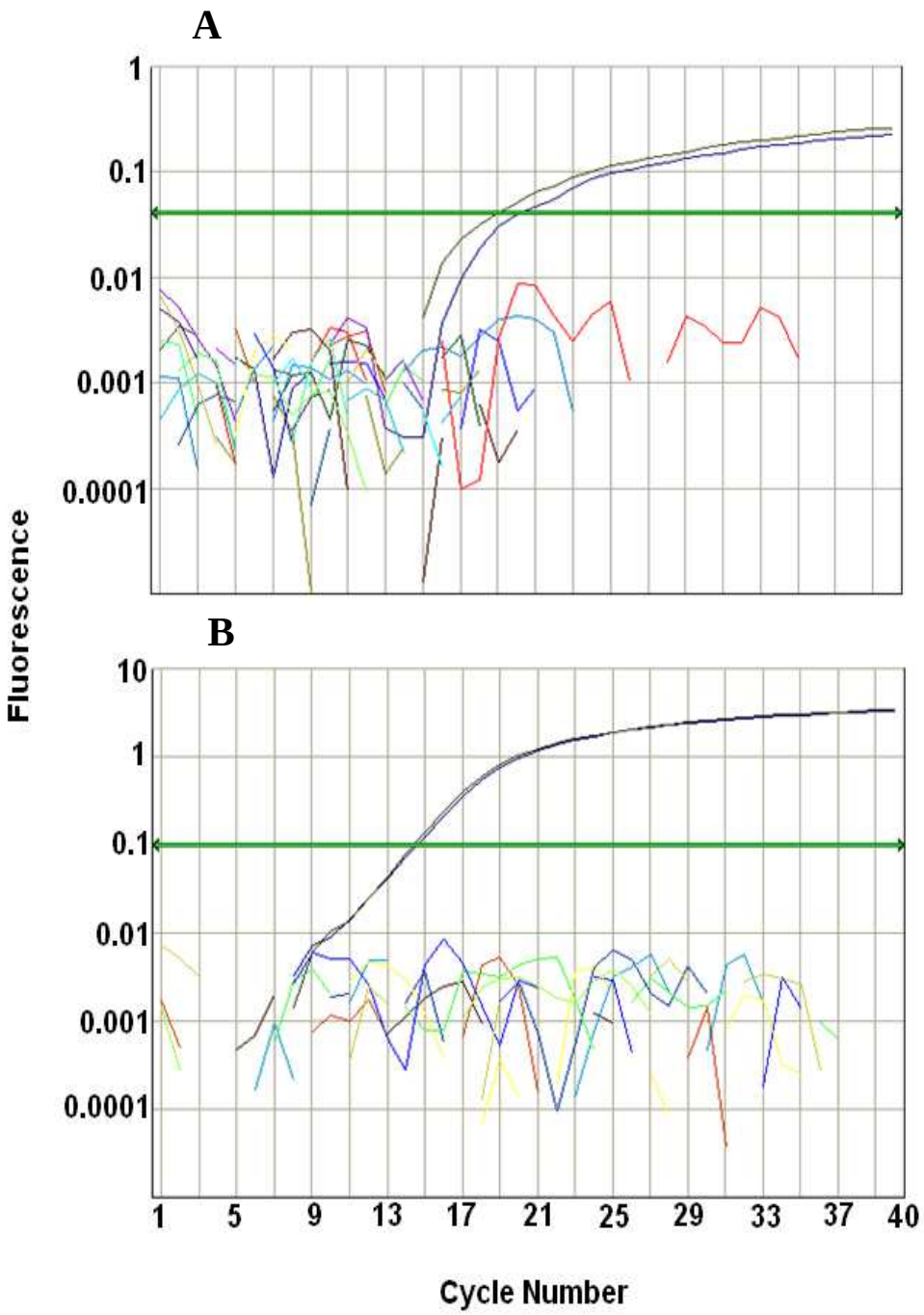


Figure 3.9 TaqMan qPCR probe specificity. (A) The amplification curve from the *Marinobacter* assay Mar160F/Mar223R/ Mar181P showing fluorescence in relation to cycle number; *Marinobacter* sp. DG879 is the only amplified species (brown and dark blue lines). *Alcanivorax* sp. DG881 = light blue and light brown, *Brachybacterium* sp. = purple and lime green, *Roseobacter* sp. DG874 = dark red and orange, *Janibacter* sp. = green and light green, negative control = blue and red; (B) The amplification curve from the *Alcanivorax* assay Alc308F/Alc368R/Alc330P showing fluorescence in relation to cycle number; *Alcanivorax* sp. DG881 (brown and dark blue line) is the only amplified species. *Marinobacter* sp. DG879= yellow and light blue, *Brachybacterium* sp. = dark red and orange, *Roseobacter* sp. DG874 = light green and dark yellow, *Janibacter* sp. = blue and grey-blue, negative control = red and lime green. The green arrow illustrates the Threshold, i.e. the line which intersection with the amplification plot determines the C_T value.

4. Screening of bacteria isolated from *G. catenatum*

Of the 31 bacterial strains isolated from the *G. catenatum* GCDE08 culture and screened with the developed probes, only 4 were amplified (Table 3.6): 2 by the *Marinobacter*-specific assay (Fig. 3.10a) and 2 by the *Alcanivorax*-specific assay (Fig. 3.10b), while the remaining 27 did not amplify. The non-specific amplification only started after approximately 30 cycles, while target amplification started after approximately 18 cycles. In comparison to the developed standard curves (Figs 3.13 and 3.15) this would correspond to approximately 10^2 cells for the non-target organism and 10^8 cells for the target despite equal concentrations of template DNA.

Table 3.6 Putative identity of four bacterial isolates amplified by *Alcanivorax*-specific and *Marinobacter*-specific TaqMan MGB assays. Nearest matching sequence identified by BLAST comparison of 16S rRNA sequences published sequences available in GenBank.

Primer-probe set	Nearest 16S rRNA sequence of bacterial isolate identified by BLAST search	% Sequence identity
Alc308F/368R/330P	*Alcanivorax borkumensis* SK2	99 %
	Roseobacter sp. DG 888	96 %
Mar 160F/223R/181P	*Roseobacter* sp. DG 874	98 %
	Uncult. *Marinobacter* sp. clone I3K-289ITS7	99 %

5. Screening of bacteria isolated from whole water

Of the 62 bacterial strains isolated from the whole water sample (Derwent Estuary) and screened with the developed probes, 13 were amplified by real-time PCR, which after sequencing were demonstrated to belong to 5 different genera (Table 3.7), all being marine organisms. Non-specific amplification started after approximately 35 cycles, while target amplification started after 18 cycles. In comparison to standard curves (Figs 3.13 and 3.15) this would correspond to approximately 10^2 cells for the non-target organism and 10^8 cells for the target at equal concentrations of template DNA.

Table 3.7 Putative identity of 13 bacterial isolates amplified by *Alcanivorax*-specific and *Marinobacter*-specific TaqMan MGB assays. Nearest matching sequence identified by BLAST comparison of 16S rRNA sequences published sequences available in GenBank. The names of the assays have been shortened to Alc set (Alc308F/368R/330P) and Mar set (Mar160F/223R/181P), + signifies amplification.

Primer-probe set amplifying bacterial isolate		Nearest 16S rRNA sequence of bacterial isolate identified by BLAST search	% sequence identity by	
Alc set	Mar set		Alc set	Mar set
+	+	*Planococcus* sp.	96 %	95 %
+	+	*Exiguobacterium* sp.	90 %	89 %
+	+	*Exiguobacterium* sp.	95 %	94 %
+	+	*Planococcus* sp.	97 %	97 %
+	+	*Planococcus* sp.	95 %	95 %
+	+	*Exiguobacterium* sp.	96 %	96 %
+	+	*Marinomonas* sp.	97 %	95 %
+	+	*Exiguobacterium* sp.	91 %	89 %
+	-	*Exiguobacterium* sp.	95 %	-
+	-	Uncultured Rhodobacteraceae	95 %	-
+	-	Uncultured *Planococcus* sp.	96 %	-
+	-	*Bacillus* sp.	95 %	-
-	+	*Exiguobacterium* sp.	-	96 %

6. Trial on uni-bacterial and multi-bacterial cultures of *G. catenatum*

When trialed on bacterial DNA extracted from uni-bacterial *G. catenatum* cultures and on algal DNA extracted from multi-bacterial cultures of *G. catenatum*, neither of the primer probe pairs were strongly inhibited by the presence of *G. catenatum* (Figs 3.10 a and b). In addition, DNA extracted from *G. catenatum* was not amplified in any of the tests. The amplification curves from the bacterial DNA extracted from the uni-bacterial *G. catenatum* cultures showed similar fluorescence to the amplification curves created when amplifying pure bacterial cultures (Figs 3.9 a and b), but in terms of the onset of logarithmic amplification, the DNA extracted from *Alcanivorax* originating from uni-bacterial *G. catenatum* cultures, started amplifyinglogarithmically approximately 5 cycles later than in comparison to DNA extracted from pure *Alcanivorax* cultures. This would lead to an approximate 20-fold under-estimation in cell concentration. This difference was not evident between the two *Marinobacter* DNA extracts.

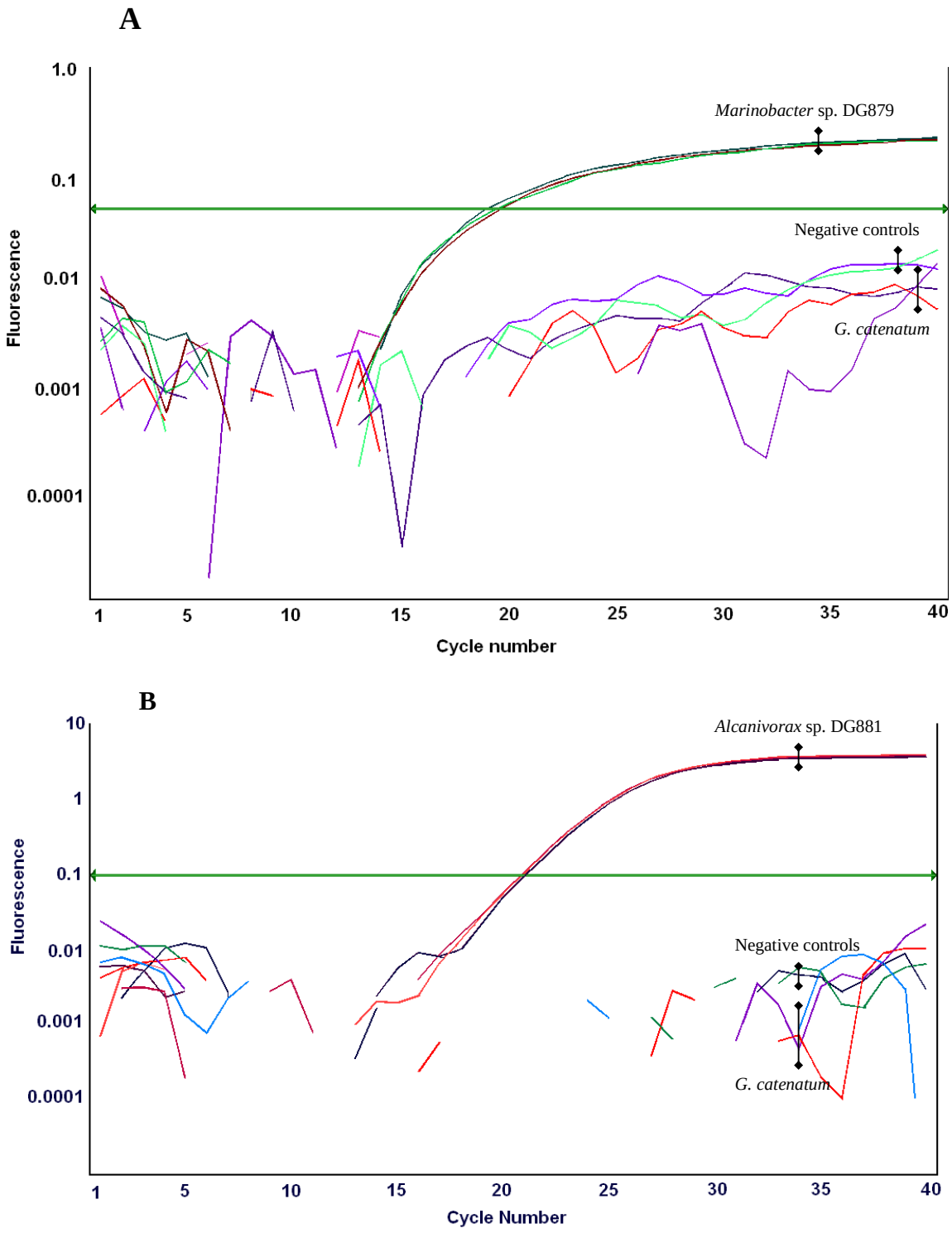


Figure 3.10 Probe specificity on bacterial DNA extracted from uni-bacterial *Gymnodinium catenatum* cultures and algal DNA extracted from multi-bacterial *G. catenatum* cultures. (a) The amplification curve from of the *Marinobacter* assay Mar160F/Mar223R/ Mar181P showing fluorescence in relation to cycle number; *Marinobacter* sp. DG879 is the only amplified species. (b) The amplification curve from the *Alcanivorax* assay Alc308F/ Alc368R/Alc330P showing fluorescence in relation to cycle number; *Alcanivorax* sp. DG881 is the only amplified species. The green arrow illustrates the Threshold, i.e. the line which intersection with the amplification plot determines the CT value.

3.3.2 Standard curves

After real-time PCR quantification of serial dilutions of bacterial genomic DNA from a known concentration of lab cultivated bacterial cells, a linear relationship between absolute bacterial cell numbers and concentration of bacterial DNA was established (Fig. 3.11).

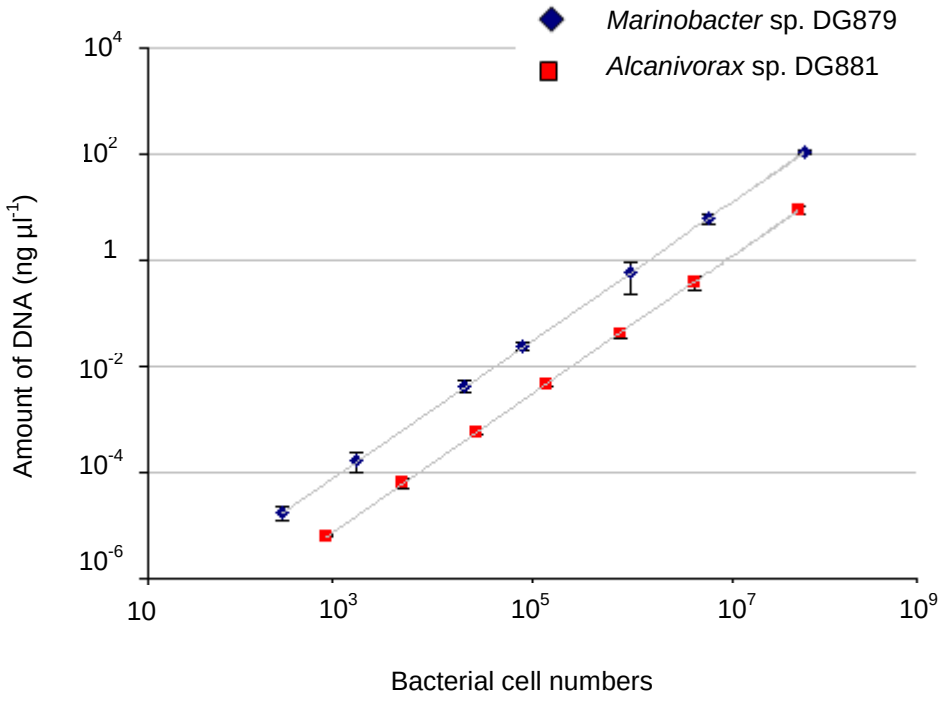


Figure 3.11 The linear relationship between the concentration of bacterial DNA and bacterial cell numbers shown for both *Alcanivorax* sp. DG881 and *Marinobacter* sp. DG879.

Standard curves were constructed with extracted DNA from pure cultures of *Alcanivorax* sp. DG881 (Fig. 3.12) and *Marinobacter* sp. DG879 (Fig. 3.14). The pure cultures, ranging from 10^8 to 10^2 cells mL^{-1}, were initially created by 10-fold serial dilutions of bacterial cell cultures. A linear relationship between the Ct and the log of starting cell concentration was evident for *Alcanivorax* sp. DG881 (r^2=0.999; Fig. 3.13) and for *Marinobacter* sp. DG879 (r^2 =0.998; Fig. 3.15).

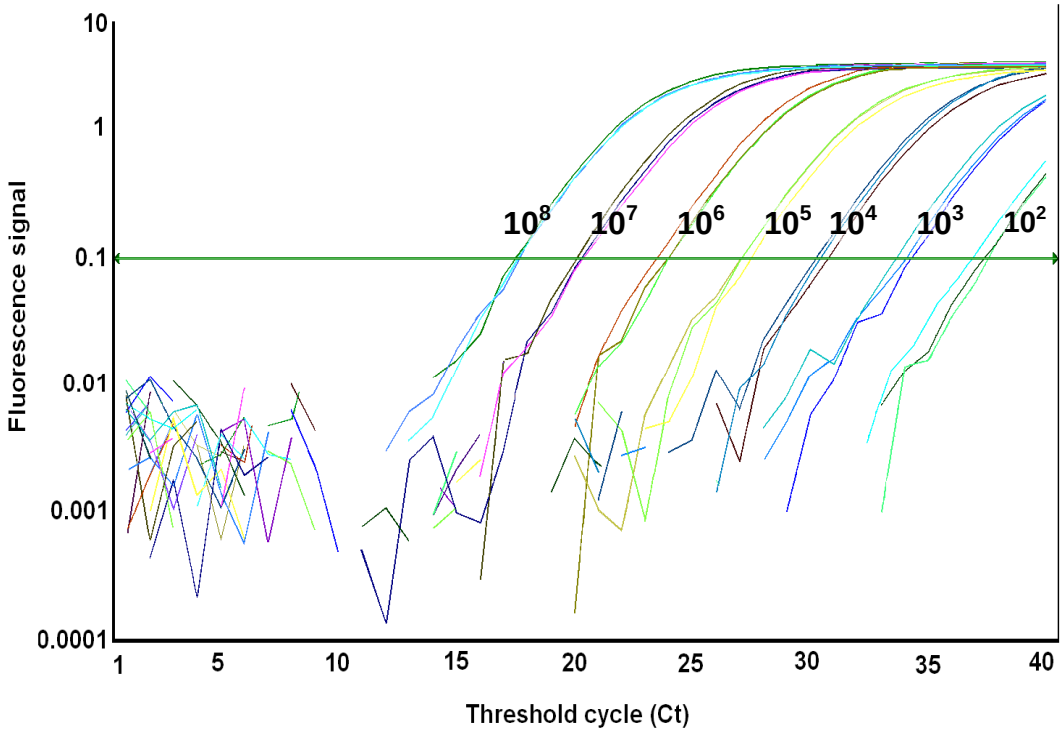


Figure 3.12 Amplification of 10-fold serial dilutions of *Alcanivorax* sp. DG881 genomic DNA, initially extracted from 10-fold serial dilutions of *Alcanivorax* sp. DG881 cells. Initial cell concentration rangied from 10^8 to 10^2 cells mL^{-1}. Amplification curves show fluorescence signal generated by the specified PCR conditions depending on the cycle number of the PCR. The green arrow illustrates the Threshold, i.e. the line which intersection with the amplification plot determines the C_T value.

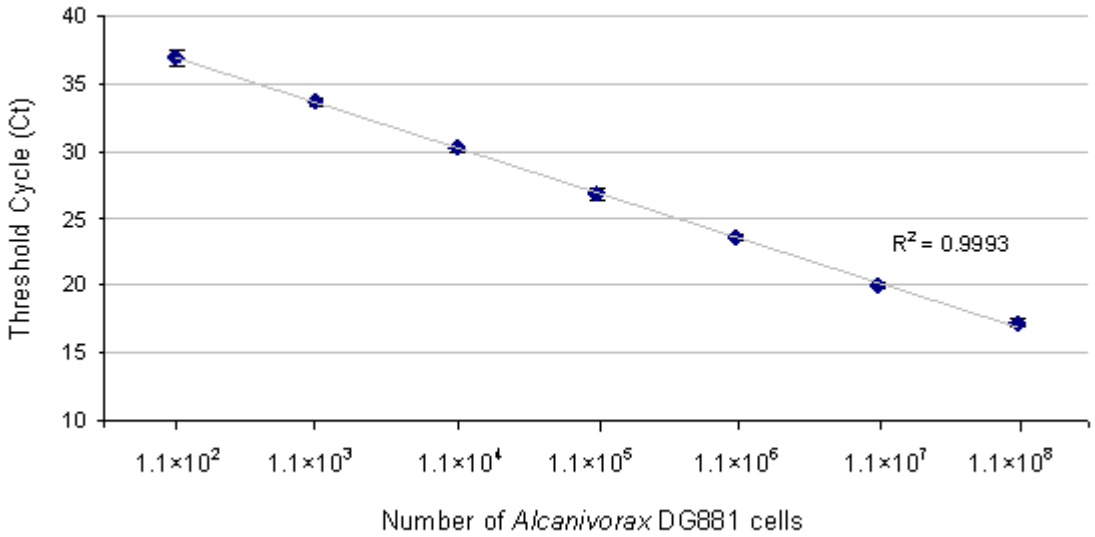


Figure 3.13 Linear relationship between threshold cycles (Ct) and cell numbers of *Alcanivorax* sp. DG881. Correlation coefficient R2= 0.999 and standard deviation from three measurements shown as error bars.

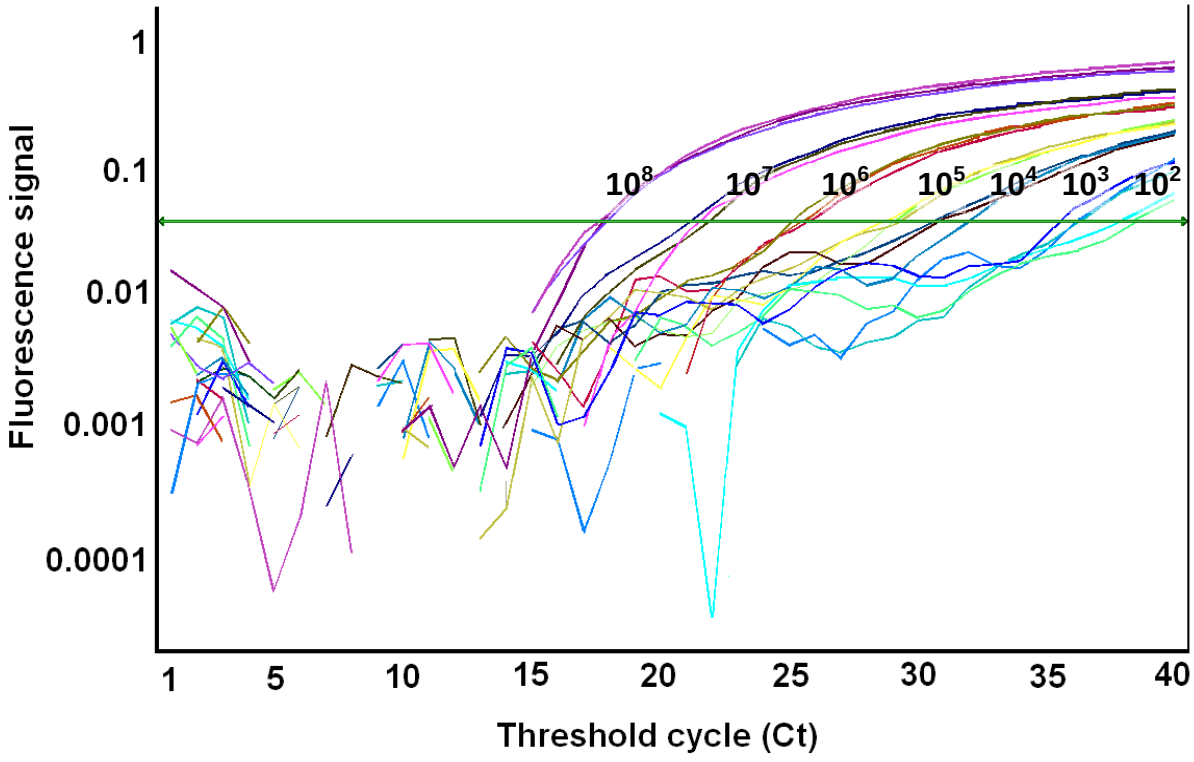


Figure 3.14 Amplification of 10-fold serial dilutions of *Marinobacter* sp. DG879 genomic DNA, ranging from 3.4× 10^8 to 3.4× 10^2 ng μL^{-1}. Amplification curves show fluorescence signal generated by the specified PCR conditions depending on the cycle number of the PCR. The green arrow illustrates the Threshold.

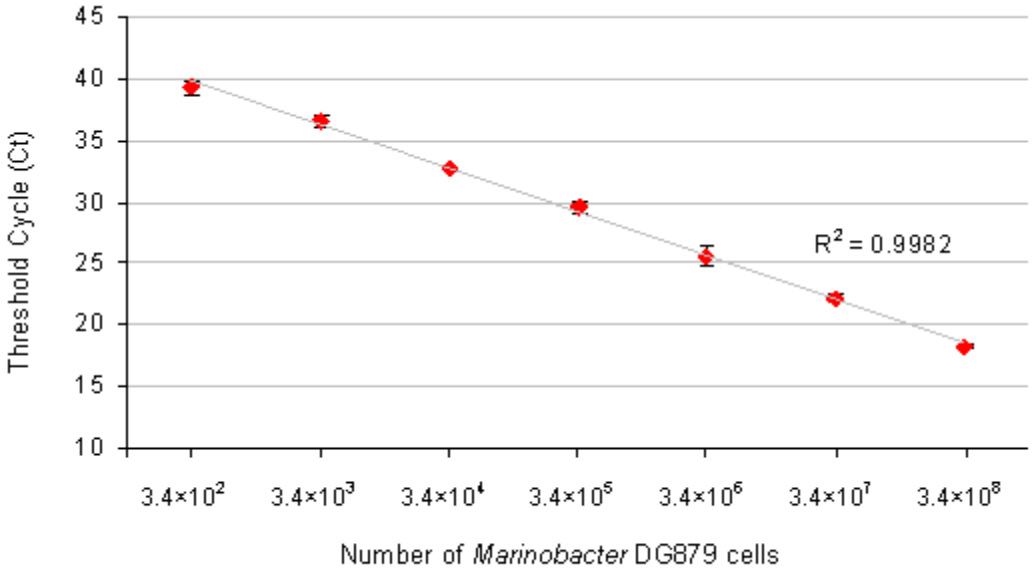


Figure 3.15 Linear relationship between threshold cycles (CT) and cell numbers of *Marinobacter* sp. DG879. Correlation coefficient R2= 0.998 and standard deviation from three measurements are shown as error bars

3.3.3 Detection limits

The *Alcanivorax* sp. DG881 assay Alc308F/Alc368R/Alc330P detected DNA concentrations down to 6.0×10^{-7} ng μL^{-1} while the *Marinobacter* sp. DG879 assay, Mar160F/ Mar223R/Mar181P, detected DNA concentrations down to 1.5×10^{-6} ng μL^{-1}. Using the standard curves generated for the two bacterial strains, these values correlate to approximately 20 cells for the *Alcanivorax* assay, and 5 cells for the *Marinobacter* assay.

3.4 Discussion

3.4.1 Comparison of TaqMan vs. SYBR Green

While screening the bacterial assays using SYBR Green, both primer sets were able to amplify some of the other tested species, however, when the primers were used in conjunction with the specific probes on the same bacterial species, the assays were shown to be strain-specific for *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881. This demonstrates the higher specificity of the TaqMan assays; where the specific hybridization between probe and target is required to generate a fluorescence signal and thereby reduce false positives. The enhanced specificity attained when using TaqMan assays have also previously been reported by a number of studies (e.g. Maeda et al., 2003; Malinen et al., 2003; Gunson and Carman, 2005).

3.4.2 qPCR sensitivity

The detection limit for the two primer/probe pairs Alc308F/Alc368R/Alc330P and Mar160F/Mar223R/Mar181P (20 and 5 cells respectively), is comparable to that of other studies using real-time PCR assays (e.g. Suzuki et al., 2000; Harms et al., 2003). As similar to studies on uni-bacterial *G. catenatum* cultures (containing either *Marinobacter* sp. DG879 or *Alcanivorax* sp. DG881; Chapter 4), show that the bacterial abundance ranges between 10^5 and 10^7 cells mL^{-1} over the course of the *G. catenatum* life cycle, these assays should theoretically be able to detect the bacteria from water samples as small as one microlitre.

The sensitivity of the primer/probe pair would also be adequate for detection of bacteria in 'normal' multi-bacterial cultures of *G. catenatum* where the gamma proteobacteria (i.e. *Marinobacter* sp. and *Alcanivorax* sp.) average at about 5 % of the bacterial community (Green et al., 2004). Naturally occurring bacterial communities in marine waters have been estimated to range between 10^4 to 10^5 cells mL^{-1} (Maier et al., 2009; Venter et al., 2004), but as the proportion of *Marinobacter* sp. and *Alcanivorax* sp. to the total bacteria is unknown, the sensitivity of these assays on environmental samples is yet to be determined. The broad detection range for the primer-probe pairs, with both pair covering 7 orders of magnitude, is also comparative to other studies (e.g. Lyons et al., 2000; Suzuki et al., 2000).

3.4.3 qPCR specificity

The higher DNA sequence variability of the 16-23S rDNA-ITS region compared to the 16S rDNA, has allowed this region to be used to distinguish bacterial species down to sub-species level (e.g. Navarro et al., 1992; Houpikian and Raoult, 2001). The real-time PCR assays developed in this study provide strain-specific detection of either *Marinobacter* sp. DG879 or *Alcanivorax* sp. DG881 in laboratory-grown cultures of uni-bacterial *G. catenatum*.

However, both SYBR Green and Taqman™ assays also detected similar genotypes (closely resembling *Alcanivorax borkumensis* SK2 and uncultured *Marinobacter* sp. clone I3K-289ITS7) and *Roseobacter* species most closely resembling *Roseobacter* sp. DG 874 (by the *Alcanivorax* assay) and *Roseobacter* sp. DG 888 (amplified by the *Marinobacter* assay). This non-target amplification did not start until considerably later in the process (after a minimum of 30 cycles) in comparison to the amplification of the target DNA (after approximately 18 cycles) despite equal concentrations of template DNA. In this case, the secondary product was detected at a cell concentration of 10^2 cells mL^{-1} while the targets were detected at 10^8 cells mL^{-1}. The results from the Taqman assay of bacteria extracted from whole water showed equal sensitivity, with non-specific amplification within the amplification reaction not starting until after 35 cycles. In this case, the effect of the non-specific secondary products (generated within the amplification reaction) on the total cell concentration

would therefore theoretically be minimal. However, in the event of non-specific amplification, the only way to confirm that it is the target that is being detected is to sequence the PCR product produced by the assay, and compare it to known sequences of the target DNA.

The assays developed are capable of reliably detecting and amplifying the target bacterial strains in uni-bacterial cultures of *G. catenatum*, but, considering their ability to also amplify non-target bacterial strains, the primer/probe pairs were regarded as inadequate for use on environmental samples. The main factor influencing the qPCR performance of both assays is primer/probe design. The primers and probes were developed using specific software (ABI PRISM Primer Express 2.0) and within guidelines of GC content, melting temperature, and sequence length of both primers, probes, and amplicons. However, smaller details in the primer/probe design could be the cause of the non-specific PCR products, such as repeated runs of identical nucleotides, having a probe sequence containing more G than C bases, or having two G bases within the last five bases at the 3' end of the probe. As the primers and probes were developed for specific thermal cycling conditions using predetermined concentrations of each component, these conditions were kept in accordance with the manufacturer's instructions. It is however possible that altered annealing temperatures may exclude non-specific PCR product (Vandesompele et al., 2002), and trials could be performed on these assays. For use on environmental samples however, the assays should be redesigned.

As mentioned earlier, insufficient assay specificity can also result from the presence of unknown related species with similar sequences to the primer/ probe sites. As the number of 16-23S ITS rDNA sequences in the NCBI GenBank is limited, sequence comparisons and determination of potential cross reactivity are made very difficult.

A way around non-specific amplification while using SYBR Green chemistry is to collect the amplification data at a temperature where the non-specific products have melted, but the specific products have not (the target products have a higher melting temperature than non-targets), in this case, the non-specific amplification would not contribute to the fluorescence recorded during amplification (Ririe et al., 1997).

3.4.4 qPCR validation of quantification

Although not undertaken in this study, the effect of filtration must be taken into account when quantifying bacterial samples that have been filtered in order to remove phytoplankton or larger cells. Bacterial attachment to phytoplankton has been documented for decades (e.g. Kogure et al., 1982; Albright et al., 1986), and, in a recent study using uni-bacterial model systems of *G. catenatum*, Subramanian (2008) found that approximately 10 % of the bacterial community (*Alcanivorax* sp. or *Marinobacter* sp.) was attached to the algal cell during the death phase of the algal culture. Consequently, bacterial enumeration on filtered samples may underestimate the bacterial abundance as filtration excludes the bacteria attached to the algal cells.

Conclusion

Real-time PCR, with accurately developed probes and primers, is more rapid and accurate than direct bacterial counts and can be used to estimate bacterial cell density and bacterial presence in studies on algal/bacterial growth dynamics. Although the primer/probe pairs developed in this study were able to detect non-target species, they are capable of detecting and quantifying the target bacteria in *G. catenatum* unibacterial experimental model systems where the bacterial diversity is known, making them utilizable for studies on the *G. catenatum*-unibacterial systems. The two bacterial species on which the assays were developed are known growth-promoters of the toxic dinoflagellate *G. catenatum* (Bolch et al., 2004), and by deciphering the complex relationship between *G. catenatum* and these bacteria, we can start to form an understanding of the ongoing interactions.

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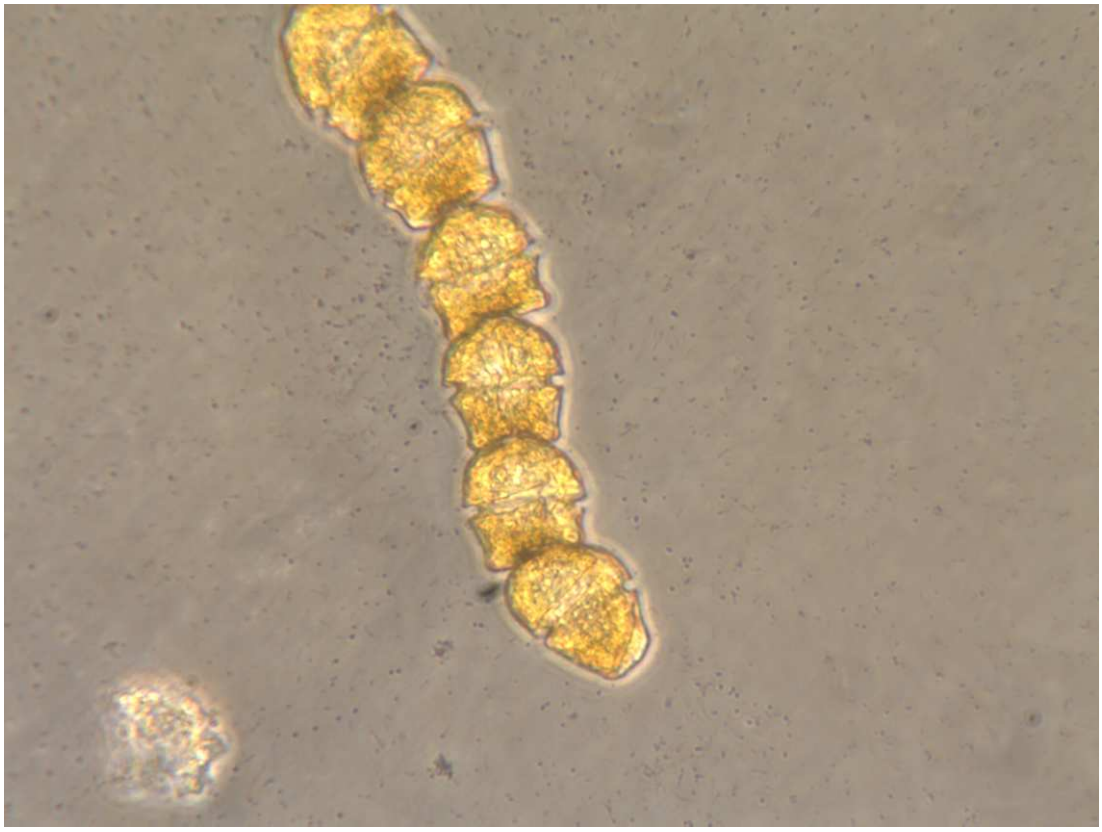
Chapter 4:

Use of real-time qPCR to track bacterial growth in uni-bacterial cultures of *Gymnodinium catenatum*



Chapter 4: Use of real-time qPCR to track bacterial growth in uni-bacterial cultures of *Gymnodinium catenatum*

4.1 Introduction

Phytoplankton and bacteria are ubiquitous in the aquatic environment and studies have shown that they influence each other on several aspects of both their existence (See review by Doucette, 1995; 1998). Just to mention a few examples, algae have been shown to affect bacteria by production of growth substrates for bacterial utilization (Bell et al., 1972 & 1974), and provide increased surface area for bacterial attachment (Cole, 1982); while in turn, bacteria are known to produce algal growth promoters (Stewart et al., 1997; Croft et al., 2005), Cytokinins (plant hormones) that promote proliferation of algae (e.g Fukami et al. 1991; Furuki and Kobayashi, 1991; Maruyama and Simidu, 1986; Iwasaki, 1979), Vitamin $_{12}$ for algal utilization (Croft et al., 2005), algal growth inhibitors (Fukami et al., 1997); and algicidal substances selecting for particular algal species (Furuki and Kobayashi, 1991; Lovejoy et al., 1998; Holmström and Kjelleberg, 1999).

Gymnodinium catenatum is a toxic dinoflagellate species that has formed recurrent blooms in southeast Tasmanian waters since the 1980s, with blooms affecting local aquaculture industry (Hallegraeff et al., 1989). The drivers of *G. catenatum* bloom dynamics are currently unknown, yet studies on the bacterial community of laboratory-grown *G. catenatum* cultures have reveled that two bacterial species, *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881, are required for survival and growth of *G. catenatum* in culture (Bolch et al., 2004).

Using experimental model systems with *G. catenatum* (uni-bacterial or mixed-bacterial cultures; see Chapter 3) Bolch et al. (2004) found both that *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 were essential for growth and survival of *G. catenatum*. As the bacteria appear to be of such importance to *G. catenatum*, the re-

sults suggest that the bacterial species may play a role in *G. catenatum* bloom formation in the natural environment.

In order to better understand population growth dynamics of bacteria and its interactions with phytoplankton, it is essential to correctly identify and quantify the specific organisms. Real-time PCR (see page 47 for more information) is an important tool in several fields including diagnostic and forensic medicine (e.g. Lyons et al., 2000; Nicklas and Buel, 2003), molecular biology (e.g. Hosoi-Tanabe and Sako, 2005), and within microbiology (e.g. Nadkarni et al., 2002; Harms et al., 2003; Torben et al., 2004). In microbiology, this tool is often used to detect pathogenic bacteria such as *Salmonella* in food (Malorny et al., 2004) or applied to identify or detect microscopic cells whose morphologic identification is impossible (e.g. Harms et al., 2003). With carefully validated probes, qPCR can allow for unambiguous counts of particular species in mixed bacterial and algal communities (e.g. Hermanson and Lindgren, 2001; Maeda et al., 2003; Handy et al., 2005), making it possible to study each organism of a community in terms of growth dynamics, and in turn determine eventual competition within that same community.

Competition for limiting resources has long been considered an important factor for regulating bacterial community structure (Grover, 2000; Fox, 2002), where resource competition in its simplest form predicts that the organism that captures most of the limiting resource to the lowest level will exclude its competitors (Fox, 2002). The importance of a single environmental factor was recognized as long ago as 1840 by Justus von Liebig, with “Liebig's Law of the Minimum” stating that the growth of a plant will be limited by whichever requisite factor is the most deficient in the local environment (Brock, 1997). In aquatic systems, bacteria and algae are competitors for limiting nutrients (Currie and Kalff 1984, Harte and Kinzig 1993), which in turn affects the growth dynamics of the two organisms (Hulot et al. 2001).

The aim of this chapter is to examine growth dynamics of *G. catenatum* and the bacteria *Alcanivorax* sp. DG881 and *Marinobacter* sp. DG879 in uni- and mixed-bacterial cultures of *G. catenatum*. This was conducted over a batch-culture cycle using real-time qPCR assays developed for the two bacterial strains (Chapter 3).

4.2 Materials and methods

4.2.1 Algal and bacterial cultures

Two strains of the dinoflagellate *Gymnodinium catenatum* (GCDE06 and GCHU11; Appendix 3) were kindly provided by the Australian National Algae Culture Collection (www.cmar.csiro.au/microalgae). These cultures were maintained at 20 °C with a 12:12 h light/dark cycle using white fluorescent lights at 65 µmol PAR m^{-2} sec^{-1} in 50 mL Erlenmeyer flasks containing 40 mL GSe medium (Blackburn et al., 2001, Appendix 1). Bacterial cultures were generously provided by Dr. David H. Green of the Scottish Association Marine Science (SAMS). The *Marinobacter* sp. DG879 culture was maintained on ZM1 agar (Zobell, 1941; Appendix 2) while *Alcanivorax* sp. DG881 was maintained on ZM1 agar with an addition of Sodium acetate (NaAc, 10 g/L media). Both cultures were held at 20 °C in total darkness, with weekly transfers.

The experimental *G. catenatum* cultures, either uni-bacterial (grown with *Alcanivorax* sp. DG881 or *Marinobacter* sp. DG879) or mixed-bacterial (grown with both bacteria), were established as described in section 3.2.1. These cultures were grown to mid-logarithmic phase in 50 mL Erlenmeyer flasks and inoculated (10 ml) into triplicate Erlenmeyer flasks containing 300 mL of GSe medium. The experimental cultures were grown at 20 ºC with a 12:12 h light/dark cycle using white fluorescent lights at 65 µmol PAR m^{-2} sec^{-1}. Cultures were sub-sampled weekly for enumeration of bacteria (5 mL volume) and algae (2 mL volume). The *G. catenatum* cell concentration was determined by triplicate cell counts from Lugol's Iodine solution (1 %) fixed samples using a Sedgwick Rafter counting chamber (Throndsen, 1995) and a Leitz Labovert FS microscope (200 × magnification).

Bacterial cell concentration was determined using the TaqMan qPCR detection approach described in Chapter 3, and briefly described here: The 5 mL sample was filtered through a 10µm Nucleopore® Polycarbonate filter (Whatman) to remove algal cells and retain bacteria attached on the algae, followed by collection of un-attached bacterial cells on a 0.2 µm cellulose acetate (Whatman) membrane filter. Collection of cell-associated bacteria was only conducted on the mixed-bacterial *G. catenatum*

cultures. Filters were frozen at -20 ºC until DNA extraction. Growth interactions were measured for a total of 230 days for the uni-bacterial *G. catenatum* cultures and 100 days for the mixed-bacterial *G. catenatum* cultures, tracking the different growth phases (Fig 4.1).

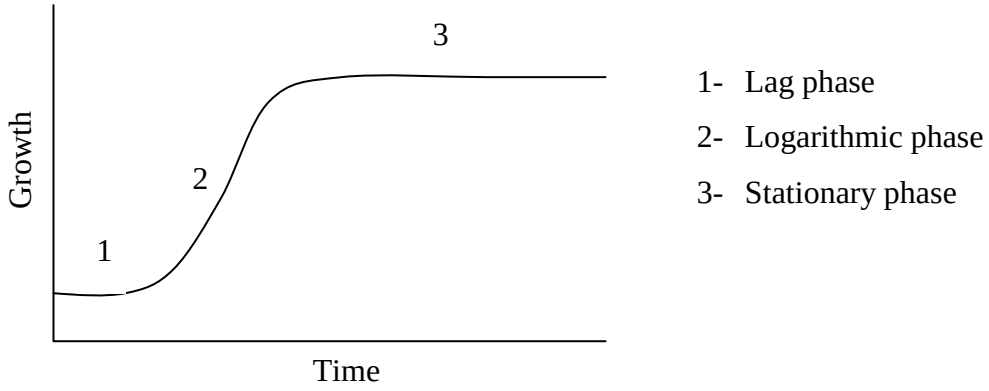


Figure 4.1 Schematic drawing of the 3 growth phases of *G. catenatum*

4.2.2 Calculations and statistical analysis

Specific growth rates (μ) of the cultures were calculated over the different growth phases using the equation:

$$\mu = Ln\ (N2 / N1) / (t2 - t1)$$

where *N1* and *N2* = cell abundabce at time 1 (*t1*) and time 2 (*t2*) respectively (Levasseur et al., 1993). Growth rate expressed as divisions day^{-1} were calculated from specific growth rate using:

$$Div.\ day^{-1} = \mu / Ln2$$

Regression statistics were used to investigate the relationship between algal and bacterial cells, and significant differences between treatments and controls for growth rates were compared using one-way ANOVA with Tukey's post hoc tests in the statistical software R, Version 2.9.0.

4.2.3 Real time PCR quantification of bacteria

Extraction of bacterial DNA from the filters was carried out as described in Chapter 3 section 3.2.2. The qPCR detection was performed on an ABI 7500 Real time PCR system (Applied Biosystems, CA, USA). Reactions were carried out using the TaqMan® Universal PCR Master Mix (Applied Biosystems, CA, USA) in 25 µL volumes according to manufacturer's instructions, containing the components listed in Table 4.1 below, with primers and probes developed from sequences listed in Table 4.2.

Table 4.1 Reagents and concentrations for use in Real-time PCR assays

Reagent	Concentration	Concentration in Thermal cycling reaction	Volume (µL) to be used in reaction
TaqMan® Universal PCR Master Mix	2 x	1 x	12.5
Primer (Forward)	9 mM	0.9 mM	2.5
Primer (Reverse)	9 mM	0.9 mM	2.5
TaqMan® Probe	2.5 mM	0.25 mM	2.5
DNA template	100 ng/ul	10 ng	2.5
Demineralised H_2O	-	-	2.5
Total			**25**

The conditions for thermal cycling consisted of 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min (AmpliTaq Gold® activation), 40 cycles of 15 s at 95 °C followed by 1 min at 60 °C for annealing and extension. At the end of each PCR cycle, fluorescence data was collected, and cycle threshold (C_T) line was automatically calculated by the instrument. Bacterial DNA concentrations were determined by comparison of the threshold cycle number to the standard curves developed in Chapter 3 (sections 3.2.6 and 3.3.3).

Table 4.2 Primers and TaqMan MGB probes used in this study for detection and quantification of *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881. FP = Forward primer, RP = Reverse primer, and P = Probe. Probes labelled with FAM at 5'-end and a minor groove binding non fluorescent quencher (MGBNFQ) at the 3'-end.

Bacterial Species	DNA target	Primer / Probe name	Sequence (5'- 3')
Marinobacter sp. DG 879	16-23 rDNA ITS	FP Mar160F	ACCCAGACCCACCAAAATTG
		RP Mar 223R	GCCCCATTTAAGCTACCTCAAA
		P Mar181P	FAM-TCAACTCCTGGAGTTGAA-MGBNFQ
Alcanivorax sp. DG 881	16-23 rDNA ITS	FP Alc308F	TCTTGCTTGCTTCTGGCTTTT
		RP Alc368R	AAAGAGCTGGCCGAATCATAAA
		P Alc330P	FAM-AGTCAGGTGCCGGAAT-MGBNFQ

4.3 Results

4.3.1 Growth dynamics

Uni-bacterial cultures

The two bacterial species had different growth-responses in the uni-bacterial *G. catenatum* cultures. The *G. catenatum* cultures grown with *Marinobacter* sp. DG879 sustained live *G. catenatum* and *Marinobacter* cells over the entire 230 day experiment (Fig. 4.2).

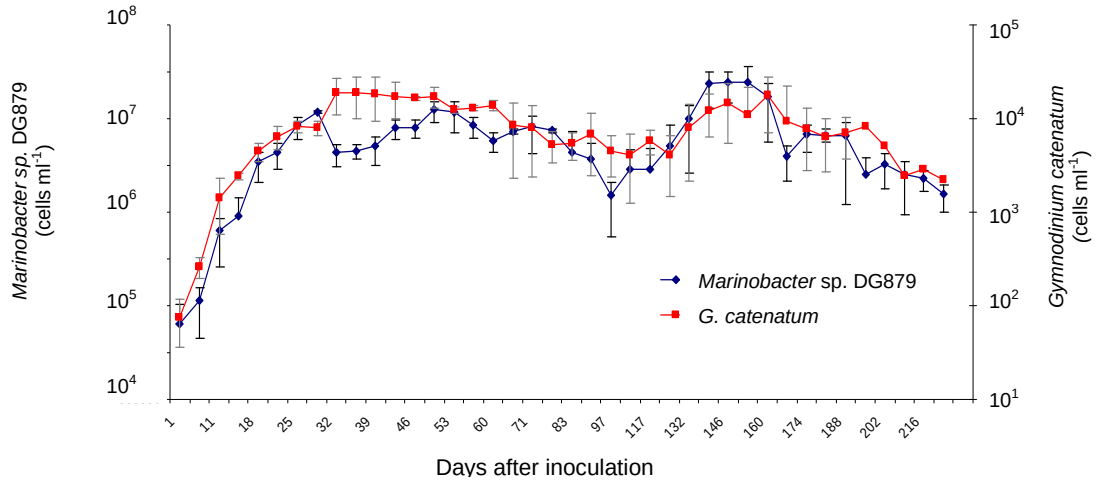


Figure 4.2 Growth of uni-bacterial *Gymnodinium catenatum* cultures grown with *Marinobacter* sp. DG879 over 230 days. Error bars= ±SD, n=3. Red line represents *G. catenatum* and blue line represents *Marinobacter* sp. DG879.

G. catenatum demonstrated an exponential growth phase which appeared to be divided in two stages; one with rapid growth between days 1 and 11 with a growth rate of 0.39 div. day^{-1}, followed by a period of slower growth (0.18 div. day^{-1}) between days 11-25. The growth rate for the entire exponential growth phase (days 1- 25) averaged 0.28 div. day^{-1}. *G. catenatum* then entered a prolonged two-staged stationary growth phase. Between days 25 and 125, *G. catenatum* went through a period of negative growth, followed by a period of cell density increase (0.05 div. day^{-1}) between days 125 to 160. From day 160 until the end of the experiment at day 230, *G. catenatum* cell concentration declined. All three cultures remained viable after the termination of data collection at day 230; and after more than 420 days in culture without any addition of nutrients, *G. catenatum* cell concentrations with 4, 1700, and 2200 cells mL^{-1} in each of the three cultures.

In comparison, *Marinobacter* sp. DG879 demonstrated a relatively stable exponential growth phase between days 1 and 29 with approx. 0.53 div. day^{-1}, followed by a rapid decrease in cell abundance over a 3 day period. The stationary growth phase of *Marinobacter* sp. DG879 varied, creating an increase in cell density between days 32 and 50 (0.17 div.day^{-1}) while *G. catenatum* remained stable, then showed a decline in bacterial cell abundance between days 50 and 97 which also followed a decline in *G. catenatum* cell abundance. This was followed by a rapid increase in bacterial cell

abundance between days 97 to 146 (0.19 div.day^{-1}), and after day 146 the *Marinobacter* sp. DG879 appeared to be entering a death phase. The increase in cell abundance was also apparent in *G. catenatum*, which subsequently entered its decline phase alongside the bacterium. Despite the oscillation of *Marinobacter* sp. DG879 abundance, there is no statistical significance ($p = 0.72$, $F = 0.14$, $df = 5$) between days 25 and 125, the period during which *G. catenatum* went through negative growth.

G. catenatum grown with *Alcanivorax* sp. DG881 showed a growth curve typical of non-axenic *G. catenatum* cultures grown with a mixed-bacterial community (compare Blackburn et al., 1989) following the three classic growth stages; exponential (days 1 -22) with a growth rate of 0.19 div.day^{-1}, a slightly declining stationary growth phase (days 22-53) and death phase (days 53-97) (Fig 4.3). During the death phase, dead *G. catenatum* cells were accumulating on the bottom of the culture vessel.

Between days 1 and 22, *Alcanivorax* sp. DG881 initially went though a slow-growing lag phase (growth rate 0.24 div.day^{-1}) before entering exponential growth at day 22 reaching a growth rate of approx. 0.79 div.day^{-1}. The exponential growth phase of *Alcanivorax* sp. DG881 lasted until day 39, and was shortly followed by a decline in *G. catenatum* cell numbers. The stationary growth phase extended from day 39 to 230, showing limited growth with a growth rate of 0.02 div. day^{-1}. There is no obvious death phase, although a drop of cell abundance can be seen from day 209 onwards (Fig. 4.3).

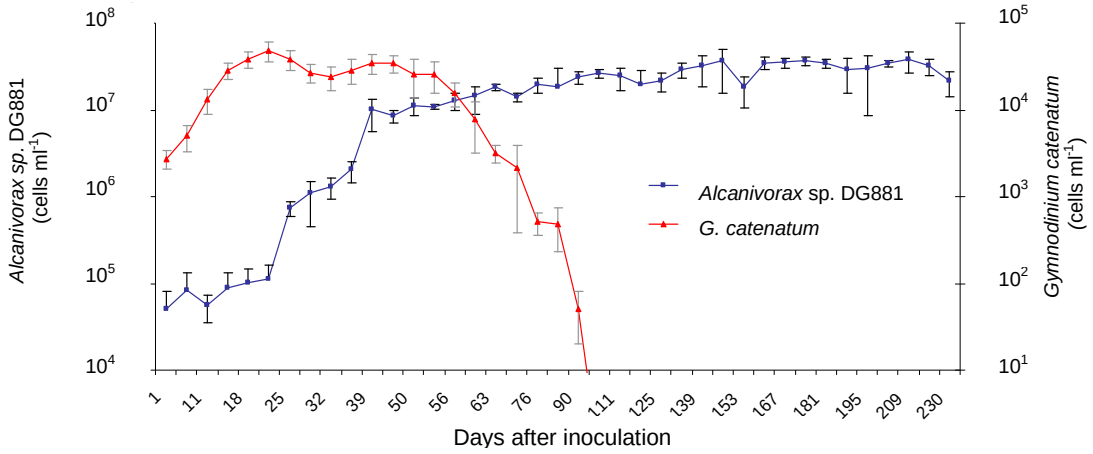


Figure 4.3 Growth of uni-bacterial *Gymnodinium catenatum* cultures grown with *Alcanivorax* sp. DG881 over the 230 day study period. ±SD, n=3. Red line represents *G. catenatum* and blue line represents *Alcanivorax* sp. DG881.

Mixed-bacterial cultures

Alcanivorax sp. DG881 displayed a growth curve with an initial peak in cell abundance between days 7-18 (Fig. 4.4), and although fluctuating, the growth curve shows a general trend of increasing cell numbers throughout the 100 days in the *G. catenatum* mixed-bacterial cultures.

In contrast to *Alcanivorax* sp. DG881, the *Marinobacter* sp. DG879 showed no initial peak in cell abundance during the first 20 days in culture, and with smaller fluctuations in cell numbers between the time points. The general trend of the growth curve is one of constant increase in cell numbers up until day 74, after which a slow decline can be noted (Fig. 4.4).

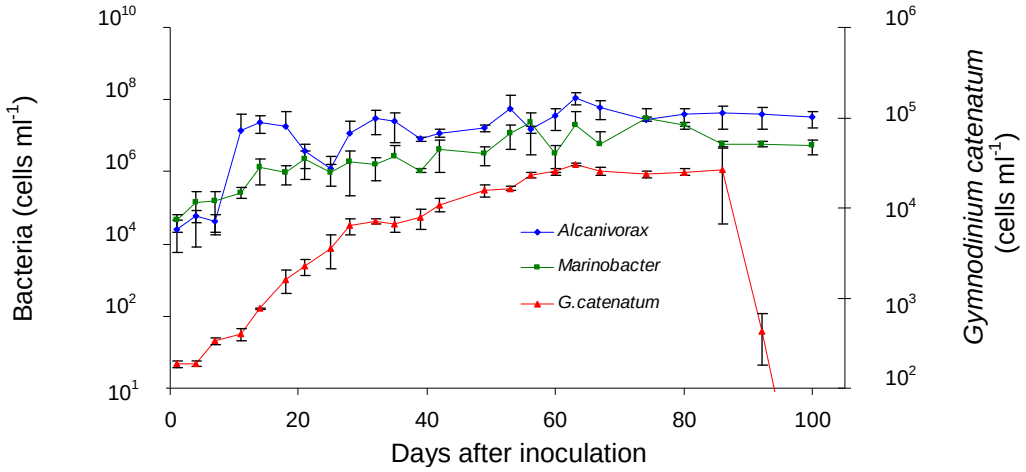


Figure 4.4 Growth of *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 in mixed bacterial *Gymnodinium catenatum* cultures. Error bars displaying ±SD with n = 3. Red line represents *G. catenatum*, blue line represents *Alcanivorax* sp. DG881 and green line represents *Marinobacter* sp. DG879.

4.3.2 Bacterial proportions

The proportion of *Alcanivorax* sp. DG881 to *Marinobacter* sp. DG879 in the mixed-bacterial *G. catenatum* cultures over the course of the mixed bacterial culture cycle is shown in Figure 4.5. Bacterial types were initially added at equal concentrations at the time of cyst germination (approximately 4 weeks prior to start of experiment). At the start of experiment (inoculation of cultures- day 1), *Marinobacter* sp. DG879 was the dominant species in the cultures and showed a higher proportion than *Alcanivorax* sp. DG881 over the first 7 days in culture. After the initial decline in relative abundance, *Alcanivorax* sp. DG881 increased proportionally by day 11 to be the dominant type over a 7 day period, (Days 7-18; Figure 4.5). With the exception of days 56 and 74, *Alcanivorax* sp. DG881 was the numerically dominant bacterial type over the course of the experiment.

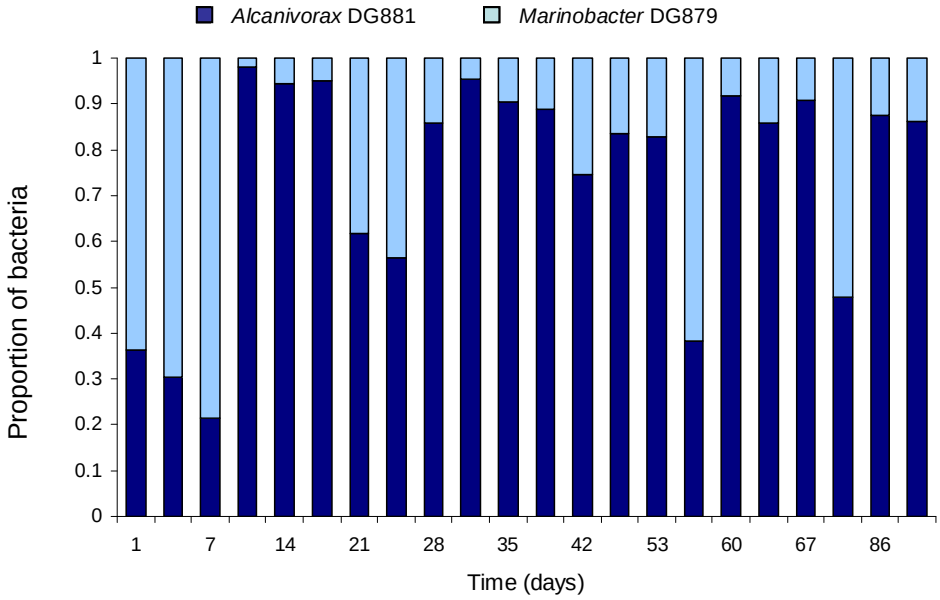


Figure 4.5 Proportion of *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 in mixed-bacterial *Gymnodinium catenatum* cultures over the 100 day study. Dark blue represents *Alcanivorax* sp. DG881 and light blue represents *Marinobacter* sp. DG879.

The proportion of *G. catenatum*-cell associated and non cell-associated bacteria was studied in both bacterial species in the mixed-bacterial *G. catenatum* cultures. Considering *Alcanivorax* sp. DG881 (Fig. 4.6), after an initial 5 day period with the non algal-associated cells being in highest proportion in the mixed-bacterial culture (~97%), *Alcanivorax* sp. DG881 displayed a rapid increase in cell association, showing close to 100% *G. catenatum*-cell association between days 11-18. As *G. catenatum* numbers were increasing after day 18, and following the rapid increase in bacterial attraction to *G. catenatum*, the proportion of *G. catenatum*-cell associated and non cell-associated bacteria changes, increasing the proportion of non cell-associated bacteria up to 98 % during days 28 to 35, and coinciding with the temporarily halted growth of *G. catenatum*. Following the initial large peak of attachment are two smaller peaks, the first starting at day 39, and the second at day 67, during which the percentage of *G. catenatum*-cell associated bacteria reached approx. 30 % of the total amount of bacteria (Fig. 4.6).

The *Marinobacter* sp. DG879 in the mixed-bacterial *G. catenatum* cultures shows a much higher proportion of non cell-associated bacteria to *G. catenatum*-cell associated bacteria (Fig. 4.6). Cell-association of *Marinobacter* sp. DG879 to *G. catenatum*

appears to be occurring only at two separate occasions of the *G. catenatum* growth cycle, first at early exponential phase (days 1-18), then at early decline phase (day 67).

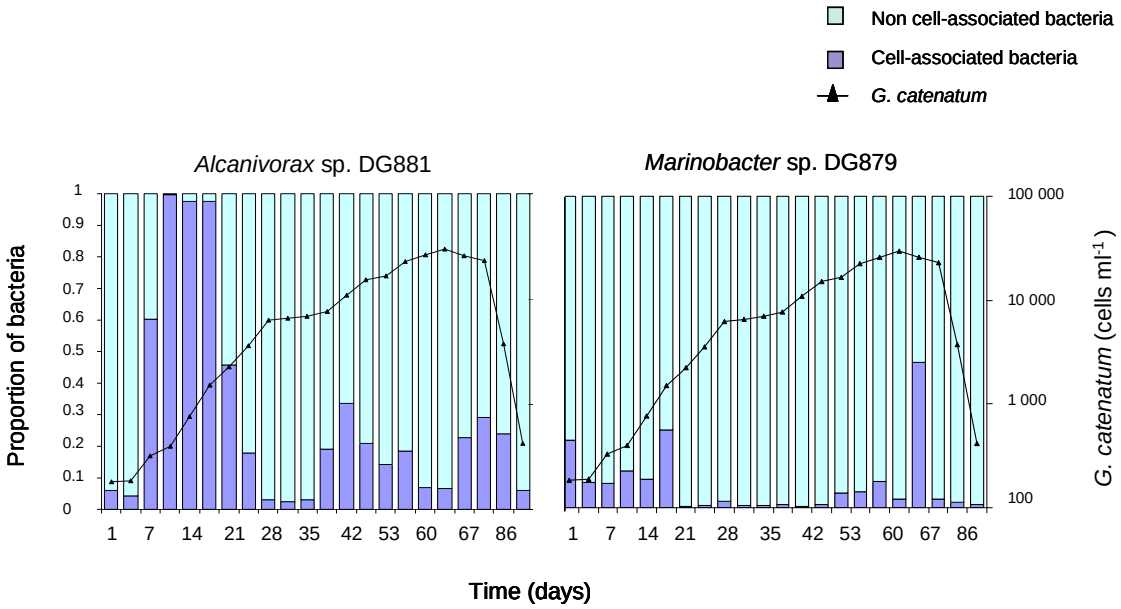


Figure 4.6 Proportion of *Gymnodinium catenatum* cell-associated and non cell-associated *Alcanivorax* sp. DG881 and *Marinobacter* sp. DG879 in the mixed-bacterial *G. catenatum* cultures. Blue represents cell-associated bacteria, light green represents non cell-associated bacteria and the line represents the average growth curve of *G. catenatum*.

4.3.3 Cell concentration correlations

Uni-bacterial cultures – Treatment with *Marinobacter* sp. DG879

Bacterial and algal growth in the uni-bacterial *G. catenatum* cultures with *Marinobacter* sp. DG879 showed a correlation over the exponential and decline phase (Figs 4.7a and c), while the cell abundances showed little correlation in the stationary growth phase (Fig. 4.7b). For statistical summary see Table 4.3.

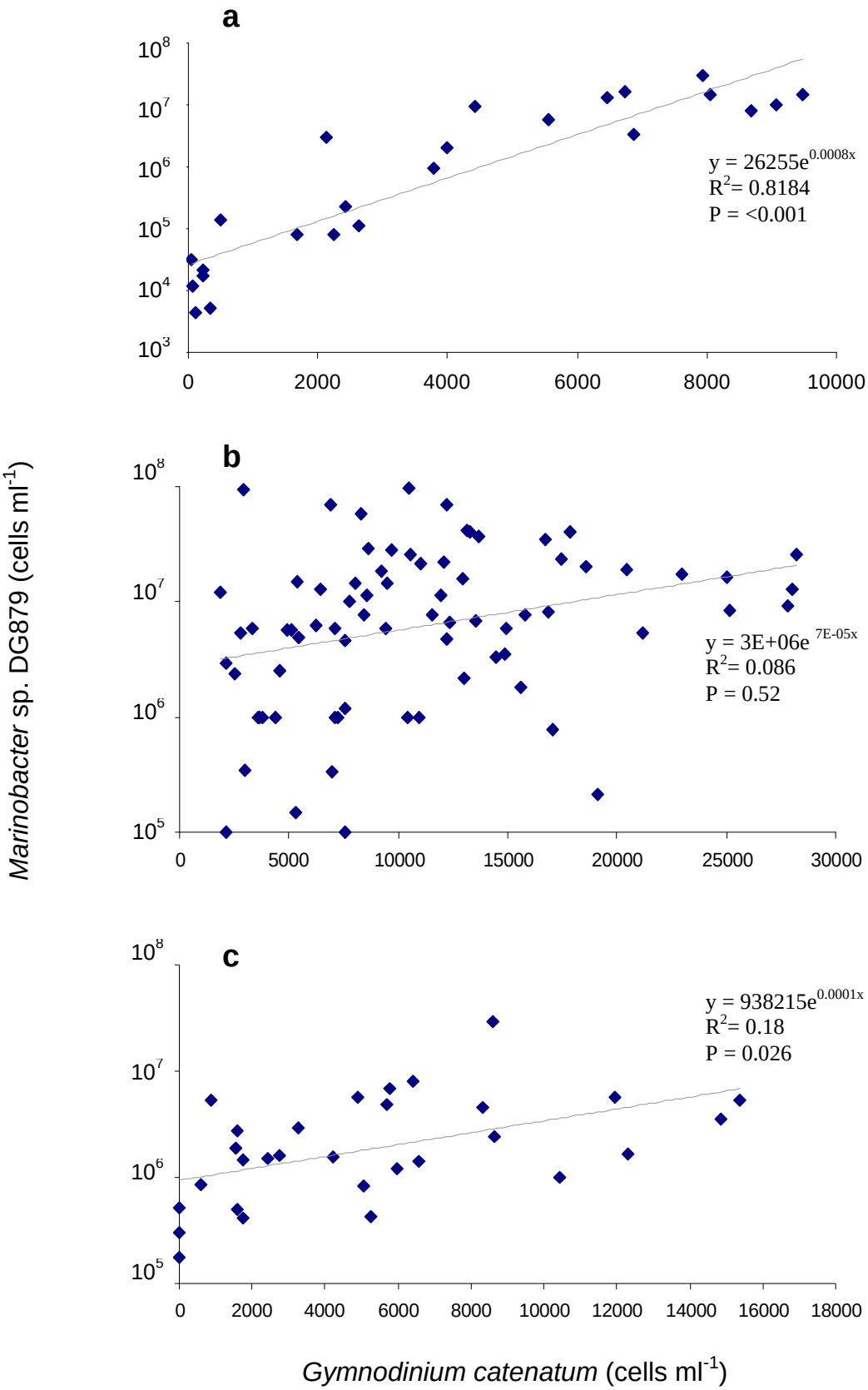


Figure 4.7 Correlation between *Marinobacter* sp. DG879 cells and *Gymnodinium catenatum* cells depending on growth phase of *G. catenatum*. a) Logarithmic growth phase - days 1-25, b) Stationary growth phase - days 25-160, and death phase - days 160-230.

Table 4.3 Regression statistics for algal and bacterial cell correlation in unibacterial cultures.

	G. catenatum with *Marinobacter* sp. DG879			*G. catenatum* with *Alcanivorax* sp. DG881		
	Logarithmic phase (days 1-25)	Stationary phase (days 25-160)	Death Phase (days 160-230)	Logarithmic Phase (days 1-22)	Stationary Phase (days 22-53)	Death Phase (days 53-90)
r	0.77	0.077	0.43	0.12	0.11	-0.48
R^2	0.82	0.08	0.25	0.22	0.024	0.28
df	23.0	71.0	26.0	17.0	26.0	20.0
F	32.88	0.42	5.63	0.24	0.29	5.56
P	<0.001	0.52	0.026	0.63	0.58	0.03

Uni-bacterial cultures – Treatment with *Alcanivorax* sp. DG881

Bacterial and algal growth in the uni-bacterial *G. catenatum* cultures with *Alcanivorax* sp. DG881 show close to no correlation over the logarithmic and stationary growth phase (R^2 = 0.015 and 0.012 respectively; Table 4.3) (Figs 4.8 a and c), while the cell abundances in the death phase showed a negative correlation (R^2 = 0.23; Table 4.3) (Fig. 4.8 b).

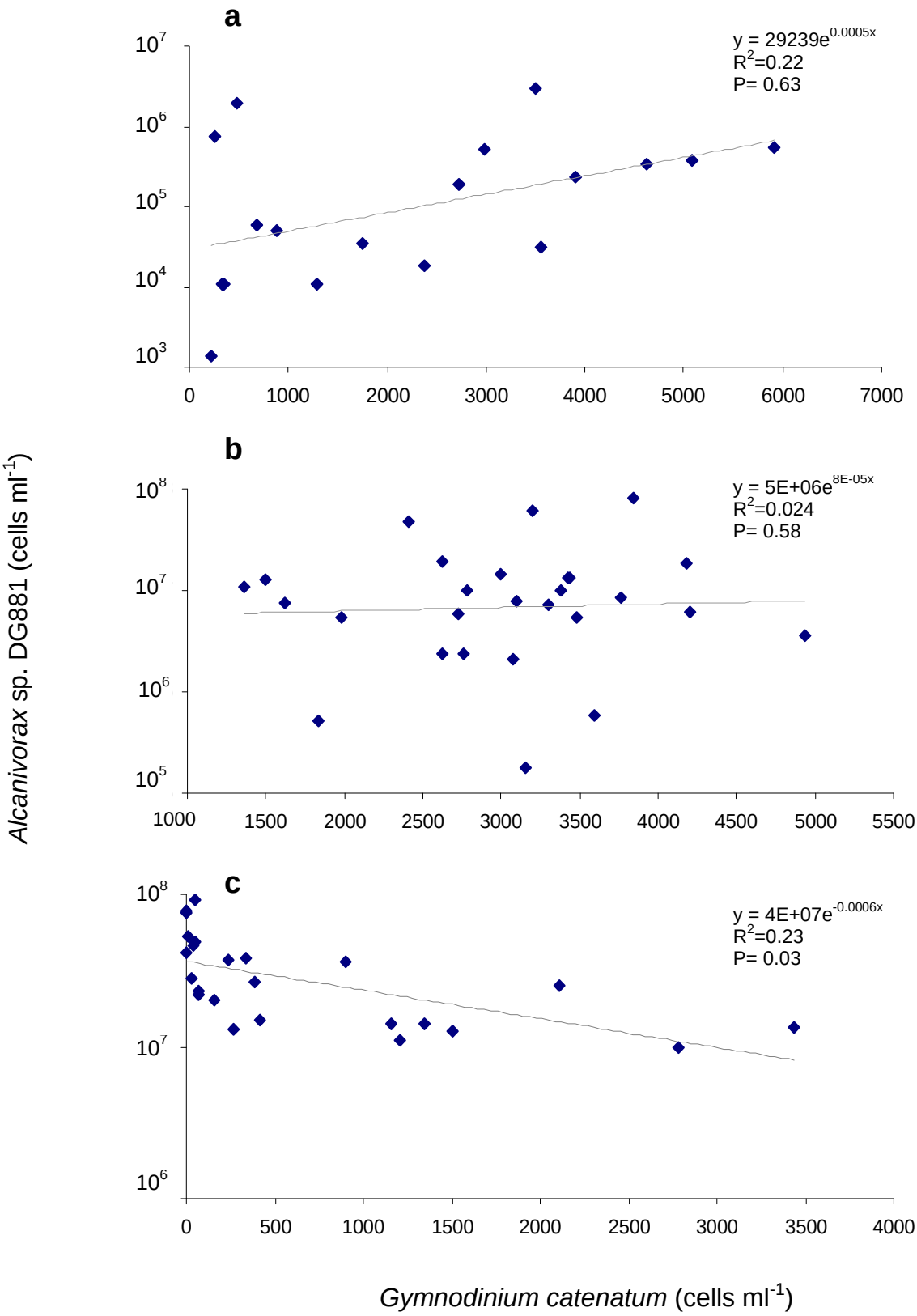


Figure 4.8 Correlation between *Alcanivorax* sp. DG881 cells and *Gymnodinium catenatum* cells depending on growth phase of *G. catenatum*. a) Logarithmic growth phase - days 1-22, b) Stationary growth phase - days 22-53, and c) death phase - days 53-90.

Mixed-bacterial cultures –Treatment with both *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881

Only a slight correlation between algal and bacterial cells can be seen during the *G. catenatum* exponential phase for *Marinobacter* sp. DG879 (R^2 = 0.27) (Fig. 4.9 a; Table 4.4), while the *Alcanivorax* sp. DG881 cell abundances during the same time showed close to no correlation at all with the *G. catenatum* cell numbers (R^2 =0.0006) (Fig. 4.9 a). During the stationary growth phase of *G. catenatum*, both *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 show close to no correlation to the alga, with R^2 values of 0.06 and 0.19 respectively (Fig. 4.9 b).

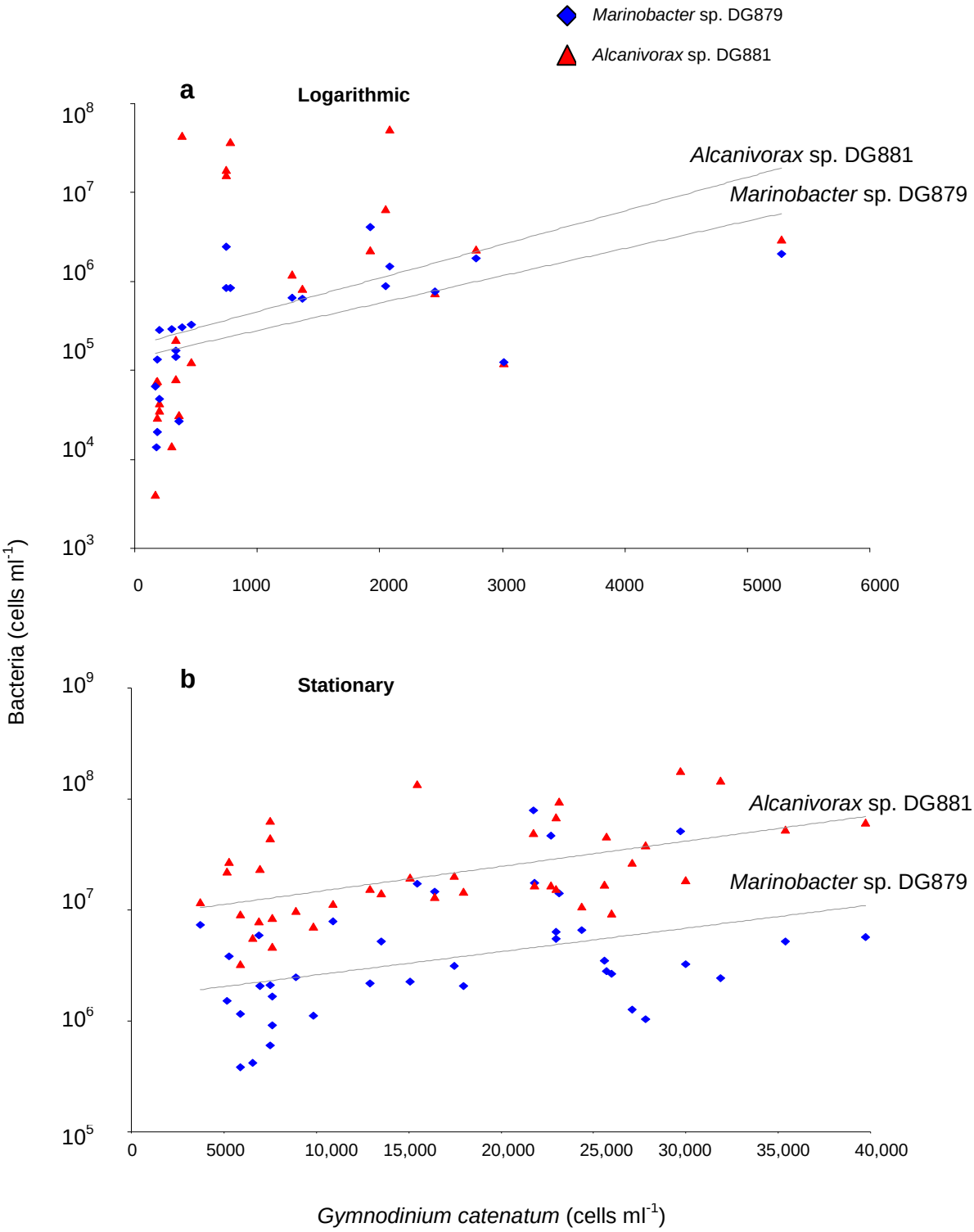


Figure 4.9 Correlation between bacterial and algal cells depending on growth phase of *Gymnodinium catenatum*. a) Logarithmic growth phase- days 1-25 and, b) stationary growth phase- days 25-86. Red triangle symbolises *Alcanivorax* sp. DG881 and blue diamond represents *Marinobacter* sp. DG879.

Table 4.4 Regression statistics for algal and bacterial cell correlation in mixed-bacterial cultures

	G. catenatum with *Marinobacter* sp. DG879		*G. catenatum* with *Alcanivorax* sp. DG881	
	Exponential phase (days 1-25)	Stationary phase (days 25-86)	Exponential phase (days 1-25)	Stationary phase (days 25-86)
r	0.52	0.25	0.023	0.43
R^2	0.27	0.06	0.0006	0.19
P	0.009	0.13	0.91	0.005
F	8.15	2.35	0.012	8.63
df	23	38	23	38

4.3.4 Growth rates

Growth rates of *G. catenatum* in the different treatments

Figure 4.10 shows a summary of the growth rates of *G. catenatum* from all cultures. The mixed-bacteria *G. catenatum* cultures displayed an average exponential growth rate slightly below that of the uni-bacterial cultures (0.18 div.day^{-1} for the mixed bacterial cultures compared to 0.28 div.day^{-1} for the *Marinobacter* sp. DG879 cultures and 0.19 div.day^{-1} for the *Alcanivorax* sp. DG881 cultures, between days 1-25 (See Fig. 4.10) There was a significant difference between the exponential growth rate of the cultures treated with *Marinobacter* sp. DG879 in comparison to the two other treatments, while there was no significant difference between the *G. catenatum* stationary growth rates of the different treatments. The death phase of the different treatments showed to vary, again with the *Marinobacter* sp. DG879 treatment being significantly different to the other two treatments.

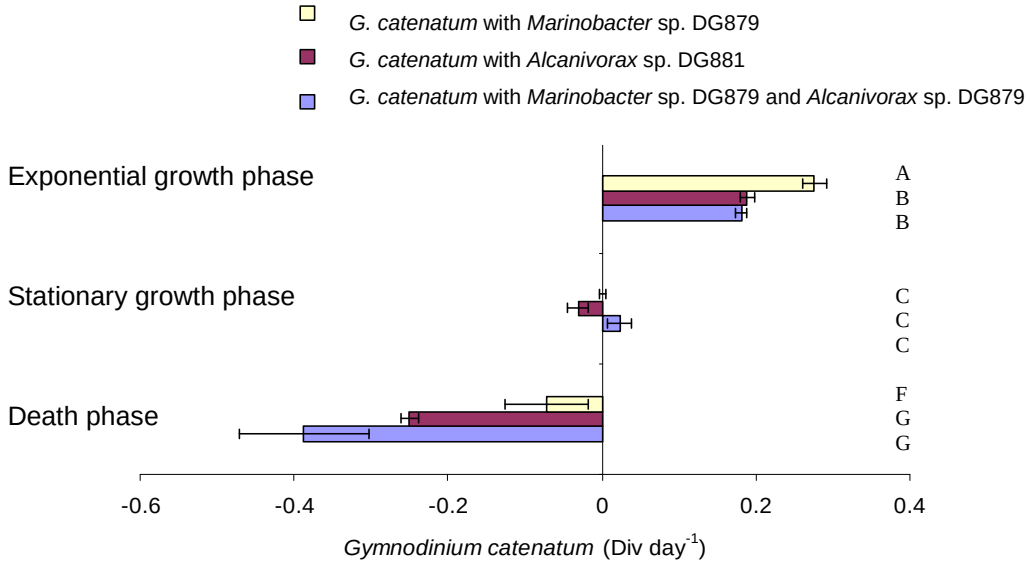


Figure 4.10 Growth rates (div day^{-1}) of *Gymnodinium catenatum* growth phases across the three studies. Significantly different means are indicated by letters on the right of figure; the same letter indicates not significantly different.

Growth rates of the bacteria in the different treatments

Statistical analysis of the bacterial growth rates in the different cultures showed that significant differences were only found in the exponential growth phases (Fig. 4.11). For the exponential growth rates, no significant difference was found in the between *Marinobacter* sp. DG879 in mixed-bacterial culture and *Marinobacter* sp. DG879 in uni-bacterial culture ($p = 0.07$, $F = 5.55$, $Df = 5$), along with *Alcanivorax* sp. DG881 in the uni-bacterial cultures and *Marinobacter* sp. DG879 in the uni-bacterial cultures ($p = 0.07$, $F = 6.14$, $Df = 5$). All others were significantly different. Table 4.5 shows a summary of the statistical values from all cultures.

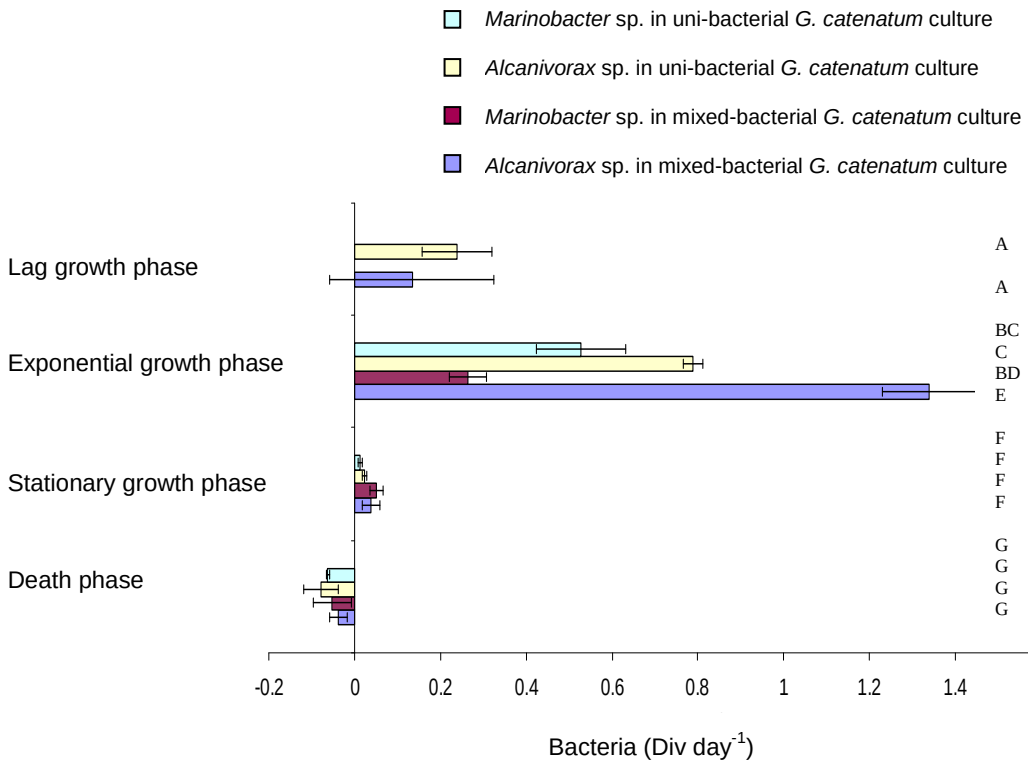


Figure 4.11 Growth rates (div day^{-1}) of *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 across the different growth phases in the three studies. Significantly different means are indicated by letters on the right of figure; the same letter indicates not significantly different.

Table 4.5 Comparison of statistical values for the bacterial (exponential) growth rates in the different uni-bacterial and mixed-bacterial *Gymnodinium catenatum* cultures.

	Marinobacter sp. in uni-bacterial culture	*Alcanivorax sp.* in uni-bacterial culture	*Marinobacter sp.* in mixed-bacterial culture	*Alcanivorax sp.* in mixed-bacterial culture
Marinobacter sp. in uni-bacterial culture		*p = 0.07* *F = 6.14* *Df = 5*	*P = 0.07* *F = 5.55* *Df = 5*	*p = 0.006* *F= 28.9* *Df = 5*
Alcanivorax sp. in uni-bacterial culture			*P = 0.0004* *F = 119.3* *Df = 5*	*p = 0.008* *F = 23.9* *Df = 5*
Marinobacter sp. in mixed-bacterial culture				*p = 0.0008* *F = 82.7* *Df = 5*
Alcanivorax sp. in mixed-bacterial culture				

4. 4 Discussion

4.4.1 Growth dynamics of *Marinobacter* sp. DG879

The growth dynamics of the two bacteria when grown in uni-bacterial cultures of *Gymnodinium catenatum* were substantially different. This suggests that the nature or mechanism of the interaction with *G. catenatum* may be fundamentally different, and could have been related to the type of direct or indirect interaction (in terms of being cell-associated or free-living.

In uni-bacterial *G. catenatum* cultures, *Marinobacter* sp. DG879 show a growth pattern closely linked to that of *G. catenatum*, suggesting that the two organisms have close interactions. How the connection between the two works is yet to be determined, however recent research (Amin et al., 2009) using experimental model systems with *Scrippsiella trochoidea* found *Marinobacter* sp. DG879 to produce Vibrioferrin, a siderophore (compound that binds iron and increases its solubility) which can promote algal assimilation of iron used for photosynthesis and respiration. Other research, however, based on field populations, suggests that there is potentially a release of algal growth-promoting compounds by the bacteria (e.g. Furuki and Koboyashi, 1991, Liu et al., 2008). It is well documented that phytoplankton DOC which serves as an energy source for microheterotrophic organisms (Larsson and Hagström, 1979). Møller-Jensen (1983) found that EOC (such as amino acids, monosaccharides, polyalcohols and volatile organic acids) released from phytoplankton are predominantly of a low molecular weight (<900 Daltons), and that bacteria show a distinct selectivity for uptake and utilisation of these small molecules. As *Marinobacter* sp. DG879 is a hydrocarbon degrader (Green et al., 2004) able to survive solely on hydrocarbons such as tetra-decane (Green et al., 2006), it is possible that production of hydrocarbons by *G. catenatum* (Hallegraeff et al., 1991) is stimulating *Marinobacter* sp. DG879 growth.

Research by Subramanian (2008) showed that re-addition of bacterial cultures (*Marinobacter* sp. DG879 and *Brachybacterium* sp.) to 32 weeks old *G. catenatum* cultures enhanced algal growth with a 47-90 % increase in cell numbers, suggesting that there is a gradual loss of growth stimulating capacity of the bacteria over time, possi-

bly as a response to a loss of production of dissolved products. Similarly, Bolch et al. (unpubl. data) have found that excystment and growth of *G. catenatum* cysts was stimulated when a 0.2 µm culture filtrate of *Marinobacter* sp. DG879 was added to *G. catenatum* cysts on a regular defined basis, allowing cyst germination and subsequent vegetative division to be ongoing for as long as the filtrate was regularly added. Results from pilot studies (results not shown) have shown that a single addition of 0.2 µm culture filtrate from a culture of *Marinobacter* sp. DG879, added to the resting cysts immediately after sterilisation, is adequate for the excystment of the resting cysts, whereas continued growth of the produced vegetative cells is not possible without it. This suggests that the growth-compound produced by the bacteria is either used or degraded by *G. catenatum* or the bacterium, or possibly degrades through some other non-biological mechanisms (e.g. light or chemical degradation, see Amin et al., 2009) and if not re-added, *G. catenatum* will not survive.

In this study, the two populations of *Marinobacter* sp. DG879 and *G. catenatum* appear to be positively influenced by one another, allowing *G. catenatum* to sustain growth in batch cultures beyond the typical batch culture life span. Blackburn et al. (1989) found batch cultures of *G. catenatum* to remain viable for up to 80 days (60 mL cultures maintained at 150 µmol PAR m^{-2} sec^{-1} at 17.5 ˚C), whereas *Marinobacter* uni-bacterial *G. catenatum* cultures remained viable for > 230 days in this study. The maximum *G. catenatum* cell densities (~ 2.0×10^4 cells mL^{-1}) found in this study while cultured with *Marinobacter* sp. DG879 are higher in comparison to those found in the Blackburn et al. (1989) study (2.0×10^3 cells mL^{-1}). One reason behind the higher *G. catenatum* cell densities found in this study could be due to the lack of antagonistic bacteria found in typical non-axenic *G. catenatum* cultures (Skerratt et al., 2002; Green et al., 2004), that are absent in the uni-bacterial culture systems used here.This is to our knowledge the first time that *G. catenatum* has been shown to remain as a viable culture for a period of >420 days without the addition of any nutrients after the initial inoculation.

4.4.2 Growth dynamics of *Alcanivorax* sp. DG881

The *G. catenatum* cultures grown with *Alcanivorax* sp. DG881 show similar stationary- and death-phase dynamics to non-axenic cultures with complex bacterial com-

munities (Blackburn et al., 1989) and also reaching maximum cell densities comparable to those found by Blackburn et al. (1989). The mechanism of interaction between *G. catenatum* and *Alcanivorax* sp. DG881 in comparison to that of *G. catenatum* and *Marinobacter* sp. DG879 is different, suggesting that *Alcanivorax* sp. DG881 does not produce growth-substances similar to those produced by *Marinobacter* sp. DG879. However, *Alcanivorax* sp. DG881 is still, on its own, capable of stimulating cyst germination and vegetative division of *G. catenatum* resembling that of a 'normal' mixed bacterial culture. In comparison to the axenic control cultures which were incapable of cyst germination, results suggest that *Alcanivorax* sp. DG881 produce a growth factor which is stimulating *G. catenatum* cell division and growth.

Considering the growth of *Alcanivorax* sp. DG881 in the uni-bacterial cultures; hypothetically, as the *G. catenatum* cells die, an increase in particulate matter could become available in the culture (Danovaro et al., 2003; Pinhassi et al., 2004). This increase may have allowed the bacteria to maintain the slowly increasing cell numbers for some time, but it appears unlikely that it would sustain growth for a period of more than 130 days, which is the case in this study. Like *Marinobacter* sp. DG879, *Alcanivorax* sp. DG881 is a known hydrocarbon degrader (Green et al, 2004); and it is possible that production of such compounds by *G. catenatum* (Hallegraeff et al., 1991) is stimulating bacterial growth. Unfortunately no measurements were taken to establish hydrocarbon production in the cultures during the experiment.

It is possible that the nature of the interaction between *G. catenatum* and *Alcanivorax* sp. DG881 changes once *G. catenatum* enters stationary phase. Figure 4.3 shows *Alcanivorax* sp. DG881 in a lag phase coinciding with *G. catenatum* exponential growth phase, and as *G. catenatum* then enters stationary growth phase, *Alcanivorax* sp. DG881 follows, entering its exponential growth phase. The same lag phase of *Alcanivorax* sp. DG881 can be seen in the mixed-bacterial *G. catenatum* culture (Figure 8), although in this case, the exponential growth of *Alcanivorax* sp. DG881 starts simultaneously to the exponential growth phase of *G. catenatum*. This change in behaviour of *Alcanivorax* sp. DG881 could be due to a change in behaviour and / or the metabolism of *Alcanivorax* sp. DG881. Concentration-dependent changes in gene expression by bacteria are well known. Quorum sensing is used by bacteria to

coordinate certain behaviors based on the local density of the bacterial population, and can regulate a host of different processes, basically serving as a communication network (Miller and Bassler, 2001). Particularly among the Proteobacteria (gram-negative bacteria), cell-cell communication uses hormone-like molecules called auto inducers, which are released externally and act as messengers between cells (Zhang & Dong 2004). As the concentration of auto inducers grow due to population growth, bacteria are able to detect when the signals reach a critical level. Bacteria are then stimulated by these signals to take action, usually by inducing or repressing certain genes (Waters and Bassler, 2005; Whitehead et al., 2001). N-acyl-homoserine lactone (AHL) signals is one of the known mechanisms by which the bacteria can communicate with each other (Fuqua and Greenberg, 2002; Daniels et al., 2004) and AHL has also been found in dinoflagellate-associated bacteria (e.g. Nakashima et al., 2006). It may be that production of specific compounds produced by *G. catenatum* after entering the stationary growth phase in turn affects *Alcanivorax* sp. DG881, which then send off chemical signals for increased bacterial growth.

It may also be that by the time *Alcanivorax* sp. DG881 enters exponential growth phase, there is a change in the bacterial gene expression, which then may alter the bacterial metabolism in a way that no longer can support *G. catenatum* growth. *Alcanivorax* sp. DG881 is therefore no longer stimulatory to *G. catenatum* throughout the whole batch culture cycle, possibly even becoming inhibitory at higher concentrations. Parallels can be found in other studies where bacteria have been shown to be capable of losing or switching off their algicidal ability, suggesting that a presumed non-algicidal bacterial species could become algicidal once the bacteria reach a specific cell density (Skerratt et al., 2002). Skerratt et al. (2002) found that many bacteria did not have the AHL mechanism or communication, but instead used an alternative pathway, the AI-2, (Schauder et al., 2001) which may play a role in switching on algicidal activity in gram-negative bacteria.

4.4.3 Growth dynamics of *G. catenatum*

G. catenatum showed different growth dynamics in the uni-bacterial systems compared to the mixed-bacterial systems. This has also been found by Subramanian (2008), indicating that the composition of the bacterial community has an influence

on the growth of the algae. Similar to the results from Subramanian (2008), the controlled mixed-bacterial *G. catenatum* cultures show an intermediate growth pattern of *G. catenatum*, suggesting that the different bacterial types can influence the growth pattern of the algae even in the presence of other bacteria.

In the case of *G. catenatum* and its relationship to *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881, further studies are required to determine the physical distribution of bacteria, perhaps using laser-scanning confocal microscopy. Research by Rooney-Varga et al. (2005) indicated that a higher intensity of interaction was found between algae and attached bacteria than between algae and free-living bacteria, and several studies have proposed that the spatial proximity between bacteria and algae may play a role in toxin production (e.g. Franca et al., 1996; Gallacher et al., 1997). Consequently, an understanding of the spatial relationship between the two may show to be useful in determining the complex nature of the interaction between algae and bacteria.

To our knowledge, research on the bacterial composition of naturally occurring *G. catenatum* blooms has not been done; however research on the bacterial community of laboratory-grown *G. catenatum* has shown a remarkable similarity to the bacterial communities of other laboratory-grown marine dinoflagellates (Alavi, et al., 2001; Hold et al., 2001; Green et al., 2004). Among the most notable members of these communities were bacteria phylogenetically affiliated with the *Roseobacter* or the *Marinobacter* clade (Kodama et al., 2006) and a recent study (Amin et al., 2009) detected *Marinobacter* spp. in 83% of the 18 dinoflagellate cultures examined. There is only a limited amount of research on algal-bacterial interactions in naturally occurring blooms, however, *Marinobacter* sp. has been found in association with blooms of *Alexandrium fundyense* (Hasegawa et al., 2007), and the evidence of *Marinobacter* spp. co-occurring with a range of laboratory-grown dinoflagellates suggests that other dinoflagellates may also have an obligate growth requirement for a certain bacteria, and possibly associated with these bacteria in the natural environment.

4.4.4 Nature of bacterial interactions with *G. catenatum*

The higher proportion of *Alcanivorax* sp. DG881 associated with the dinoflagellate cell may be due to *Alcanivorax* sp. being a non-motile bacterium that uses "twitching motility", a special kind of bacterial surface translocation using the fimbriae to attach and colonise surfaces (Henrichsen, 1983; Yakimov et al., 1998). The initial increase of attachment shown by *Alcanivorax* sp. DG881 could be a response to increasing *G. catenatum* numbers, but most likely as a response to the inoculation of the culture. The bacterial cells were transferred from a potentially nutrient-rich environment with a sufficient amount of organics from *G. catenatum* growing in late logarithmic phase, to a system where the organics are diluted and the *G. catenatum* concentration is an order of magnitude lower. Bacterial chemotaxis similar to this, when bacteria are being subjected to low organic nutrient availability, has been found in several other bacterial species; e.g. *Pseudoalteromonas* sp. attracted to *Heterosigma akashiwo* (Seymour et al., 2009) and *Pseudoalteromonas haloplanktis* showing chemotaxis towards *Dunaliella tertiolecta* (Stocker, et al., 2008). *Alcanivorax* sp. tend to congregate at the oil/water interfaces (Martins dos Santos et al., 2010), so it appears likely that possible exopolysaccarides produced by *G. catenatum* creates a surface suitable for *Alcanivorax* sp., in which a higher concentration of hydrocarbons are available for bacterial utilisation. As the concentration of organics in the experimental cultures theoretically increase over time, *Alcanivorax* sp. DG881 appear to be reverting to a more pelagic existence, no longer closely associated to the *G. catenatum* cells. This could be as a response to the now more sufficient concentration of organic compounds available in the culture media.

Marinobacter sp. DG879 has a flagellum for motility (Gauthier et al., 1992), which may be an explanation for the generally high number of non cell-associated *Marinobacter* sp. DG879 seen in Figure 4.7. Like *Alcanivorax* sp. DG881, *Marinobacter* sp. DG879 show an increase in attachment at the beginning of the experiment, but in comparison to *Alcanivorax* sp. DG881, the amount of cell-associated cells is much less and the period during which attachment is seen is much shorter. The early-on attachment seen in both bacterial species could be a response to low production and release of DOM by the algae, necessitating the bacteria to come in close and attach.

Alternatively, it might be that the algae encourage the bacteria to attach, similar to the process observed in legume-*Rhizobium* symbiosis during initiation of root nodule formation (Garg and Garg, 2007). Although it might not be signalling, the algal cell may limit release of certain DOC fractions in order to bring the bacteria closer, allowing the algal cell to benefit from bacterial siderophore production (Amin et al., 2009). After the period of close to no cell-associated bacterial cells, a slow increase in attachment is notable, starting at day 53. This could perhaps be a response to a reduced release of compounds essential to the *Marinobacter* sp. DG879 cells (like in *Alcanivorax* sp. DG881), forcing *Marinobacter* sp. DG879 to come in closer. As *G. catenatum* enters the declining growth phase, lysis of the cells release organic compounds into the media, allowing for an increase in the non cell-associated bacteria (Cole, 1982), while a loss of surface areas also lead to less algal cell-associated bacteria.

4.4.5 Algal and bacterial cell concentration correlations

In the uni-bacterial cultures, there was a correlation between the concentration of *G. catenatum* and *Marinobacter* sp. DG879 cells at the exponential and death growth phases. This could indicate that the bacterial growth is reliant upon something produced by living, intact *G. catenatum* cells, rather than some constituent that is released during cell lysis and death (like for *Alcanivorax* sp. DG881). Exudates from actively growing phytoplankton are often dominated by a wide range of low molecular weight compounds, including amino acids with high amounts of nitrogen (Mague et al., 1980; Søndergaard and Schierup, 1982), so it seems likely that a production of these compounds could be what stimulates growth of *Marinobacter* sp. DG879 (Huu et al., 1999; Shieh et al., 2003). The gradient of the slopes of the correlation graphs are indicative of the magnitude of the impact between the algae and bacteria, where a larger gradient indicate a higher bacterial to algal density ratio. Bacterial density correlating to algal density has previously been shown by other studies (e.g. Gurung et al., 1999), in which they found the ratio to be substantially changed depending on light and nutrient availability, with bacterial: algal ratio increasing with increasing light intensity. These results are comparable to the logarithmic and stationary growth phases of *G. catenatum* grown with *Marinobacter* sp. DG879, where we see a higher

bacterial: algal ratio in the non light-limited logarithmic cultures, in comparison to the possibly light-limited cultures in stationary growth phase.

Contrary to the *Marinobacter* sp. DG879 treatment, the *G. catenatum* cultures treated with *Alcanivorax* sp. DG881 show a negative correlation in the death phase, indicating that bacterial growth is supported by something produced or exuded during cell lysis. This trend of a peak in microbial activity occurring during the death-phase of an algal bloom or culture has been known for decades (e.g. Cole, 1982) and has been found between several organisms, for example the ciliate *Mesodinium rubrum* and *Vibrio* sp. (Romalde et al., 1990). Studies on *G. catenatum* with *Alcanivorax* sp. DG881 (Subramanian, 2008) showed the same trend of increasing bacterial biomass with decreasing algal concentration in stationary phase. The increase of bacterial growth leading to a decline of phytoplankton cells could be associated with bacteria competing with the algal cells for nutrients (Bratbak and Thingstad, 1985).

4.4.6 Growth rates of *G. catenatum* and bacteria

The exponential growth rates of *G. catenatum* in all three treatments were slightly higher than what was reported earlier by Subramanian (2008). This study found *Marinobacter* sp. DG879 treated *G. catenatum* to reach a growth rate of on average 0.28 day^{-1}, *G. catenatum* treated with *Alcanivorax* sp. DG881 to reach approx. 0.19 day^{-1}, and the mixed bacterial treatment approx. 0.18 day^{-1}, this in comparison to the previous study were growth rates were 0.18, 0.12, and 0.15 day^{-1} respectively. The methods of the two studies were close to identical, although Subramanian (2008) used a *G. catenatum* strain cross between GCDE08 and GCHU11 and maintained cultures at a light intensity of 90 ± 10 µmol PAR m^{-2} s^{-1}, while this study used GCDE06 and GCHU11 and 65 µmol PAR m^{-2} sec^{-1}, which may possibly explain the differing growth rates. The growth rate of the *Marinobacter* sp. DG879 treated cultures is also comparable to that found by Blackburn et al. (1989) of approx. 0.25-0.3 div. day^{-1}, at optimal growth, for the GCDE06 culture.

Both the exponential growth rate and the death rate of the *Marinobacter* sp. DG879 treated *G. catenatum* was significantly different in comparison to the other two treatments, again demonstrating that *Marinobacter* sp. DG879 have a clear effect on

the growth dynamics of *G. catenatum*. The high rates of the death phase of the *G. catenatum* cultures, particularly in the mixed bacterial cultures, could be a result of the high amount of bacteria in the cultures, possibly reaching levels were they become toxic, or simply outcompeting *G. catenatum* in terms of nutrients. Bratbak and Thingstad (1985) found that a mineral nutrient limitation stimulated the excretion of extracellular organic carbon (EOC) by the algae, and that the subsequent bacterial growth on the excreted EOC in turn required an additional uptake of mineral nutrients by the bacteria. As a result, the algae being stressed by lack of mineral nutrients responded in a manner whereby they stimulated their competitors for the lacking nutrients. Possibly, in the mixed - bacterial *G. catenatum* cultures, the sheer amount of bacteria is utilising the remaining available mineral nutrients at a very high rate, thereby causing the rapid death of *G. catenatum*.

Bacterial growth rates in this study were shown to vary greatly in comparison to the growth rates found by Subramanian (2008). The lag growth phase of *Alcanivorax* sp. DG881 that was seen in both the uni-bacterial and mixed-bacterial cultures in both studies reached a rate of 0.02 - 0.05 day^{-1} (between days 1-28) in the study by Subramanian, while this study found a rate of 0.13 - 0.24 day^{-1} (between days 1-22 in the uni-bacterial cultures, and days 1-7 in the mixed-bacterial cultures). Also the exponential growth rates differed, with Subramanian finding rates between 0.16 - 0.23 day^{-1} across all her treatments, while this study displayed a larger variation between treatments, showing exponential growth rates between 0.24 and 1.34 day^{-1}. The differing growth rates between the two studies could possibly be explained by a range of factors; 1) Bacterial concentration was in this study determined by real-time PCR while Subramanian (2008) estimated bacterial numbers (CFU mL^{-1}) from serial-dilution plating, 2) The cell concentrations of *G. catenatum* were different in the two experiments, likely affecting both the bacterial and algal growth rates; and 3) This study used the *G. catenatum* strain GCDE06 while Subramanian (2008) used GCDE08, suggesting that the two algal strains are affecting the bacterial growth in different ways.

Conclusion

This study found a considerable variation in the growth dynamics of *G. catenatum* depending on the associated bacterial species, and showed *Marinobacter* sp. DG879 to be supporting *G. catenatum* single batch culture growth for what I believe to be the longest period of time on record. Because of the tight linkage between growth and cell concentration of *G. catenatum* and *Marinobacter* sp. DG879, and the absolute requirement for *Marinobacter* sp. DG879 for *G. catenatum* growth in the laboratory (Bolch et al., 2004), it is possible that detection of *G. catenatum*-associated bacteria such as *Marinobacter* sp. may facilitate early detection of *G. catenatum* blooms. The next step will be to confirm the presence and dynamics of *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 associated with naturally occurring blooms of *G. catenatum* (Chapter 5).

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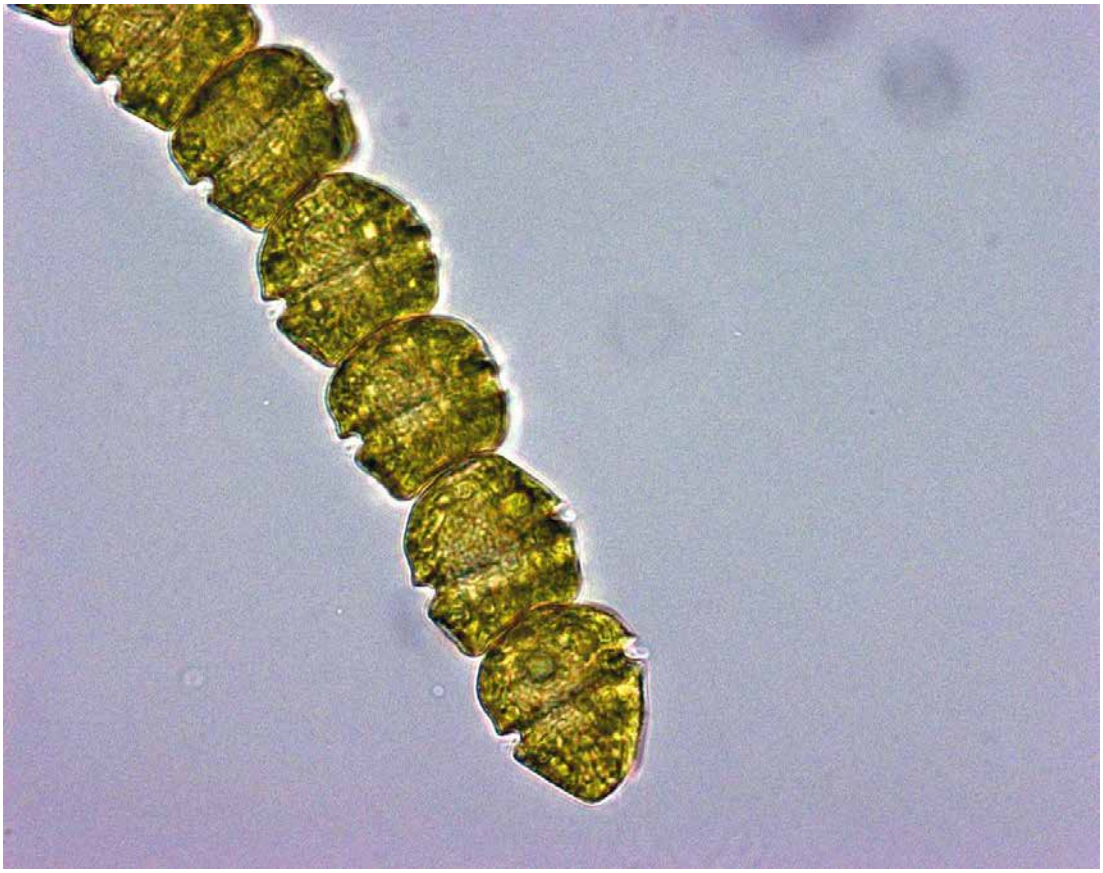
Chapter 5:

Use of real-time PCR probes for detection of *Gymnodinium catenatum* growth-promoting bacteria in field samples



Chapter 5: Use of real-time PCR assays for detection of *Gymnodinium catenatum* growth-promoting bacteria in field samples

5.1 Introduction

Blooms of the paralytic shellfish toxin (PST) producing dinoflagellate *Gymnodinium catenatum* have been occurring in Tasmanian waters since the 1980s (Hallegraeff et al., 1989), and since then, the local shellfish industry has undergone a Shellfish Quality Assurance Program with farm closures due to elevated toxin levels in the shellfish meat, causing financial loss to the industry.

Studies of bloom dynamics along with the discovery of extensive cyst beds in the Derwent and Huon Estuaries (Bolch and Hallegraeff, 1990) suggests that blooms are autochthonous, i.e originating from within the area (Hallegraeff et al., 1995). Blooms of *G. catenatum* have been associated with a range of environmental variables, and the study by Hallegraeff et al. (1995) suggests that blooms can only develop within certain environmental constraints. These constraints include: 1) a seasonal temperature window from January to June where water temperatures must exceed 14˚C for initation and development of blooms; 2) extended periods of low wind stress maintaining a calm stable water column; 3) the incidence of freshwater input after rainfall contributing organic and inorganic growth factors, and; 4) the capacity of *G. catenatum* to vertically migrate. The relationship between wind data and water column stability leading to bloom formation is however complex. Contrary to Hallegraeff et al. (1995), the CSIRO Huon Estuary Study Team (2000) instead suggested that strong winds may resuspend sediments from shallow regions of the estuaries and thereby enhance resting cyst germination and subsequent blooms by increasing the oxygen levels they are exposed to. Nevertheless, neither the findings of the CSIRO Huon Estuary Study Team, nor those of Hallegraeff et al. (1995) were able to determine the factors or conditions that in some years sporadically appeared to be prohibiting the *G. catenatum* blooms.

Successful prediction of algal blooms, in terms of location, magniture and duration, would greatly assist in mitigating their effect on aquaculture industry; and by using biogeochemical modeling, which examines the physical circulation of water coupled with biotic and abiotic elements in the specific ecosystem, prediction of algal blooms can be achieved to a certain degree (Wild-Allen et al., 2010). However, research has shown that interactions between algae and bacteria play a significant role in the life cycles of the algae (for reviews see Doucette, 1995; Doucette et al., 1998), but due to the limited knowledge of these complex biological interactions, they are not yet being incorporated into models. By studying the relationship between algae and bacteria, both in natural and laboratory systems, it should be possible to decipher the interactions, making the biological models more robust, and, in turn, enhance the predictability of approaching bloom events.

Laboratory-grown cultures of *G. catenatum*, initially isolated from Tasmanian waters, co-exist with complex marine bacterial communities that appear to support growth of the dinoflagellate (Bolch et al., 2004). Within this community, two particular strains of bacteria, *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 are capable of supporting the growth of the dinoflagellate in the absence of other bacteria (Bolch et al., 2004; Green et al., 2004) suggesting that these bacteria may also play a potentially important role in the naturally occurring *G. catenatum* populations of southeastern Tasmania. Using the qPCR approaches developed in Chapter 3, this study examined the abundance of *Alcanivorax* and *Marinobacter* genotypes associated with natural *G. catenatum* populations in southern Tasmania.

5.2 Materials and methods

5.2.1 Field sampling

Water samples were collected from five different locations (Fig. 5.1). Hobart CSIRO Wharf station in the Derwent Estuary was sampled weekly from October 2008 to March 2009, then again between May 2009 and July 2009. Three replicates of both surface water and net samples were taken at each sampling. Four other locations in the D'Entrecasteaux Channel and Huon Estuary were sampled once (triplicate samples) (Port Esperance 4th May 2008, Deep Bay 16th May 2008, Fleurtys Point 24th

May 2008, and Woodbridge 24th May 2008), following notification of *G. catenatum* blooms from the Tasmanian Shellfish Quality Assurance Program (TSQAP) (Figs 5.1). Woodbridge was not part of the TSQAP sampling regime, but was sampled simultaneously with the Fleurtys Point station as a *G. catenatum* bloom was known to move northwards through the D'Entrecasteaux Channel at the time (A. Turnbull, Pers. comm.)

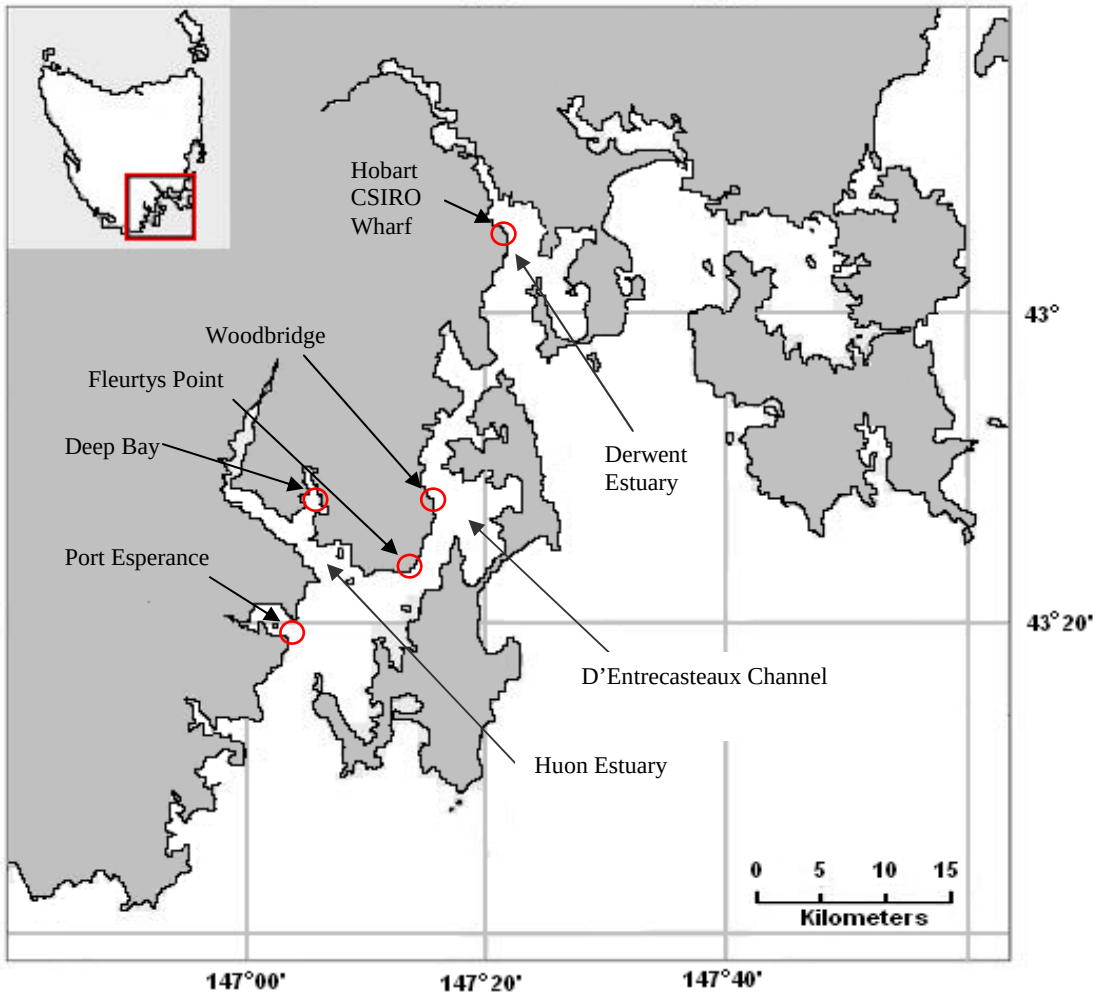


Figure 5.1 Sites sampled in southeastern Tasmania, Australia used in the current study

At each site, water samples for general algal species identification were collected using both a 10µm mesh, 22 cm diameter plankton net with attached 50 mL collection container from approx. 5 metres depth, while whole water samples for algal and associated bacterial enumeration were taken from the surface (top 2 meters) using a bucket. All samples were collected at between 10 and 11am. The samples collected by TSQAP (Fig 5.3), were all collected as 10m integrated samples, giving 3 replicates each of 1.5 L, and all collected between 10am and 3pm.

5.2.2 Sample preparation and filtration and cell counts

To get a general overview of the dominant species composition, triplicate plankton net samples (10 µm mesh size) were scanned in 10 mL Petri dishes using a Leitz Labovert FS microscope (200× magnification). Cell counts on the whole water samples were carried out in triplicates on 10 mL Lugol's iodine preserved whole water samples (Throndsen, 1978) which had previously been concentrated to 1 mL volume using the Utermöhl settling method (Utermöhl 1931). Counts were carried out using a Sedgwick-Rafter counting chamber and examined with a Leitz Labovert FS microscope at 200× magnification. Samples from TSQAP were analysed by Analytical Services Tasmania (AST). Two samples were taken from each site, one was fixed with Lugol's iodine solution and one was untreated. The untreated sample was examined for species identification while the samples fixed with Lugol's iodine solution was concentrated ten times by settling using the Utermöhl settling method then counted at either 100× or 400× magnification, depending on what algal species were present.

For bacterial enumeration, triplicate samples of 250 mL of whole water, was filtered through 10 µm Nucleopore® polycarbonate filters to remove the algae, and the filtrate collected onto 0.2 µm cellulose acetate filters (Whatman®), and frozen at -20°C until DNA extraction.

5.2.3 DNA extraction, Real time PCR assay conditions and sequencing

Extraction of bacterial DNA from 0.2 um filters was carried out as described in Chapter 3 section 3.2.2 and stored at -20 °C until the samples were prepared for qPCR. Real time PCR assays were performed on an ABI 7500 Real time PCR system (Applied Biosystems). The qPCR reactions were carried out in duplicates in 25 µL volumes with reagents and concentration, including primer and probe sequences, as described in Chapter 4, section 4.2.3.

The conditions for thermal cycling consisted of 1 cycle at 50 °C for 2 min, 1 cycle at 95 °C for 10 min, 40 cycles of 15 s at 95 °C followed by 1 min at 60 °C for annealing

and extension. At the end of each qPCR cycle, fluorescence data was collected, and the cycle threshold (C_t) line automatically calculated by the instrument. Bacterial DNA concentrations were determined by comparison of the threshold cycle number to the standard curves developed in Chapter 3 (sections 3.2.6 and 3.3.3).

In order to determine the dominant sequences in the bacterial abundance peaks, positive qPCR products were sequenced using primers described in Chapter 3, section 3.2.3, and ABI-Prism 'BigDye' terminator chemistry (Applied Biosystems, USA) according to manufacturer's standard protocols. The sequences were then identified by comparison with bacterial rDNA sequences available on the NCBI GenBank database.

5.2.4 Statistical analysis

Regression statistics (Microsoft Excel) were used to investigate the correlation between algal and bacterial cells. Significant differences between sampling sites were compared using one-way ANOVA with Tukey's post hoc tests in the statistical software R, Version 2.9.0.

5.3 Results

5.3.1 Samples from CSIRO Wharf Station

Figure 5.2 shows the abundance of *Marinobacter* sp. and *Alcanivorax* sp. (detected by the qPCR assays) in comparison to total phytoplankton, dinoflagellates, diatoms and *G. catenatum* abundance from samples collected at the CSIRO Wharf Station.

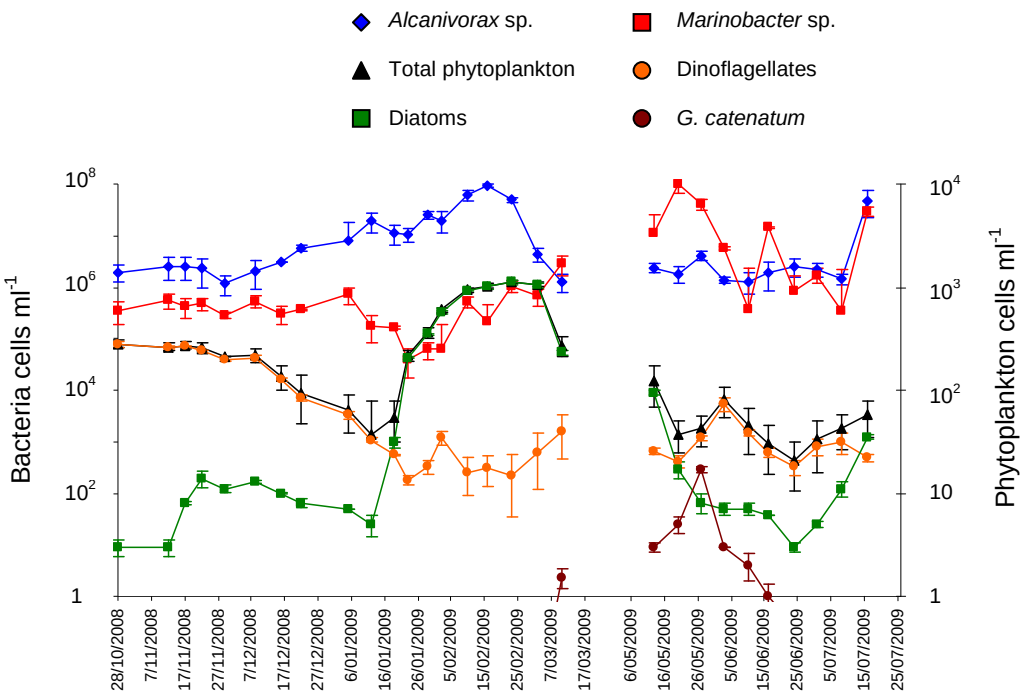


Figure 5.2 *Marinobacter* and *Alcanivorax* abundance detected by the qPCR assays in comparison to total phytoplankton, dinoflagellates, diatoms and *Gymnodinium catenatum* abundance from samples collected at the Hobart CSIRO Wharf Station in October 2008 and July 2009. Error bars showing standard error, n = 3

There was no clear correlation between bacterial and total phytoplankton numbers over the study period (Fig. 5.2). Total phytoplankton abundance declined from late October 2008 until mid January 2009 (from an average of 290 to 50 cells mL^{-1} over the period, dominated by dinoflagellates) after which a peak in cell abundance (dominated by diatoms) was found between mid January and early March 2009, reaching peak densities of approximately 1200 cells mL^{-1}. The phytoplankton composition was dominated by either dinoflagellates or diatoms depending on the time of sampling. Dinoflagellates showed highest abundance (approx 250 cells mL^{-1}, consisting mainly of *Ceratium tripos*, *C. furca*, and *C. fusus*) in late October 2008, which was followed by a slow decrease over the following 3 months, and with a smaller peak (of approximately 20 cells mL^{-1}, mainly *Gymnodinium catenatum*) noted on the 3rd of June 2009. From mid January 2009, diatoms increased from close to zero to a peak of 1100 cells mL^{-1} in late February 2009, and this was followed by a 3 month decline in cell numbers, bringing the diatoms down to <5 cells mL^{-1} over the follow-

ing months. The diatom population consisted almost entirely of *Skeletonema* spp. and *Pseudonitzschia* spp., with a smaller amount (<5%) of *Chaetoceros* spp.

Starting on the 3rd of March 2009, *G. catenatum* numbers increased from 0 to 17 000 cells L^{-1}, reaching the peak density on the 27th of May, which was followed by a rapid decline. A second increase of *G. catenatum* cells (approx. 10 000 cells L^{-1}) also started shortly after the end of this study in late July (I. Jameson, pers. comm.). The bacteria detected by the *Alcanivorax* sp. probe slowly increased (as calculated from the probe detection) over the course of almost 3 months, from late November 2008 until mid February 2009, peaking at approximately 10^8 cells mL^{-1} between 2nd of February to 10th of March 2009, and followed by a second increase in *Alcanivorax* sp. cell abundance in mid July 2009. The bacteria detected by the *Marinobacter* sp. probe showed a peak in cell abundance between mid May and early June 2009, during which, the bacterial abundance increased from ~6.0×10^5 – 2.0×10^8 cells mL^{-1}. This was followed by a smaller peak between 10th and 24th of June and another starting on the 8th of July.

Sequencing of qPCR products from the bacterial abundance peaks on the 20th of May and 16th of July showed the DNA to be dominated by a bacterium closely resembling the uncultured *Marinobacter* sp. clone I3K-289ITS4 (99 % sequence resemblance in the NCBI GenBank), followed by other *Marinobacter* sp. clone strains. The PCR products from the 16th of February were dominated by bacteria resembling *Roseobacter* sp. DG794 (98 % sequence resemblance) and a *Synechococcus* sp. (98 % sequence resemblance). This demonstrated that the probe was not species-specific to *Alcanivorax* sp. DG881. The use of the qPCR assay for detection of *Alcanivorax* sp. was therefore terminated from this point on.

5.3.2 Bloom samples

The TSQAP sampling regime followed the changes in cell concentration in *G. catenatum* between the 23rd of March to 11th of July 2008 at Deep Bay, Fleurtys Point, and Port Esperance (Fig. 5.3). The *G. catenatum* blooms from the three sites showed different profiles in terms of cell abundance and life spans. The Deep Bay site demonstrated both a slow increase and decrease of *G. catenatum* lasting from early April

to mid June with a maximum of 3400 cells L^{-1}. The bloom at Fleurtys Point was only established between 12th and 27th of May reaching 3700 cell L^{-1}, while the Port Esperance bloom reached cell densities of 27 000 cells L^{-1}, and covered the period mid April to mid June (Fig. 5.3).

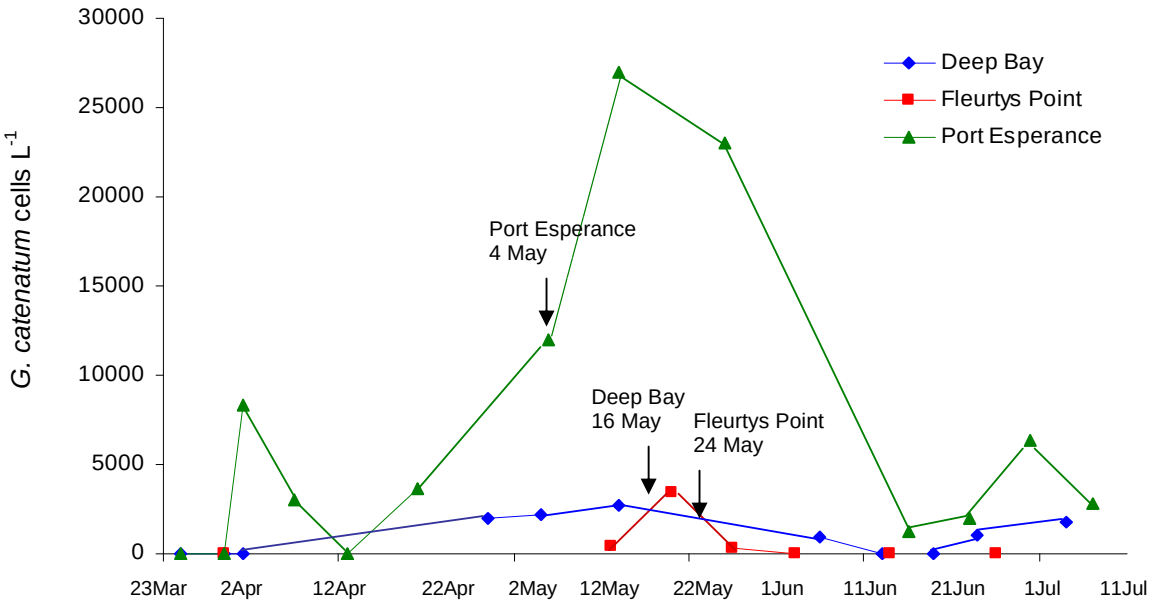


Figure 5.3 Changes in cell concentration of *Gymnodinium catenatum* at the three sites Deep Bay, Fleurtys Point, and Port Esperance between the 23rd of March to 11th of July 2008. Arrows indicate when the samples for qPCR were collected from each site.

Figure 5.4 shows the correlation between all bloom samples (including the samples from the Hobart CSIRO Wharf Station) with stage of the bloom cycle indicated by a ■ for proliferating bloom or ▲ for declining. No clear correlation was found between the *G. catenatum* and the bacteria ($p=0.241$, $F=1.426$, $R^2= 0.044$, $Df = 31$). When the blooms are plotted in regards to their stage of the bloom cycle (as proliferating or declining; Fig. 5.4) proliferating blooms appear to be associated to higher bacterial numbers, however this is not significant ($p= 0.099$, $F= 2.93$, $Df =24$).

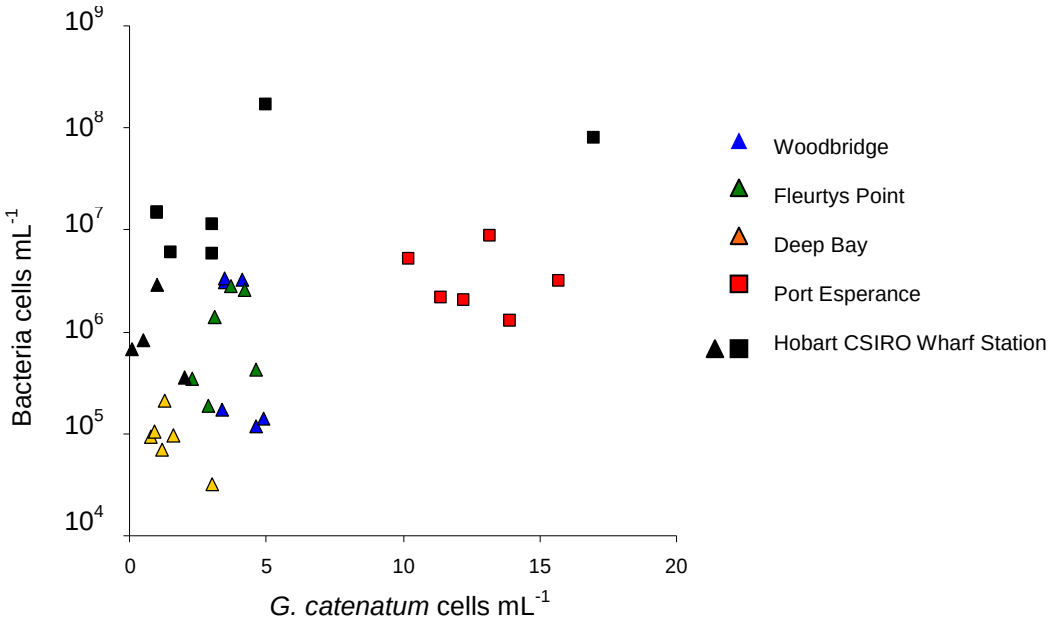


Figure 5.4 Correlation of bacterial and *Gymnodinium catenatum* numbers detected by the *Marinobacter* sp. qPCR assay from all sampling stations: Hobart CSIRO Wharf Station, Woodbridge, Fleurtys Point, Deep Bay and Port Esperance. The colour of the marker indicates the location, while ■ indicates proliferating and ▲ indicates declining bloom

The amount of *Marinobacter* sp. cells per *G. catenatum* cell is plotted for all sampling sites in Figure 5.5. There is no significant difference in bacterial abundance between the proliferating and declining *G. catenatum* cells by the Hobart CSIRO Wharf Station ($p= 0.053581$, $F= 4.784228$, $Df = 11$), but the bacterial abundance at the Hobart CSIRO Wharf Station is significantly different to the rest of the sampling sites. The three sampling sites in the D'Entrecasteaux Channel (Woodbridge, Fleurtys Point, and Port Esperance) were not significantly different from each other ($p= 0.15$, $F= 2.14$, $Df = 17$), while the Deep Bay site in the Huon Estuary was significantly different from the rest of the D'Entrecasteaux sampling sites ($p= 0.046$, $F= 5.17$, $Df= 23$)

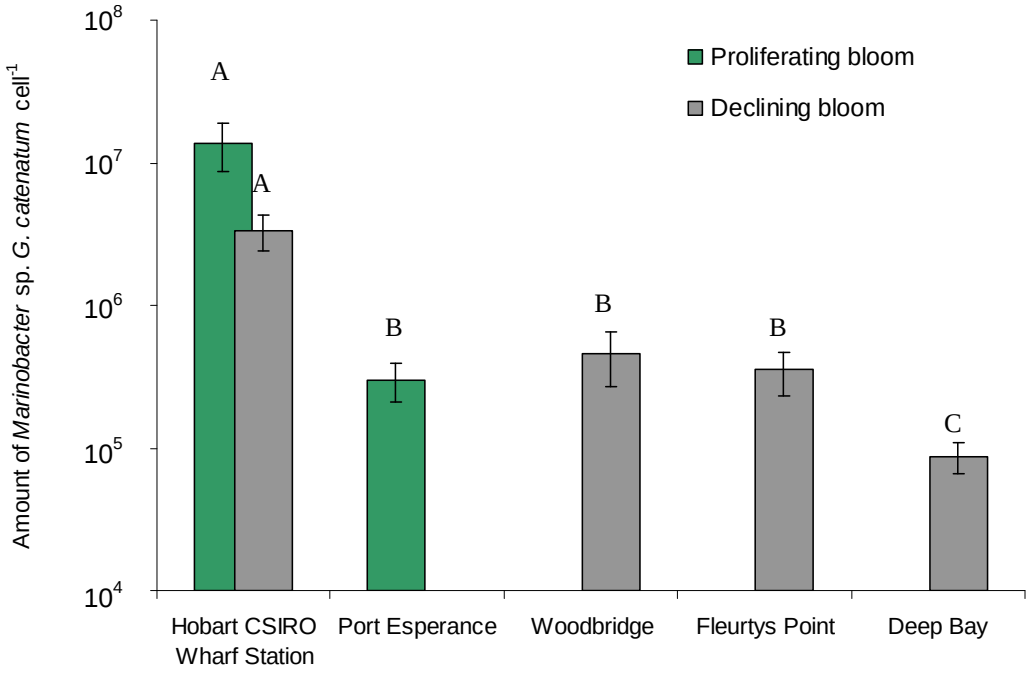


Figure 5.5 Comparison of the amount of *Marinobacter* sp. per *Gymnodinium catenatum* cell^{-1} between proliferating and declining blooms at the different sampling stations. Subscripts indicate significant differences; superscripts with the same letter are not significantly different. Error bars indicate SE, n = 6.

5.4 Discussion

Detection and enumeration of bacteria from field samples can be challenging. The pure complexity of the natural environment, where naturally occurring phytoplankton communities commonly co-exist with 10s to 100s of different bacterial types (e.g. Groben et al., 2000; Alavi, et al., 2001; Green et al., 2004), create a situation where the detection and enumeration of a single species requires highly specific real-time PCR probes. The probes used in this study were originally developed for detection of *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 in laboratory-grown cultures of *Gymnodinium catenatum*, but were shown to be capable of non-specific amplification (Chapter 3). This non-specific amplification, however, only occurred very late in the qPCR cycles (around cycle 30), so was therefore only going to be significant if the target group was at very low abundance (i.e. when the Ct approaches cycle 30), and the effect of the non-specific secondary products on the total cell concentration would theoretically be very small. However, after sequencing

analysis of the positive qPCR products from the two bacterial 'peaks' on the 16th of February (*Alcanivorax* sp. assay) and 20th of May (*Marinobacter* sp. assay) the *Alcanivorax* sp. assay was shown to be amplifying non-target organisms, rendering it inadequate for detection of *Alcanivorax* sp. in field samples, and demonstrating the importance of accurate validation of the assays before use.

5.4.1 Samples from Hobart CSIRO Wharf Station

The peaks in *Marinobacter* sp. abundance appearing just prior to the bloom of *G. catenatum* at the Hoabrt CSIRO Wharf Station on the 20th of May, and then again on the 16th of July, suggested that *Marinobacter* sp. possibly could be involved in the onset of the two *G. catenatum* blooms. Other studies have found *Marinobacter* sp. to be part of the bacterial community associated with dinoflagellate blooms: analysis of the bacterial population associated with a bloom of *Lingulodinium polyedrum* demonstrated the presence of *Marinobacter* sp. in the free-living fraction of the bacterial population (Fandino et al., 2001), and an early study by Ogata et al. (1989) found a bacterium, PCOB-2, was associated with a bloom of *Alexandrium cohorticula*. This strain was subsequently recognized as belonging to the *Marinobacter* sp. *(M. hydrocarbonoclasticus* group, GenBank accession AJ000647). So far, studies on algal-*Marinobacter* associations from dinoflagellate blooms are limited, while investigations from laboratory cultures are more frequent. Jasti et al. (2005) showed a close relative of *Marinobacter* sp. PCOB-2 (100% sequence similarity) to be associated with a range of phytoplankton such as *Alexandrium tamarense*, *A. fundyense*, *Scrippsiella* sp. and *Pseudopedinella elastica*, while Hold et al. (2001) found a relative of *Marinobacter aquaeolei* in *A. tamarense*, *A. lucitanicum*, and *S. trochoidea* (Fig. 5.6). Another culture study, by Alavi et al. (2001), suggests that a relative of *M. hydrocarbonoclasticus*, found to be in association with *Pfiesteria piscicida*, may be mediating the toxicity of these dinoflagellates based on the assumption that *Marinobacter* sp. can utilise dinoflagellate secondary metabolites as carbon and energy sources, while these metabolites also can serve as chemical precursors of certain dinoflagellate toxins.

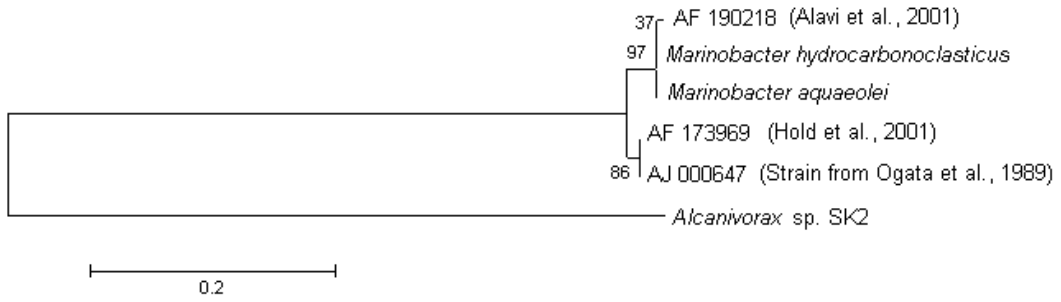


Figure 5.6 Phylogenetic tree of 16S rRNA gene sequences from the *Marinobacter hydrocarbonoclasticus* and *M. aquaeolei* with their related strains. Distances were calculated using the maximum-likelihood method, and the tree was constructed using the neighbour-joining method. *Alcanivorax* sp. SK2 was used as out-group.

Population dynamics of phytoplankton blooms are governed by a range of complex interactions, and in the case of bacterial effect on these dynamics, growth rate and mortality of the algae may be affected by the interactions between the algae and bacteria. Similar to this study, Furuki and Kobayashi (1991) presented data suggesting that the presence of a bacterial assemblage was promoting the growth of *Chattonella antiqua* on regular 5-year intervals, and earlier work by Riquelme et al. (1987; in Fukami et al., 1997) observed that high densities of the bacterium *Pseudomonas* sp. 022 appeared just prior to a bloom of the diatom *Asterionella glacialis*. In the case of *A. glacialis*, further studies (Riquelme et al., 1988 in Fukami et al., 1997) revealed that the growth factor produced by *Pseudomonas* sp. 022 was a glycoprotein. The identity of the growth stimulant is in most cases unknown, although a few suggestions have been brought forward. Iwasaki (1979) found that kinetin, one of the hormones of the cytokinin group accelerated growth of *C. antiqua*, and later studies by Takahashi and Fukazawa (1982) and Maruyama et al. (1986) suggested cytokinin to be a possible growth-promoting substance after studies on formation of red tides in Tanigawa harbour, Japan.

5.4.2 *Gymnodinium catenatum* bloom samples

The lack of correlation between *G. catenatum* and *Marinobacter* sp. abundance in the *G. catenatum* bloom samples could be a result of numerous factors. Shifts in

composition and abundance of the bacterial community over the progression of phytoplankton blooms are well documented (e.g. Fukami et al., 1981; Romalde et al., 1990; Fukami et al., 1991; Fandino et al., 2001). The bloom samples examined in this study were taken at different phases of bloom development and decline. The bacterial community composition may have changed, however, and as total bacterial abundance was not measured it is difficult to ascertain whether *Marinobacter* sp. is simply increasing in abundance along with total bacterial abundance, or is increasing or decreasing as a proportion of the total bacterial abundance.

Although no significant difference was found between proliferating and declining blooms in terms of bacterial numbers, the high bacterial load per *G. catenatum* cell in the samples Hobart CSIRO Wharf Station makes for an interesting observation. A possible explanation could be the proximity to sewage and run off from the city of Hobart (population of 204 000 people including large industrial areas) in comparison to the other four sites which are only affected by rural communities. However, nutrient data from previous studies of the areas suggest that there is little variation between the sites in terms of nitrates and phosphates. The Derwent Estuary Program (Whitehead et al., 2010) found phosphate and nitrate, in June 2004, to reach concentrations of 12 μg/L and 30 μg/L, respectively, adjacent to the Hobart CSIRO station, while during the same time, the D'Entrecasteaux Channel showed an average concentration of 13 μg/L phosphate and 49 μg/L nitrate (Volkman et al., 2009). Additionally to nitrates and phosphates, there is a large possibility that other macro- or micro nutrients are involved in controlling the bacterial growth dynamics, although as research on these have been made at different times of the year, they cannot be compared.

Studies on hydrocarbons have not been made in either of the Huon Estuary, the D'Entrecasteaux Channel, or the Derwent Estuary, thereby not making a comparison possible between the sites. However, it appears likely that there would be a higher amount of hydrocarbons in the Derwent Estuary, considering the highly boat-trafficated region and land run-off (E. Butler, pers. comm.), suggesting that the higher *Marinobacter* sp. load could be a result of hydrocarbon pollution in the region. The Huon Estuary (Deep Bay) has a higher level of dissolved organic carbon (DOC 10-15 mg L^{-1}) in comparison to the Derwent Estuary (Hobart CSIRO Wharf

Station; DOC 2-8 mg L^{-1}) (Butler et al., 2000), suggesting that, contrary to the results from this study, the Deep Bay station would have a higher bacterial load.

The diversity of the blooms could also possibly play a role. The blooms in the Huon Estuary and in the D'Entrecasteaux Channel were monospecific; while during the *G. catenatum* bloom at the Hobart CSIRO Wharf Station, there were also other dinoflagellates (*Ceratium furca*, *C. tripos* and *C. fusus*) present. Dinoflagellates are known to have a similar composition of associated bacteria (e.g. Alavi et al., 2001; Hold et al., 2001; Green et al., 2004); possibly suggesting that the higher bacterial load at the Hobart CSIRO Wharf Station could be a result of the higher number of dinoflagellates at the location. The phytoplankton species composition of the Derwent Estuary is also more diverse than the Huon Estuary (Hallegraeff and Westwood, 1994; Jameson and Hallegraeff, 1994), hypothetically allowing for a larger bacterial population.

Conclusion

Although more research is required in order to establish the relationships, the close interactions found in laboratory cultures of *G. catenatum* and *Marinobacter* sp. DG879 (Chapter 4), coupled with the primary results from this environmental study, suggest that *Marinobacter* sp. DG879 possibly can serve as indicator-species for *G. catenatum* in Tasmanian waters. This study found *Marinobacter* sp. at all sites experiencing *G. catenatum* blooms, and the *Marinobacter* abundance peak seen prior to the *G. catenatum* bloom at the CSIRO wharf station, suggests *Marinobacter* could be a pre-requisite for *G. catenatum* bloom formation. By deciphering the complex web of interactions that are occurring in the marine environment, biological models can then consecutively be made more robust, and in turn enhance predictability of bloom events.

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Chapter 6:

The effect of bacteria on the toxin production of *Gymnodinium catenatum*

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6.1 Introduction

Algal-bacterial interactions can influence microalgal bloom dynamics in a variety of ways (for review see Doucette, 1998). One facet of the association is the role played by bacteria in the production of phycotoxins usually attributed to harmful microalgal species. The theory of a bacterial origin of paralytic shellfish toxins (PSTs) was first suggested by Silva (1982) due primarily to the occurrence of bacteria-like particles within dinoflagellate cells. Subsequently, a PST-producing intracellular bacterium *Moraxella* sp. was isolated from *Alexandrium tamarense* (Kodama et al, 1988) and bacteria were later observed within the dinoflagellate nucleus (Kodama et al., 1990a).

There is evidence to suggest a role of bacteria in phycotoxin production. This includes: (1) the ability of bacteria to either enhance or decrease toxin production of dinoflagellates (e.g. Hold et al., 2001), (2) phylogenetically diverse organisms have been shown to produce the same toxic compounds (Gallacher and Smith, 1999), (3) there can be a substantial variation in toxicity within an algal species (Kim et al., 1993), (4) the occurrence of toxic events in the apparent absence of algal blooms (Kodama et al, 1990b), and (5), the production of phycotoxin-like compounds (e.g. tetrodotoxin) by bacteria occurring in organisms previously considered to be the source of the toxin (Gallacher et al., 1996).

On the other hand, several studies have indicated that axenic dinoflagellate cultures can retain the ability to produce toxins at levels similar to those of non-axenic cultures (e.g. Kim et al., 1993; Dantzer and Levin, 1997, and Uribe and Espejo, 2003). In fact, some axenic cultures are more prolific producer of PSTs, Singh et al. (1982) demonstrating that bacteria may not be influencing toxin production. There is also evidence suggesting that bacteria can be indirectly involved in phycotoxin produc-

tion. For example, indirect enhancement of algal toxin yields by bacteria that themselves are not considered to be toxigenic, has been demonstrated in the domoic acid (DA) producing diatom *Pseudo-nitzschia multiseries* (Bates et al., 1995), suggesting that bacteria, although not essential for the synthesis of DA production, profoundly influences the production by the algae. Since the effects were observed with bacteria isolated from a non-toxic diatom, and none of the bacteria were shown to be able to produce DA, the enhanced toxicity likely results from a more indirect contribution such as DA precursor molecules. A similar case of positive influence of bacteria on phycotoxin production was shown by Tosteson et al. (1986), where peak toxicities in cultures of the ciguatoxic dinoflagellate *Ostreopsis lenticularis* were positively related to the abundance of the non-toxic bacterium *Nocardia* sp. Bacterial biotransformation of PSTs is another potential mechanism by which these organisms may influence dinoflagellate toxicity. PST consists of saxitoxin (STX) and at least 22 derivatives (See Chapter 1, section 1.3.4), where each derivative has its own potency, with some being more toxic than others (Shimizu, 1993). In early reports by Kotaki et al. (1985a and b), marine bacteria were demonstrated to be capable of interconverting the PST neurotoxins, and more recently Smith et al. (2001) demonstrated that bacteria isolated from a variety of shellfish possess the capacity to carry out side-chain modifications of PST.

Gymnodinium catenatum is a well-known PST producer, and is the only unarmoured dinoflagellate known to produce these toxins. Dinoflagellates can have bacteria transiently present (fleeting association) or persistently associated (assumed symbionts), where the persistently associated bacteria that are specific to dinoflagellates would be the most likely candidates to be either toxin producers or to have an effect on the toxin production of the dinoflagellate (Pérez-Guzmán et al., 2008). As *G. catenatum* has been shown to have very close interactions with two γ-proteobacteria, *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881, each of which have been shown to be essential for survival and growth (Bolch et al., 2004). The strict requirement of these strains for *G. catenatum* raises the possibility that the two bacteria may also influence aspects of toxin production by *G. catenatum*.

This study aimed to investigate the potential effects of *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 on the toxin production of *G. catenatum*. In order to achieve

this, uni-bacterial cultures of *G. catenatum* were first established from resting cysts with different bacteria added, then the toxicity of the cultures was established using high pressure liquid chromatography (HPLC). The uni-bacterial cultures were then compared with the two non-axenic parent *G. catenatum* strains with mixed-bacterial communities.

6.2 Materials and Methods

6.2.1 Algal and bacterial cultures

The pre-experimental *G. catenatum* cultures (Table 6.1) were maintained in 50 mL Erlenmeyer flasks in approx. 40 mL GSe medium (Blackburn et al., 2001, Appendix 1) at 18 ºC, 65 µmol PAR m^{-2} sec^{-1}, and with an 18:6h light: dark light regime from cool-white fluorescent lights. All cultures were transferred at three week intervals, and handled aseptically to prevent cross-contamination and bacterial contamination of the cultures. Both bacterial species, *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 were maintained on ZoBell Marine (ZM1) agar (Zobell, 1941; Appendix 2) at 20 ºC in total darkness. Transfers were made every 2 weeks.

The experimental *G. catenatum* cultures (see section 6.2.3) were maintained in 175 mL Erlenmeyer flasks under the same light and temperature conditions as outlined above.

6.2.2 Production of resting cysts and unibacterial cultures

In order to produce resting cysts of *G. catenatum*, the two toxin-producing, Tasmanian strains, GCDE06 and GCLV01 (see Appendix 3 for more information), provided by the Australian National Algae Culture Collection (ANACC- formerly known as the CSIRO Collection of Living Microalgae, www.cmar.csiro.au/ microalgae) were crossed. Cyst production was based on the techniques described in section 2.2.2. Resting cysts were harvested by micropipetting after approximately 3 weeks, and were then washed and surface-sterilised using H_2O_2 (Bolch and Hallegraeff, 1993) (refer to section 2.2.5 of Chapter 2). Successful surface-sterilisation was

checked by spread-plating 10 µL of the sterilised sample onto ZM1 agar which was incubated at 24 °C for 4 days. If bacterial growth was seen after incubation, the media and cysts were discarded.

Ten to fifteen sterilised cysts were placed using micropipette in 35 mm diameter sterile polystyrene Petri dishes containing 10 mL GSe medium, and to which different bacterial communities were aseptically added: (1) Pure *Marinobacter* sp. DG879 (1 mL of 10^5 cells mL^{-1}); (2) pure *Alcanivorax* sp. DG881 (1 ml of 10^5 cells mL^{-1}); (3) 1 mL of a 5 µm bacterial filtrate from the *G. catenatum* culture DE06; (4) 1 mL of a 5 µm bacterial filtrate from the *G. catenatum* culture LV01; and (5) a positive control with cysts unsterilized and allowed to excyst with a 'normal' multi-bacterial community. A negative control contained sterile cysts and sterile filtered (0.2 µm) seawater without bacteria added. As *G. catenatum* cannot be grown axenically, axenic cultures were not included in the study.

After the excystment of resting cysts, cultures of either uni-bacterial or multi-bacterial *G. catenatum* were established from single cells or chains to produce clonal cultures. The original cultures were diluted with sterile GSe medium to a concentration of approximately 1 cell or a single chain of cells mL^{-1} (2-8 cells long), and from there, 1 mL was transferred to each of the wells of a 24-multiwell plate containing 2 mL sterile GSe medium. By using a Leitz Labovert FS inverted microscope (200× magnification), wells shown to contain only one cell or cell chain were labelled and the plate incubated under the culture conditions previously described for 1 week. Clonal cultures were established from the labelled wells, transferred into 50 mL Erlenmeyer flasks containing 25 mL fresh GSe medium and when sufficient cell concentration was achieved these were transferred to 50 mL flasks containing 40 mL GSe medium. Five clonal cultures from each bacterial treatment together with 5 clonal cultures of each of the two parent *G. catenatum* cultures made up the 7 treatments used in this study (Table 6.1).

To determine the period of logarithmic-phase growth under experimental conditions, all cultures were grown under identical conditions for 50 days prior to the experiment, and growth examined using cell counts (Fig. 6.1). All cultures were subse-

quently harvested at Day 0 and at Day 25 which corresponded to late-logarithmic phase.

Table 6.1 Summary of established *Gymnodinium catenatum* / bacterial cultures used in this study

Culture name	*G. catenatum* details	Bacterial addition
DE06	*G. catenatum* strain GCDE06	---
LV01	*G. catenatum* strain GCLV01	---
Gc/Mar	Cyst derived from DE06 × LV01 strain cross	*Marinobacter* sp. DG879
Gc/Alc	Cyst derived from DE06 × LV01 strain cross	*Alcanivorax* sp. DG881
DE06 filtrate	Cyst derived from DE06 × LV01 strain cross	Bacterial filtrate from DE06
LV01 filtrate	Cyst derived from DE06 × LV01 strain cross	Bacterial filtrate from LV01
Positive control	Cyst derived from DE06 × LV01 strain cross	Non-sterilised, natural bacterial assemblage

6.2.3 Sample preparation for toxin analysis

At the initiation of the experiment (Day 0), 10 mL of the established cultures in late logarithmic growth phase were each inoculated into 150 mL of sterile GSe medium, 5 replicates of each. Cell counts were conducted on the inoculum culture (Leitz Labovert FS microscope, 200× magnification) and 100 mL of the culture filtered through precombusted (400 °C, 4 h) 47 mm GF/C (Whatman) filters, placed in 15 mL screw-cap polycarbonate centrifuge tubes containing 5 mL of 0.05 M acetic acid, and frozen at -20 °C until toxin analysis could be completed. At late-logarithmic phase, (Day 25, determined from earlier studies), a 5 mL cell count sample was taken from each culture and 100 mL of culture filtered as described above and frozen at -20 °C until toxin analysis was carried out.

6.2.4 Calculations and statistics

Specific growth rates (μ) of the cultures from Day 0 to Day 25 were calculated over the exponential growth phase (Day 0 to Day 25) using the equation:

$$\mu = \ln (N2 / N1) / (t2 - t1) \quad (1)$$

where *N1* and *N2* = cell concentration at time 1 (*t1*) and time 2 (*t2*) respectively (Levasseur et al., 1993), divisions per day can then be calculated once the specific growth rate is known using:

$$Div.\ day^{-1} = \mu / Ln2 \quad (2)$$

The specific toxin production rate μ_{tox} between Day 0 and 25 was calculated using similar equations as described by Anderson et al. (1990). STX content of cells was determined at Day 0 and at Day 25 using HPLC analysis (fmol cell^{-1}) and multiplied by N_t (cell concentration at time *t*) to yield T_t, the total toxin concentration (fmol STX mL^{-1} culture) at time *t.* Toxin concentrations (fmol STX mL^{-1}) at Day 0 were calculated from the STX content (fmol cell^{-1}) of the inocula and included a correction for a 10 mL into 160 mL dilution after inoculation.

Values of *T* were then used instead of cell concentrations *N* in equation 1 to calculate μ_{tox} over each interval:

$$\mu_{tox} = \ln (T_2 / T_1) / (t_2 - t_1) \quad (3)$$

The net toxin production rate R_{tox} (fmol toxin cell^{-1} d^{-1}) was determined using the equation:

$$R_{tox} = (T_2 - T_1) / ((N') (\Delta t)) \quad (4)$$

where *N'* is the *ln* average of the cell concentration:

$$N' = (N_2 - N_1) / (\ln N_2) - (\ln N_1) \quad (5)$$

and Δt (= $t_2 - t_1$) the interval between Day 0 and Day 25. It is necessary to use the *ln* average concentrations because the dinoflagellate is growing exponentially between t_2 and t_1, and *ln* therefore accounts for the fact that more cells will produce toxin at a higher rate (Anderson et al., 1990).

Regression statistics were used to investigate the correlation between the specific toxin production rate and algal growth rate, and significant differences between toxin production of the treatments and controls were compared using one-way ANOVA with Tukey's post hoc tests in the statistical software R, Version 2.9.0. Cluster analysis and subsequent similarity analysis of the toxin profiles was based on Euclidean distance resemblance calculated on standardised data, with a UPGMA algorithm (unweighted pair-grouping averaging), using Primer Version 6. Primer-E (Plymouth, UK). The toxin profile data was standardised because the variances of GTX1+4 and GTX2+3 are much smaller than the other toxin fractions, and would have almost no influence on the cluster analysis. By standardising the data all toxin fractions are allowed to make an equal contribution to the cluster analysis.

6.2.5 HPLC analysis

While still in the tubes containing 5 mL of 0.05 M Acetic acid, the filters were sonicated for 30s on ice several times using an ultrasonic cell disruptor (150W, 5 mm probe). The tubes were then centrifuged at 5000 × g for 5 min and the supernatants filtered to 0.45 µm then frozen at -20°C until analysis. PSTs including C-toxins, gonyautoxins (GTX) and the hydroxybenzoate (GC) toxins were analysed by HPLC using the methods of Negri and Jones (1995) and Negri et al. (2003). Briefly, toxins were separated using a Waters 600 HPLC, with post-column reactor (Pickering PCX 5100) using a 5 µm, 250 mm × 4.6 mm Alltima ODS column (Alltech, IL, USA) with a flow rate of 0.8 mL min^{-1}. Post-column oxidation was performed according to the method of Oshima et al. (1993). Derivative PST fluorescence was detected with a Linear LC305 spectrofluorometric detector (excitation at 330 nm and emission at 290 nm). The retention times and fluorescent intensity of the PSTs were compared with PST standards (NRC, Canada) and identity of PST compounds confirmed by sample spiking experiments and removing post-column oxidation and observing the disappearance of peaks. The quantification of toxins was achieved by comparing peak ar-

eas with those of authentic standards and combining this data with cell counts from the time of harvest then converting to total PSTs (fmol) per cell.

6.3 Results

6.3.1 Growth of cultures

All cultures, with the exception of LV01, showed growth curves typical of *G. catenatum* over the 50 day growth period (Blackburn et al., 1989; 2001) (Fig. 6.1). LV01 grew more slowly than the other cultures with a slow increase in cell numbers between days 5 and 45 only declining slightly on the last day of sampling. There was however no significant difference between the different growth rates ($p = 0.092$, $F = 1.96$, $Df = 6$). The inoculum of LV01 was one order of magnitude lower then the rest of the cultures, a result of a pre-experimental culture in poor health. These results of slow growth are also reflected in the rate of divisions day^{-1} (Table 6.2) where LV01 has the lowest rate (0.10 div. day^{-1}), while *G. catenatum* with added *Alcanivorax* sp. DG881 (Gc/Alc) reached the highest rate of 0.18 div. day^{-1}.

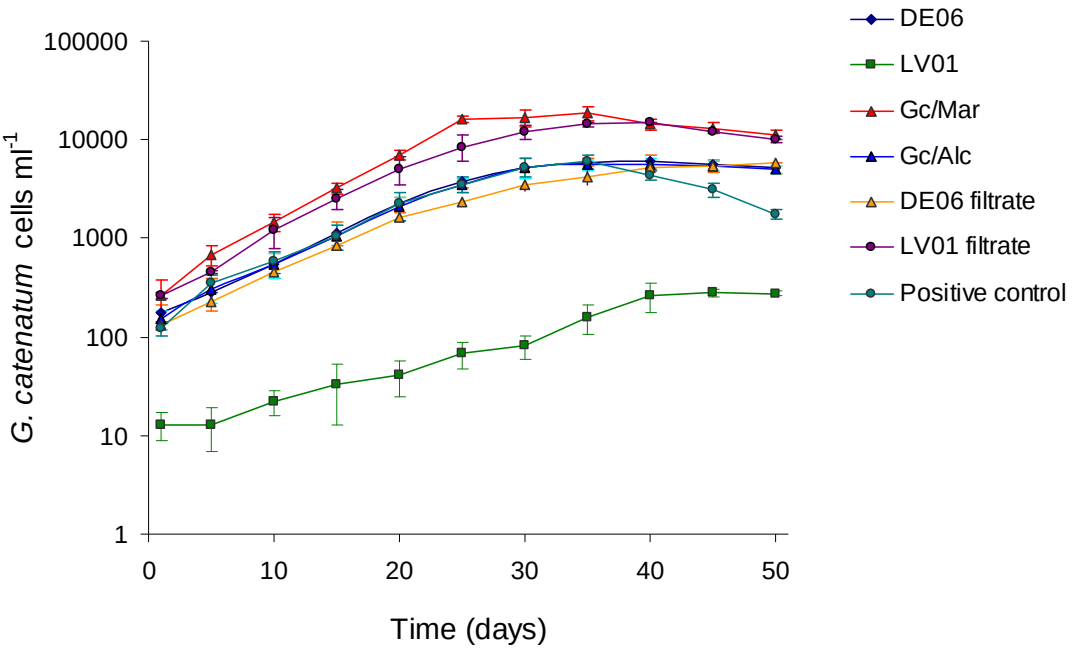

Figure 6.1 Growth of the established *Gymnodinium catenatum* cultures over a 50 day period. N = 5 +/- SE

Table 6.2 Mean logarithmic growth rate and divisions day^{-1} of *Gymnodinium catenatum* (between Day 0 and 25) when grown with different microbial communities. Standard error shown in brackets.

Culture	Growth rate (K') = ln (N2 / N1) / (t2 - t1) (±SE)	Divisions day^{-1} = K'/ln2 (±SE)
DE06	0.11 (±0.005)	0.16 (±0.008)
LV01	0.07 (±0.006)	0.10 (±0.010)
Gc/Mar	0.09 (±0.004)	0.13 (±0.006)
Gc/Alc	0.13 (±0.008)	0.18 (±0.012)
DE06 filtrate	0.10 (±0.005)	0.15 (±0.007)
LV01 filtrate	0.08 (±0.004)	0.12 (±0.005)
Positive control	0.08 (±0.006)	0.11 (±0.009)

6.3.2 Toxin analysis

The analyses of total toxin content per cell at day 0 (inoculation) and day 25 (harvest) showed some variation between the treatments, however, in 5 of 7 treatments, toxin content per cell increased during the study (Fig. 6.2). Table 6.3 provides a summary of the statistical analysis with the significant differences ($p < 0.05$) in bold.

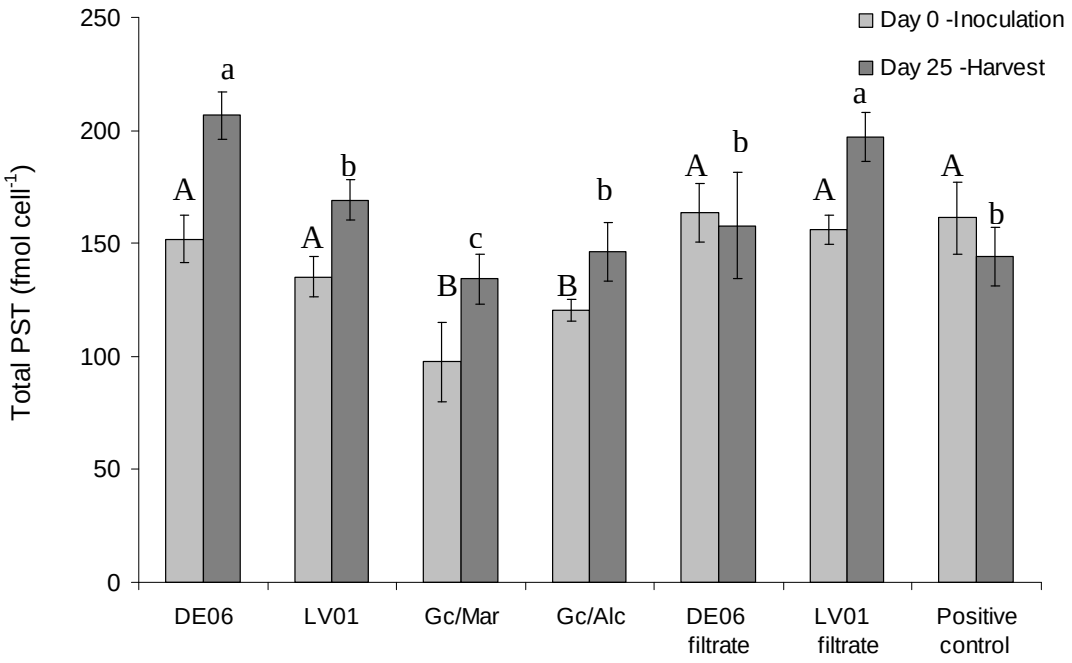

Figure 6.2 Changes in toxicity from inoculation at Day 0 to harvest at Day 25. Superscripts indicate significant differences ($p<0.05$), means with the same letter are not significantly different. Error bars +/- SE, n=5

Table 6.3 Summary of pair wise significant differences in toxin content cell^{-1} of *Gymnodinium catenatum* cultures grown with different microbial communities among treatments (one-way ANOVA with Tukey's Post hoc test), from inoculation at Day 0 to harvest at Day 25. Upper number = Day 0, bottom number = Day 25. F = 5.19 and Df = 6 for all samples at Day 0. F = 4.05 and Df = 6 for all samples at Day 25. Significant differences ($p < 0.05$) between treatments in bold.

	DE06	LV01	Gc/Mar	Gc/Alc	DE06 filtrate	LV01 filtrate	Positive control
DE06		0.915 0.475	**0.016** **0.013**	0.377 0.057	0.999 0.998	0.985 0.194	0.995 **0.043**
LV01			0.192 0.559	0.954 0.898	0.797 0.771	0.495 0.997	0.591 0.849
Gc/Mar				0.723 0.995	**0.008** **0.042**	**0.002** 0.878	**0.003** 0.998
Gc/Alc					0.243 0.157	0.093 0.996	0.128 1.000
DE06 filtrate						0.998 0.422	0.999 0.124
LV01 filtrate							0.999 0.991
Positive control							

There was no significant overall correlation of cellular toxin content (fmol cell^{-1}) and the growth rates of the *G. catenatum* cultures (R^2 = 0.10, p = 0.06, F = 3.68, Df = 34) (Fig. 6.3); however, clustering due to strain type was evident.

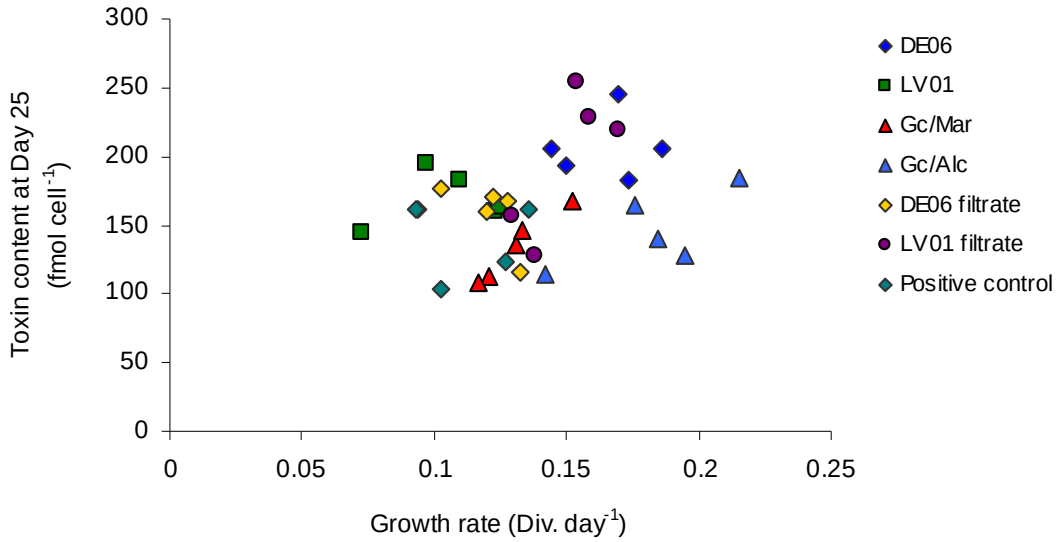

Figure 6.3 Correlation between toxin content per cell at Day 25 (fmol cell^{-1}) and growth rate (div.day^{-1}) in each of the 35 cultures.

The specific toxin production rates (fmol day^{-1}) varied both between the treatments and within the treatments, with the rate of toxin production varying between 0.004 and 0.165 fmol day^{-1} (Fig. 6.4). ANOVA showed significant differences between the different treatments (p = 0.0003, F = 6.35, Df =34), and a summary of the Tukey's post hoc test is shown in Table 6.4.

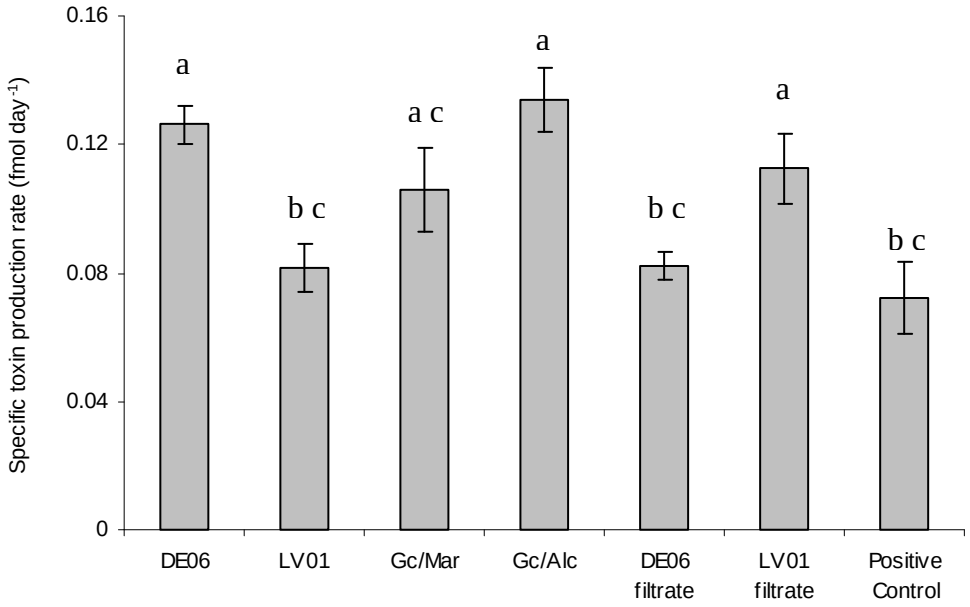

Figure 6.4 Specific toxin production rate (fmol day-1) between Day 0 and Day 25 of *Gymnodinium catenatum* grown with different bacterial communities. Superscripts indicate significant differences ($p<0.05$), means with the same letter are not significantly different.

Table 6.4 Summary of pair wise significant differences in specific toxin production of *Gymnodinium catenatum* cultures grown with different microbial communities among treatments (one-way ANOVA with Tukey's Post hoc test), from inoculation at Day 0 to harvest at Day 25. Significant differences ($p < 0.05$) between treatments in bold. P = 8.11 and Df = 9 for all samples.

	DE06	LV01	Gc/Mar	Gc/Alc	DE06 filtrate	LV01 filtrate	Positive control
DE06		**<0.001**	0.199	0.521	**<0.001**	0.300	**0.003**
LV01			0.146	**0.003**	0.961	**0.046**	0.508
Gc/Mar				0.129	0.121	0.709	0.088
Gc/Alc					**0.001**	0.184	**0.004**
DE06 filtrate						**0.029**	0.440
LV01 filtrate							**0.033**
Positive control							

There is a clear correlation between the specific rates of toxin production and the growth rates of the cultures between days 0 and 25 (R^2 = 0.82, p = <0.001, Df = 34). The highest mean growth rate and highest mean specific toxin production rate was shown by the *G. catenatum* cultures grown with *Alcanivorax* sp. DG881 (Gc/Alc), although not significantly different from cultures DE06, Gc/Mar and LV01 filtrate (Fig. 6.5).

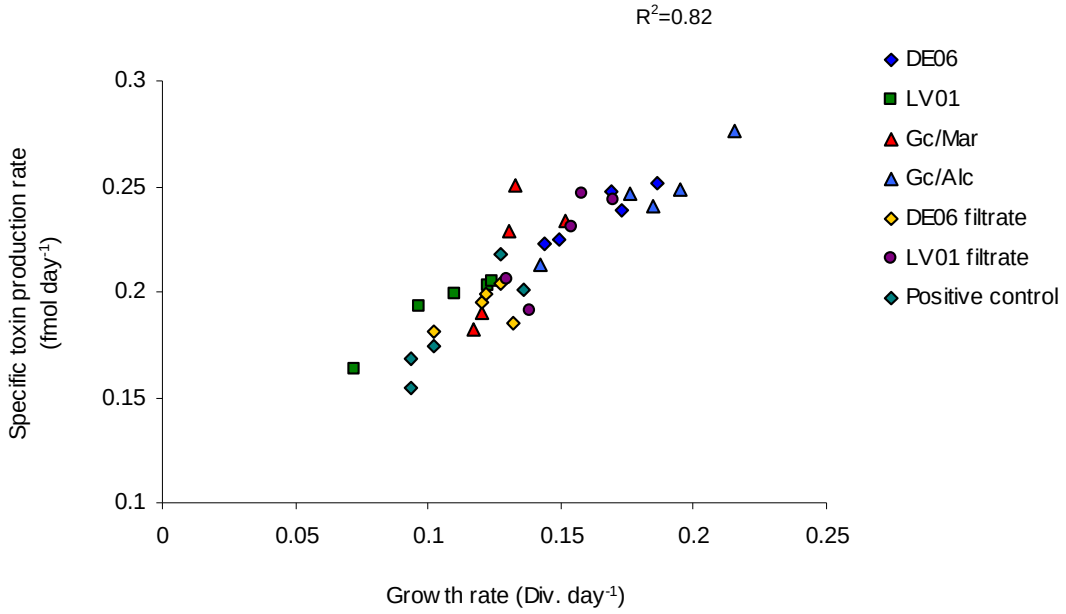

Figure 6.5 The relationship between growth rate (div. day-1) and specific toxin production rate (fmol day-1) of 35 cultures of *Gymnodinium catenatum* in comparison to measured between day 0 and day 25.

The net rates of toxin production (fmol toxin cell^{-1} day^{-1}) and the growth rates of the cultures showed a random pattern with no distinct correlations (Fig. 6.6). Within the same treatment, between Day 0 and Day 25, some cultures showed a positive per cell toxin production while others were negative. The *G. catenatum* cultures grown with *Alcanivorax* sp. DG881, and cultures with mixed bacterial communities (DE06 and LV01), all had a positive net toxin production across all five replicates. One of the five replicates grown with mixed bacterial filtrate from LV01 showed negative toxin production, while cultures grown with *Marinobacter* sp. DG879 and DE06 mixed bacterial filtrate had two of five replicates showing negative net toxin production. Four of the five replicates grown with the bacteria associated with unsterilised rest-

ing cysts (positive control), displayed a negative net toxin production over the time period. There was no significant difference between the net production rates of the different cultures (p=0.064, f = 2.44, Df = 6).

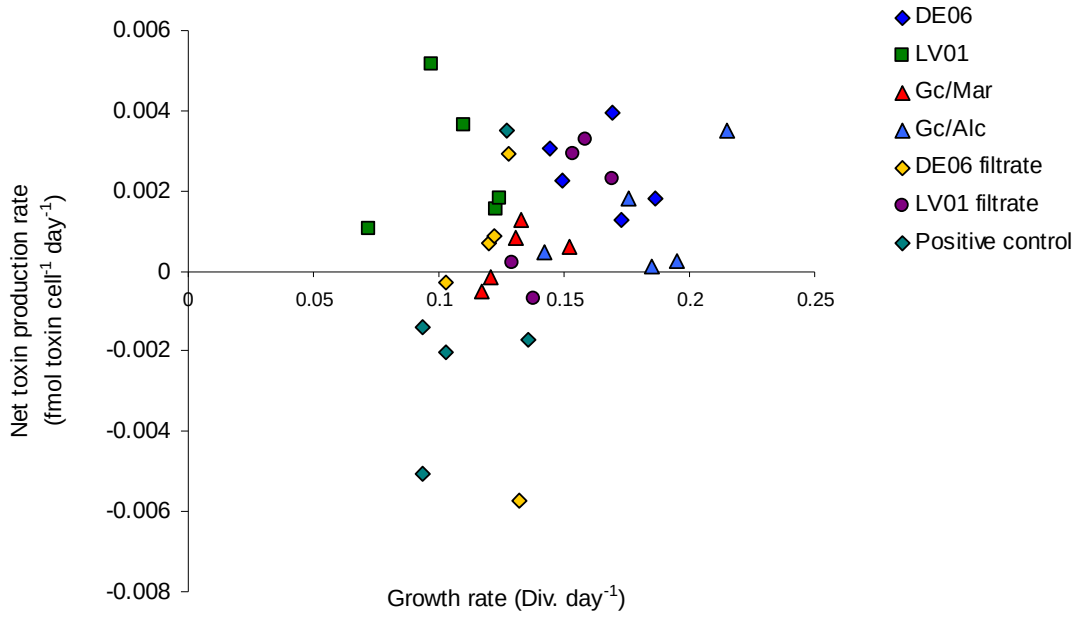

Figure 6.6 The relationship between net toxin production rate per cell (fmol toxin cell-1 day-1) and growth rate (div. day-1) for each of the 35 cultures of *Gymnodinium catenatum* in comparison to measured between day 0 and day 25.

The toxin profiles for all treatments are shown in Figure 6.7 as means of triplicates. As the proportion of α-epimers often is very small and some interconversion is expected, the concentrations of epimer pairs were combined (an epimer is one of two molecules that differ only in the spatial arrangement around a single carbon atom). Toxin analysis revealed that the β-epimers (C2, C4, dcGTX3 and GC2) were the dominant toxin species (Fig. 6.7a-d), and none of the analysed cultures contained detectable concentrations of dcSTX, STX or GC3. The parent strains (DE06 and LV01) used for cyst production and to establish the cultures with different bacterial communities, possessed different toxin profiles. Strain DE06 produced higher proportions of C1+2 and GC1+2, while LV01 produced a higher proportion of C3+4 (Figs 6.7a-c). The cultures of the four treatments Gc/Alc, DE06 filtrate, LV01 filtrate, and the positive control all showed similar toxin composition (Figs 6.7a-f),

however Gc/Mar exhibited a lower proportion of C1+2 and C3+4 (Figs 6.7a, b), and greater proportions of dcGTX2 + dcGTX3 at Day 25 (harvest) (Figs 6.7a, b, d and 6.8).

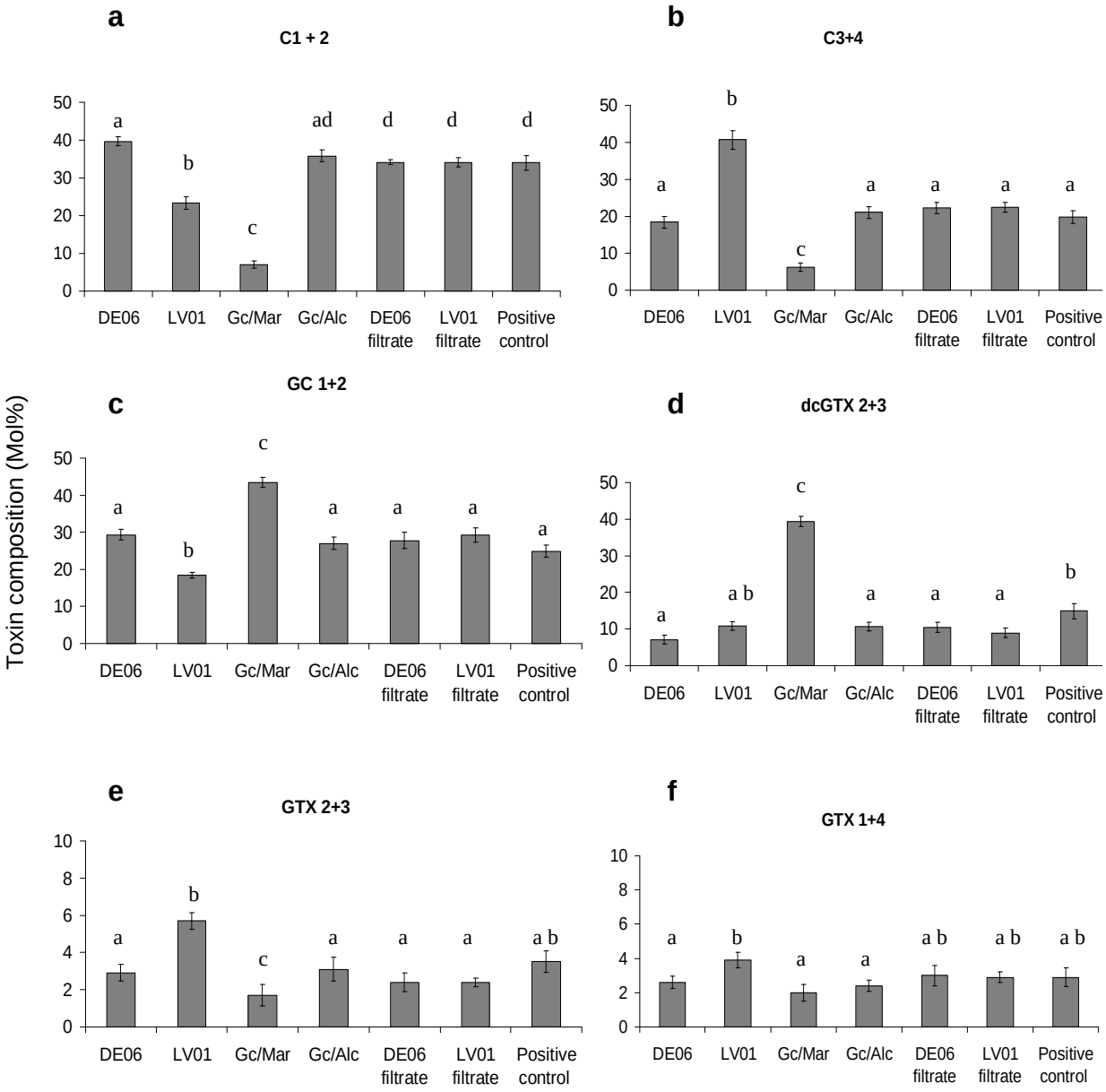

Figure 6.7 Profiles (mol %) of each toxin group identified from HPLC analysis on cultures at Day 25 (harvest), Error bars ± SD. Superscripts indicate significant differences ($p<0.05$), means with the same letter are not significantly different.

The different toxin composition of the cultures treated with *Marinobacter* sp. DG879 (Gc/Mar) is clearly demonstrated in the chromatogram of the toxins of the positive control cultures (that have a mixed natural bacterial assemblage) and Gc/Mar (Fig.

6.8). The toxin C2 dominates the positive control cultures; however this toxin is only a minor component in the Gc/Mar treatment (Fig. 6.8 b). The trend is reversed for dcGTX3, a minor component of the positive control cultures but a major contributor to the profiles of Gc/Mar cultures.

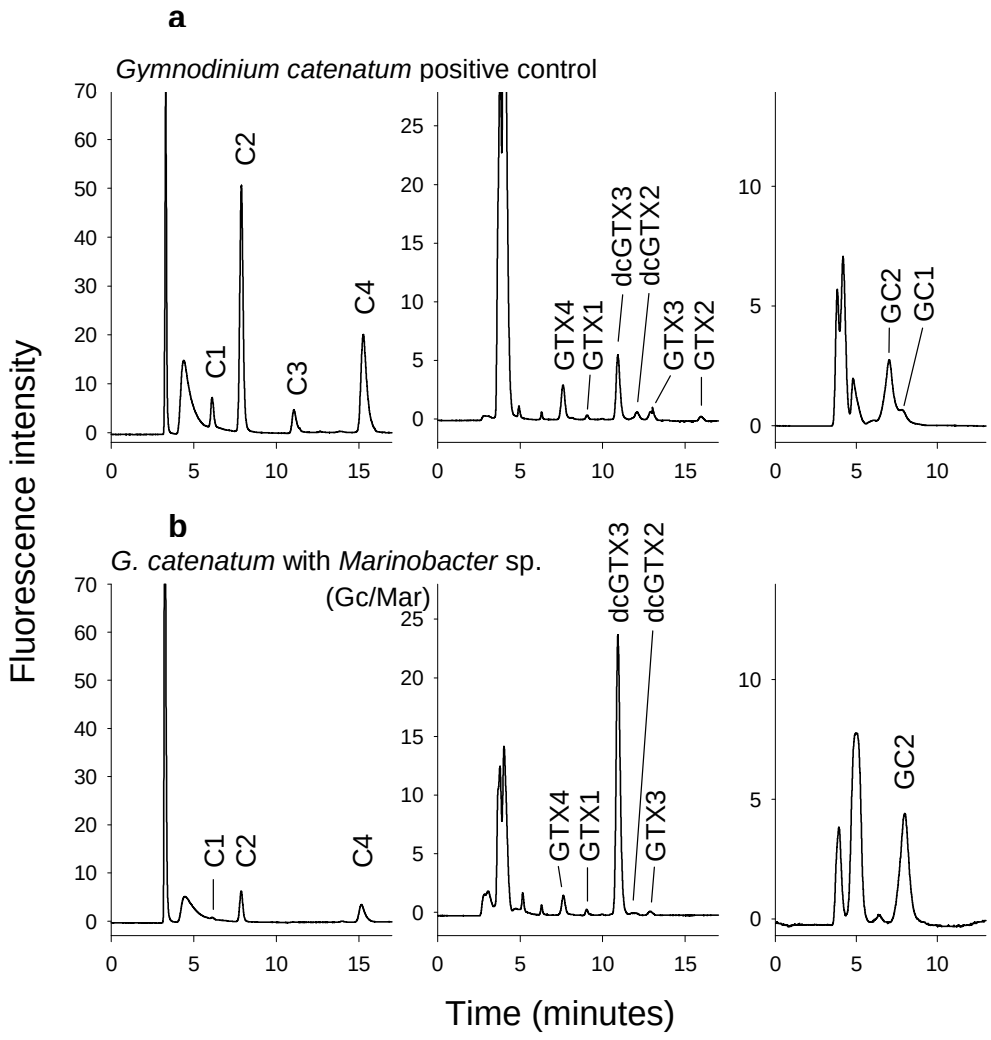

Figure 6.8 HPLC chromatograms of (a) *Gymnodinium catenatum* positive control (DE06 x LV01 cross with natural bacterial assemblage) and (b) with the addition of *Marinobacter* sp. DG879 (Gc/Mar).

The similarity among the toxin profiles of the different treatments based on cluster analysis of Euclidean distances show that cultures grown with the *Marinobacter* sp.

DG879 (Gc/Mar) and also parent culture LV01 cluster as two separate groups, while the remaining 25 cultures are interspersed (Fig. 6.9).

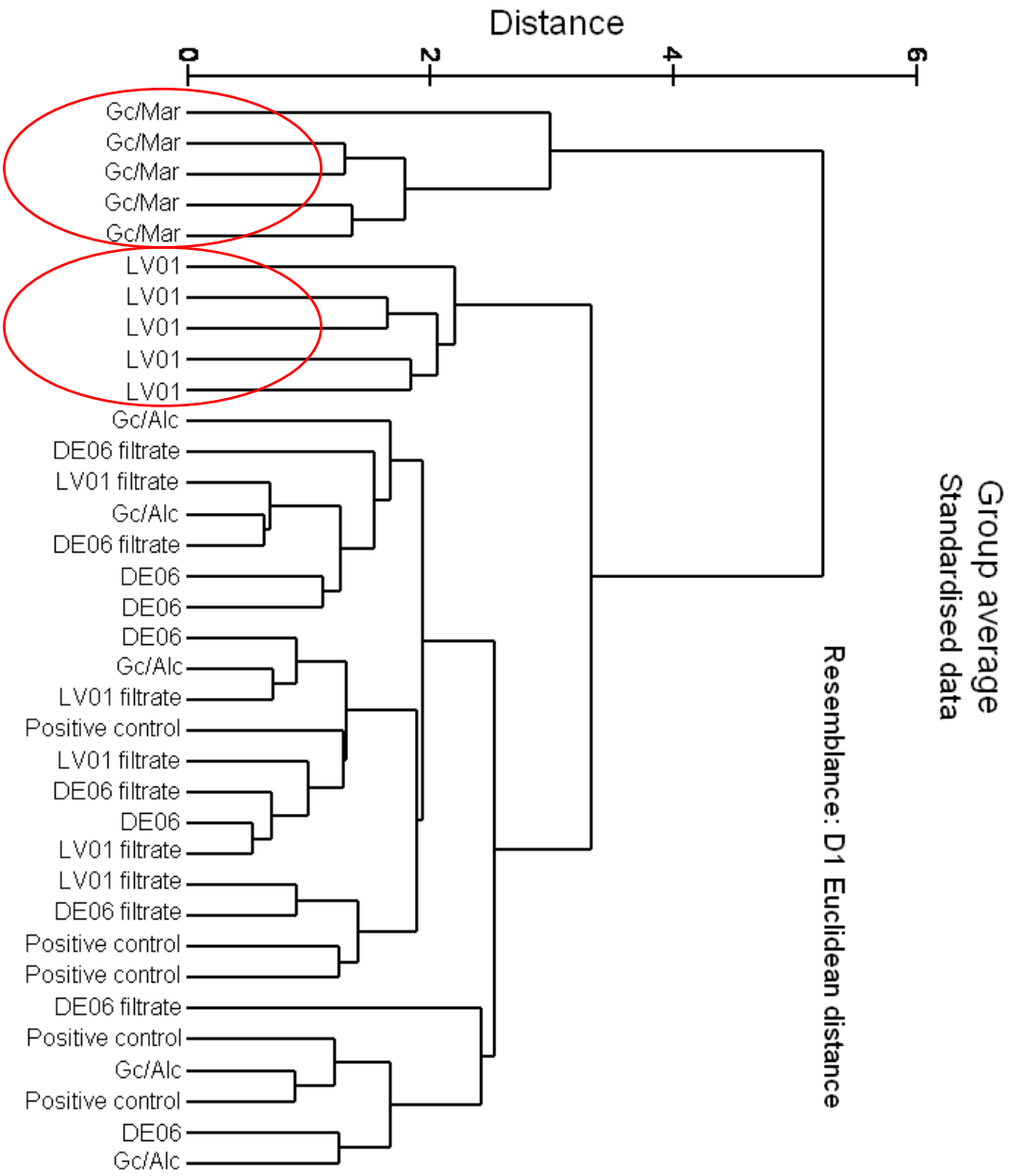

Figure 6.9 Cluster analysis of toxin profiles of 35 *Gymnodinium catenatum* cultures grown with different microbial communities. Clustering based on Euclidean distances calculated from standardised data of the PST fractions. For codes see Table 6.1.

6.4 Discussion

6.4.1 Total amount of toxins in *G. catenatum*

In this study, across all analysed cultures, the total cellular toxin content varied between approximately 95 and 205 fmole cell^{-1}, values comparable to previous research which indicated that the toxicity of vegetative cells of *G. catenatum* can vary considerably, usually ranging between 10 – 250 fmole cell^{-1} (e.g. Oshima et al., 1993; Negri et al., 2001; 2007), depending on cell physiology, growth medium, isolation methods and bloom location (e.g. Flynn et al., 1996; Negri et al., 2007).

This study also found that cyst-derived cultures produced a similar amount of cellular toxin to the non cyst-derived cultures. This is contrary to earlier studies which have reported that cyst-derived cultures produce less toxin (Negri et al., 2001; Bolch et al., 2001). The lower toxin content has been considered a response to the removal of biological factors that are prevalent in the natural environment (i.e. bacteria, viruses and their biochemical influences) which may affect algal cell physiology and PST production (e.g. Bolch et al., 2001; Uribe and Espejo, 2003). A possible reason to why the cyst-derived cultures in this study (all but the DE06 and LV01 parent cultures) did not show a difference in total toxin content in comparison to the non-cyst derived DE06 and LV01 may be because of the added bacterial cultures and the resulting influence of this interaction on algal biochemistry (including toxin production). This however does not explain why the positive control attained similar amounts of total toxin per cell as there were no additional bacterial cultures or filtrates added to the cysts, and the only bacteria present would theoretically be the bacteria still attached to the outer cyst wall after washing. Bolch et al. (2001) found that germination of washed cysts in the laboratory, resulted in strains with a significantly reduced PST content (>40-fold reduction in STX cell^{-1}) and PST profile, however, Bolch et al. (2001) used sonication as a step in the cyst isolation process, possibly increasing the effectiveness of this washing step. The most likely reason behind the comparably high toxin content of the positive control in the present study would therefore be that surface-attached bacteria have persisted through germination and early culture development, facilitating “normal” toxin biosynthesis.

Another factor to consider is genetic variation. The positive control has, in theory, the same kind of genetic combination of strains as the other treatments, so assuming that genotype plays a role, the experimental cultures with the different bacterial assemblages could be expected to be more similar to each other, than to the two parent strains (DE06 and LV01). A study by Subramanian (2008) found that the bacterial community can to some degree select the bacterial types that establish in a culture. In which case, it would not be surprising if the genotypes established from the mixed bacterial communities would be rather similar to each other, and quite distinct from either DE06 or LV01. In terms of toxin content, the fact that there was not a lot of difference among the treatments could indicate that the genotype is a stronger factor in toxin production in comparison to the composition of the bacterial community.

6.4.2 Toxin production rate

Specific toxin production rates have not previously been reported for *G. catenatum*; however, the values attained in this study are comparable to those found for another dinoflagellate, *Alexandrium fundyense* (Anderson et al., 1990) where, like in this study, a growth rate of 0.15 div. day^{-1} corresponds to a specific toxin production rate of 0.10 fmol day^{-1}.

This study found a strong linear correlation of specific toxin production rate μ_{tox} with specific growth rate comparable to findings by Anderson et al. (1990) for *Alexandrium fundyense*. The poor correlation between the net toxin production rate R_{tox} and the specific growth rate is also similar Anderson et al. (1990), showing that toxin synthesis in batch-cultures may be un-coupled from cell division (possibly as a result of nutritional deficiencies), leading to highly variable rates of toxin accumulation. In the present study, nutritional limitation was minimised by harvesting the cultures while still in their logarithmic growth phase. The lack of correlation may instead result from differences in cell physiology which varies with the age of the cells. While toxins are being synthesised from low molecular weight metabolites (Cembella, 1998), cell growth is a more complex process depending on factors such as temperature, light, salinity, nutrient concentrations along with accumulation of allelochemical (bio-chemicals that influence the growth and development of other organisms) and autoinhibitory metabolites (Cembella, 1998; Cembella and John, 2006). Another

factor influencing toxin biosynthesis is genetic regulation (Cembella, 1998). While in this study I attempted to control for genetic influences by using clonal cultures, each replicate is potentially containing a different combination of discrete genotypes. This is derived from the fact that the progeny cultures (i.e. the experimental cultures with altered bacterial assemblage) are populations of related genotypes; equivalent to a collection of 'brothers and sisters' from a single set of parents. As Subramanian (2008) found the bacterial community to select for particular genotypes, it may be possible that the genotypes selected by the specific bacterial assemblages in this study have an influence on the toxin production.

6.4.3 Toxin profile

The toxin profiles of all treatments were dominated by the C-toxins together with GC1+2, which has also previously been described for strains from the Derwent Estuary by Oshima et al. (1993) and Negri et al. (2003). The lack of dcSTX, STX or GC3 detected in this study differs from the findings of Negri et al. (2007) where all three toxin species were minor components detected in cultures of DE06. It is possible that the presence of toxins without the 11-hydroxysulfate residues may due to specific bacterial associations and/or culture conditions.

Results from the analysis of the DE06 cultures at day 25 (late logarithmic growth phase) are comparable to those found by Negri et al. (2007) and Oshima et al. (1993) who also performed the toxin analysis on DE06 at late logarithmic growth phase. All three studies found C1+2 and C3+4 to be the dominating toxin species, while Negri et al. (2007) and this current study also considered the hydroxybenzoate containing GC1+2. A comparison of the STX profile of strain DE06 with previous studies is shown in Table 6.5.

Table 6.5 Comparison of DE06 toxin composition (mol %) between this study (mean of cultures), Negri et al. (2007) and Oshima et al. (1993). ND = Not Detected, NT = Not Tested.

	Toxin composition (mol%) of DE06									
	C1+2	**C3+4**	**GC1+2**	**GC3**	**GTX1+4**	**GTX2+3**	**GTX5+6**	**dcGTX2+3**	**dcSTX**	**STX**
This study	39.7	18.4	29.3	ND	2.6	2.9	ND	7.1	ND	ND
Negri et al. 2007	10.7	27.4	40.5	14.2	0.03	0.1	1.2	4.1	1.7	0.1
Oshima et al. 1993	20.9	70.3	NT	NT	0.1	0	7.5	0.1	1.2	NT

Although toxin composition (i.e. PST profile) of *G. catenatum* is generally stable and consistent within a population, different global populations commonly exhibit minor differences in the profiles (Oshima et al., 1990, 1993; Negri et al., 2007). Past analyses of DE06 revealed similar toxin profiles to this study, although in this study we detected GTX1+4 whereas in the past, GTX5+6 were detected. However, these toxins were minor toxins in all cases. These variations in toxin chemistry may again be due to nutrient limitation and aging of dinoflagellate cultures, something earlier shown by Boyer et al. (1986), Anderson et al. (1990), Taroncher-Oldenburg, (1997), and Lippemeier et al. (2003).

The composition of the microbial communities had no significant effect on toxin composition of the cyst-derived cultures of *G. catenatum*, except for the cultures treated with *Marinobacter* sp. DG 879. These cultures exhibited significantly lower C1+2 and C3+4 and greater dcGTX2+3 and GC1+2. This change in profile may be due to either direct transformation of the toxins by the bacteria, or to the presence of the bacteria affecting the toxin synthesis. Other studies have shown bacteria to be capable of transforming PSTs by enzymatic reactions. Kotaki et al. (1985a, 1985b, 1989) focussed on the conversion of the gonyautoxins GTX1 and GTX4 and the highly toxic saxitoxins by marine invertebrates, while other studies focussed on specific bacterial groups capable of degrading PSTs (Donovan et al., 2009). In the case of the *Marinobacter* sp. DG879 treated *G. catenatum* cultures (Gc/Mar); it is possible that the shift in toxin composition is due to *Marinobacter* sp. DG879 converting the C1+2 into their corresponding decarbamoyl derivatives dcGTX2+3. This type of

bacterial bio-conversion has previously been shown by both Choi et al. (2003) and Sullivan (1983) from studies on bacteria associated to bivalves, and Smith et al. (2002) found dinoflagellate-associated bacteria to be able to transform PST using both oxidase and reductase activity.

The reason for the elevated levels of GC1+2 in the *Marinobacter* sp. DG879 treatment (Gc/Mar), i.e. the pathway leading to biosynthesis of GC-toxins, is unknown. However, Negri et al. (2003) showed GC1 and GC2 to be *p*-hydroxybenzoate analogues of the carbamate toxins GTX2 and GTX3, respectively. All cultures tested in this study, including those treated with *Marinobacter* sp. DG879, however, showed equally low proportions of GTX2 and GTX3 (2-7 mol %) indicating that the elevated levels of GC1 and GC2 (approx. 45 mol %) in the *Marinobacter* sp. DG879 treatment, were unlikely to have been converted by the available GTX2 and GTX3 in the cultures as this in limited supply for biosynthesis of GC1 and GC2. However, the C2-toxins can be converted to GTX3 in the presence of natural reductants (Laycock et al., 1995) and/or by enzymatic transformations (Cembella et al., 1993; 1994). Conversely, the β-epimers C2 and GTX3 can be easily converted into the more stable α-epimers C1 and GTX2, by epimerization at the C-11 hydroxysulfate at neutral pH (Oshima, 1995) thereby allowing production of GC1 and GC2 from the GTX2 and GTX3 derived from C1 and C2.

Bacterial influences on toxin synthesis have been found in several microalgal species e.g. *Ostreopsis lenticularis* (Pérez-Guzmán et al., 2008), *Alexandrium* spp. (Jasti et al., (2005), and *Heterosigma carterae* (Carrasquero-Verde, 1999), and several studies have suggested autonomous production of PST toxins by bacteria (e.g. Gallacher et al., 1996; 1997; and Töbe et al., 2001). However *Marinobacter* sp. DG879 is not capable of autonomous production of any PST-like toxins (Green et al., 2004), indicating that if it is the bacteria causing the differing toxin composition in the Gc/Mar cultures, then it may instead be a result of biochemical interactions between the alga and the bacteria, rather than autonomous production of any PSP-like toxins. However, as this study only focussed on intracellular PST content, biotransformation of the PSTs seems unlikely unless *Marinobacter* sp. DG879 (or the biological agent of the biotransformation activity) could be expressed within the *G. catenatum* cells.

Alternatively there could be other indirect physiological mechanisms, like a bacterial production of compounds influencing the phycotoxin production of the algae. Bates et al. (1995) found that when bacterial strains, isolated from two non-axenic *P. multiseries* clones, were reintroduced individually into cultures of three axenic *P. multiseries* strains, the bacteria caused a 2- to 115-fold enhancement of domoic acid (DA) production (per cell basis) relative to axenic cultures. As none of the isolated bacteria were capable of autonomous production of DA, it suggests that the bacteria instead may produce precursors that are used directly in DA synthesis, regenerate nutrients, provide essential dissolved organic matter (e.g. elicitors such as polysaccharides, small proteins, or lipids), or increase the carbon dioxide content of the growth medium, which would provide carbon for the DA synthesis. A similar study was performed using an initially axenic non-toxic strain of *Heterosigma carterae*, in which toxicity was induced by addition of *Vibrio natriegens* or *Alteromanas haloplanktis* (both non-toxic marine bacteria) (Carrasquero-Verde, 1999). As on their own, both *H. carterae* and the bacteria were non-toxic, Carrasquero-Verde suggested that the bacteria could provide *H. carterae* with both some kind of stressor element and/or a precursor molecule which would triggers toxin synthesis.

The GC-toxins have to date only been found in *G. catenatum*, and represent an important contribution of the toxin profile found in strains from a range of countries such as Australia, Portugal, China and Spain (Negri et al., 2007; Vale, 2008a; 2008b), suggesting that production of toxins might be genetically driven (Flynn et al., 1996). Earlier studies have also showed genetically driven toxin production. Sako et al. (1992) and Ishida et al. (1993) reported on a chromosomal localisation of the saxitoxin biosynthetic genes where the F1 progeny (Mendelian inheritance) separated 2:2 with respect to the parental toxin phenotype. One parental strain produced gonyutoxin 4 and C4, while the other produced neosaxitoxin and saxitoxin. The F1 progeny showed one parental toxin composition and segregated independently with the mating type.

Results from this study suggest that the genotype (i.e. the genetic constitution) of the algae is a strong factor in toxin production. The study found that the STX content and the toxin profile of the cyst-derived offspring both appear to predominantly be an average between the parental ranges, indicating that the STX content and the establishment of the toxin pro-

file is genetically driven. However, considering that all cultures contain populations of related genotypes, this is not an unlikely outcome. Bacterial communities have been shown to select for particular genotypes in *G. catenatum* (Subramanian, 2008), and this in turn suggests that the genotypes selected by the specific bacterial assemblages in this study could have an influence on the toxin production. Although this indicates that the genotype is a stronger factor in toxin production than the bacterial community composition, the results from the STX profile data of *G. catenatum* with *Marinobacter* sp. DG879 still demonstrate that the bacterial community can have a significant effect on the profile of the STX produced.

Conclusion

The results of this study indicate that *Marinobacter* sp. DG879 may play a role in PST production of *G. catenatum*, most likely as a result of biochemical interactions between the alga and the bacteria. *Alcanivorax* sp. DG881 did not appear to have an effect on toxin production, showing total amounts of toxin per cell and toxin profiles similar to both multi-bacterial parental strains and uni-bacterial cultures (excluding *Marinobacter* sp. DG879). However, the results also suggest that comparing PST production and content on its own may be misleading, as growth rates and physiological stress may strongly influence toxin production. There is still much scope for further investigations into the specific relationships of bacteria and dinoflagellates in *G. catenatum* toxin production, particularly in terms of determining the genetic and environmental factors that are involved.

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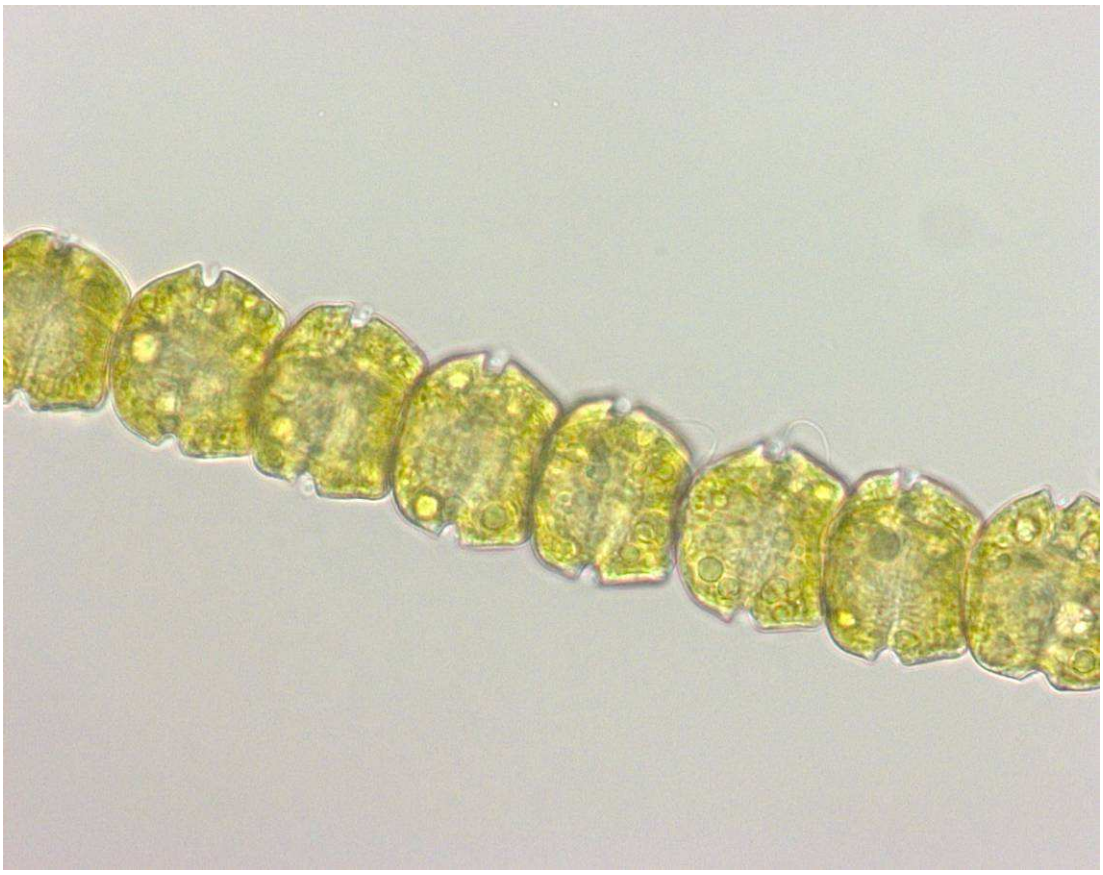
Chapter 7:
Summary and Conclusions



Chapter 7: Summary and conclusion

The increased frequency and global expansion of harmful algal blooms (HABs), coupled with the growing economic impacts, are the large driving forces behind the desire to understand the factors that influence algal blooms. Although both abiotic and biotic factors are known to influence HABs, the effect of microbial interactions, including algal-bacterial interactions, although recognized as important factors since the 1980s, has had limited investigation. The results from this thesis have added to the current knowledge on algal-bacterial interactions, showing growth enhancement and altered toxicity of algae as a result of bacterial involvement, thereby playing a potentially significant role in controlling the algal bloom dynamics. Below is a conceptual diagram based on the review by Cole (1982), modified with red arrows indicating the areas where this thesis has contributed new data and understanding to the interactions of algae and bacteria (Fig. 7.1).

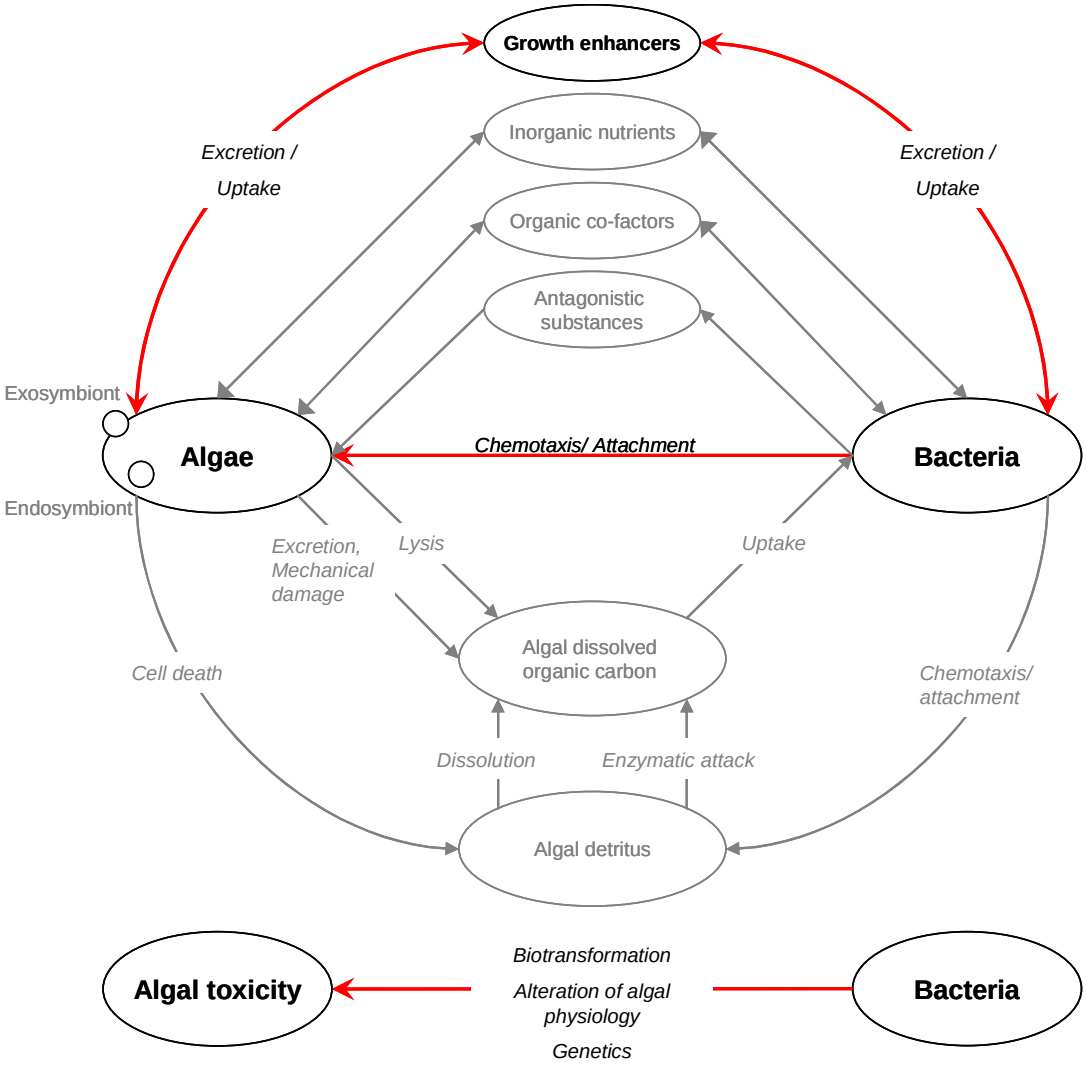


Figure 7.1 The phycosphere and some of the interactions that occur within it (based on review by Cole, 1982). Red arrows indicate the areas where this thesis has contributed with new data.

The specific outcomes of each chapter are summarised in the following sections.

7.1 Reproductive compatibility and the requirement for bacteria

The toxic HAB dinoflagellate *Gymnodinium catenatum* has been cultured and studied in the laboratory since the 1980s, with a number of strains having been cultured for more than two decades, and with sexual reproduction and resting cyst production reasonably readily achieved in culture (Blackburn et al., 1989). As long-term culturing has been shown to influence sexual reproduction (Destombe and

Cembella, 1990) a trial was set up where ten Tasmanian *G. catenatum* strains were pair-wise crossed (intercrosses testing for heterothallism) as well as self-crosses (testing for homothallism) in order to determine the reproductive compatibility between the strains. The most successful strain cross was shown to be between the heterothallic strains GCHU11 and GCHA01, which showed the highest production of cysts in the shortest timeframe. This study demonstrated that GCDE08 is both homothallic and heterothallic, i.e. capable of producing cysts in both self-crosses and inter-crosses. It appears that GCDE08 homothallism has evolved over time in culture as studies by Blackburn et al. (1989; 2001) did not find GCDE08 to self-cross while it did in later studies by Parker (2002) and Vincent (2003). The progeny produced by the GCDE08 self-cross were, however, non-viable, not surviving beyond the 8-cell stage (Blackburn et al., 2001). The successful strain-cross GCHU11 × GCHA01 was used for large-scale cyst production, with the cysts in turn being used to establish experimental model systems of *G. catenatum* with specific bacterial assemblages. The experimental model systems developed in this thesis were established with two bacterial strains, *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881, either as uni-bacterial or mixed-bacterial cultures of *G. catenatum*.

7.2 Real-time PCR assays for bacterial enumeration

In order to study the interactions between *G. catenatum* and its two growth-promoting bacterial associates, *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881, quantitative real-time PCR probes (TaqMan MGB format) were developed from the 16-23S rDNA Intergenic Transcribed Spacer region (ITS). The ITS region has been frequently used to discriminate bacterial strains at the species and intraspecies levels (e.g. Syutsubo et al., 2001; Lee et al., 2002; Fuhrman et al., 2008), allowing for a more rapid detection and enumeration in comparison to traditional methods of microscopy and culturing. Probes and primers were validated in a variety of ways; (1) Assessing cross-reactivity by comparison of the primer and probe sequences to bacterial sequences available in the NCBI GenBank; (2) Trialling primers against five bacterial strains, *Roseobacter* sp. DG874, *Janibacter* sp., *Brachybacterium* sp., *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 using SYBR Green Chemistry;

(3) Trialling developed qPCR assays against five bacterial strains, *Roseobacter* sp. DG874, *Janibacter* sp., *Brachybacterium* sp., *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881; (4) Screening 45 bacterial isolates from *G. catenatum* using the developed TaqMan qPCR assays; (5) Screening 61 bacterial strains isolated from whole water using the qPCR assays, and; (6) Trialling uni-bacterial and multi-bacterial cultures of *G. catenatum* in order to determine whether the presence of *G. catenatum* would affect the specificity of the PCR. The developed assays were shown to be capable of non-specific amplification, although the non-specific amplification occurred very late in the PCR (only after >30 cycles), while target organisms were amplified at 15-18 cycles (using equal DNA concentrations of target and non-target organisms). The non-specific amplification would therefore be significant only when the target DNA was at low abundance. The qPCR assays developed were able to distinguish between *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 in uni-bacterial or mixed bacterial systems containing only the two bacteria, allowing the assays to be used in further investigations of the interactions between *G. catenatum* and the two bacterial strains in the experimental model systems used in this research.

7.3 Bacterial influence on growth dynamics

By using the developed molecular detection probes described in the former chapter, growth dynamics of *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881 were studied in the experimental model systems where the bacteria are in close interaction with *G. catenatum*, either on their own or in a mixed bacterial community. The results illustrate that, although both bacteria are shown to be growth promoting, they affect *G. catenatum* growth in entirely different ways. The *G. catenatum* / *Marinobacter* sp. DG879 model system show that *Marinobacter* sp. DG879 was able to sustain growth of *G. catenatum*, without any addition of nutrients beyond what was in the initial culture medium, over a period longer then ever recorded (>420 days). The two organisms have a close growth correlation to one another, with *Marinobacter* sp. DG879 cell abundance closely matching the growth phases of the alga. *Alcanivorax* sp. DG881 did not have the ability to maintain prolonged *G. catenatum* growth like *Marinobacter* sp. DG879. However, *Alcanivorax* sp. DG881 was still capable of maintaining a growth pattern of *G. catenatum* comparable to that

which is seen in 'normal' non-axenic *G. catenatum* cultures (i.e. *G. catenatum* as isolated from the natural environment with its natural microbial consortium). *G. catenatum* grown with *Alcanivorax* sp. DG881 displayed three distinct growth phases and a culture life span of approximately 90 days. This is in agreement with earlier studies that *Alcanivorax* sp. DG881, in its own right, can act as a growth promoter for *G. catenatum* (Bolch et al., 2004). When *G. catenatum* was grown in the mixed-bacterial model systems, the resulting growth curve did not display the clear growth phases seen in the uni-bacterial model systems with *Alcanivorax* sp. DG881, but instead a prolonged 2-step logarithmic growth phase, followed by a rapid death phase.

The results from the uni-bacterial model systems suggest that *Marinobacter* sp. DG879 produces a compound which influences a significant positive growth response in *G. catenatum*. Additionally, it is also possible that the bacteria may be a factor in carbon recycling or nitrogen fixation, in turn allowing for the prolonged growth of the cultures. The presence of *Alcanivorax* sp. DG881 with *G. catenatum* supported excystment and vegetative cell division of *G. catenatum*, but lacked the ability to sustain prolonged growth of *G. catenatum* (as was the case for *Marinobacter* sp. DG879). This could be due to changes in the bacterial gene expression, for example, possibly brought on by an increasing bacterial cell concentration or changes in the surrounding media. A change in gene expression could cause *Alcanivorax* sp. DG881 to change from growth stimulating to growth inhibitory, thereby no longer stimulating *G. catenatum* growth. The different growth dynamics of *G. catenatum* found in the mixed bacterial systems may be due to changing bacterial dynamics and a competition between different bacteria. The *G. catenatum* growth in the mixed bacterial systems were found to be an intermediate between the two uni-bacterial systems, suggesting that the bacteria may individually influence the alga even in the presence of the other bacterium.

Bacterial chemotaxis was found in both the uni-bacterial model systems, and could be a response to low production and release of DOM by the algae, necessitating the bacteria to draw near and attach (Garg and Garg, 2007). Alternatively, the algae could be encouraging bacteria to approach by limiting the release of certain DOM fractions, thereby possibly allowing the algal cell to benefit from bacterial siderophore production (Amin et al., 2009).

7.4 Growth-promoting bacteria in field samples

As the bacterial species have been found to be of such importance to laboratory-grown cultures of *G. catenatum*, there was reason to consider that they would be significant in the growth dynamics of naturally occurring *G. catenatum*.

The developed qPCR assays were applied to a range of field water samples in order to detect bacterial presence in *G. catenatum* bloom and non-bloom conditions. The samples originated from four locally occurring *G. catenatum* blooms in southeast Tasmania (Deep Bay, Fleurtys Point, Port Esperance and Woodbridge) together with samples taken over a 6 month period between October -08 to March -09, from an area commonly affected by blooms during some parts of the year (Hobart CSIRO Wharf station - Derwent Estuary). The qPCR assay developed for detection of *Marinobacter* sp. DG879 detected an increased *Marinobacter* abundance just prior to the onset of a *G. catenatum* bloom in the Derwent Estuary (CSIRO Wharf station), whereas from mid February to mid May 2009, *Marinobacter* abundance (as calculated from the probe detection) increased from ~10^5 – 10^8 cells mL^{-1}, whilst during the same time, *G. catenatum* abundance increased from 0 to 17 000 cells L^{-1}. Also, in the Derwent Estuary, the qPCR assay developed for *Alcanivorax* sp. DG881 detected elevated levels of bacteria which were followed by a diatom bloom of *Skeletonema* spp. and *Pseudonitzschia* spp. However; gene sequencing of representative qPCR products indicated that the qPCR assay was detecting a *Roseobacter* species rather than the *Alcanivorax* target, and use of the *Alcanivorax* sp. assay was terminated.

With regards to the *G. catenatum* bloom samples, *Marinobacter* sp. was detected at all bloom sites, but there did not appear to be a clear correlation between *Marinobacter* sp. and *G. catenatum* abundance. This could be a result of numerous factors influencing the bloom, in particular biological factors, such as the stage of development and the age of the bloom, and also physical factors, the location and currents, and also depending on the development, specificity and the detection limits of the qPCR assay. As the total bacterial abundance was not measured during the study, the effects of bacterial fluctuations are difficult to determine but could be an influencing factor. When comparing blooms from all the sites no significant difference in the *Marinobacter* sp. abundance was found between proliferating and declining

blooms, although the proliferating blooms showed a slightly higher *Marinobacter* sp. abundance. In the case of *Marinobacter* sp. the bacteria reached higher numbers prior to the onset of *G. catenatum* blooms (results from the CSIRO Wharf station), thereby suggesting that *Marinobacter* sp. could possibly play a role in the initiation of *G. catenatum* blooms.

7.5 The effect of bacteria on toxin production

The type of bacteria associated with toxic dinoflagellates has been shown to play a role in dinoflagellate toxicity (Gallacher and Smith, 1999; Hold et al., 2001; Uribe and Espejo, 2003). Considering the very close relationship between laboratory-grown *G. catenatum* and the bacteria *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881, I hypothesised that these two bacteria could influence *G. catenatum* toxin production and composition. By using the uni-bacterial model systems I set out to determine whether there could be a difference in *G. catenatum* between uni-bacterial (containing either *Marinobacter* sp. DG879 or *Alcanivorax* sp. DG881) and 'normal' multi-bacterial *G. catenatum* cultures (i.e. as isolated from the natural environment). HPLC analysis was used to determine toxin species and content of the different treatments.

The study compared seven different *G. catenatum* cultures, out of which two strains, (1) GCDE06 and (2) GCLV01, were used as clonal (independent) controls. The experimental model system approach was then employed to generate genetically similar non-clonal *G. catenatum* cultures (by crossing GCDE06 and GCLV01) to which specific bacterial assemblages were added in order to study the effect of bacteria. The remaining five cultures were made up of: (3) cyst-derived cultures with *Marinobacter* sp. DG879; (4) Cyst-derived cultures with *Alcanivorax* sp. DG881; (5) Cyst-derived cultures with a bacterial filtrate from GCDE06; (6) Cyst-derived cultures with a bacterial filtrate from GCLV01; and (7) acting as a positive control, a cyst-derived culture with a natural bacterial assemblage from the GCDE06 × GCLV01 inter-cross culture.

Analyses found toxin content cell^{-1} (at harvest, Day 25) to be within the range of what has been found in previous studies and the rate of saxitoxin (STX) production

was not significantly altered by the bacterial community composition. However, the proportions of the different STX compounds differed significantly. The *G. catenatum* cultures containing *Marinobacter* sp. DG879 exhibited lower amounts of C1+C2 and C3+C4 and greater amounts of dcGTX2+dcGTX3 and GC1+GC2 than the other treatments and the positive control. This differing toxin composition could be due to *Marinobacter* sp. DG879 influencing toxin synthesis indirectly through its effect on dinoflagellate physiology, or perhaps by biotransformation of the PST compounds. However, because this study focussed on intracellular toxins, and as biotransformation most commonly operate on extracellular STX, in this case biotransformation would seem unlikely. The experimental design tried to minimise genetic variation among treatments. Nevertheless, different clones may have been advantaged by the different bacterial communities selecting for particular genotypes in each treatment, in turn leading to changes in the toxin profile. Although the exact cause of the altered toxin profile in the *G. catenatum*/ *Marinobacter* sp. DG879 culture systems is unclear, that there is a significant alteration is clear, with the most likely explanation appear to be the one of *Marinobacter* sp. DG879 influencing toxin synthesis through its effect on *G. catenatum* physiology.

7.6 Conclusions and aspects for future research

As we are facing an ever growing problem of algal blooms affecting not only the natural environment and its inhabitants but also aquaculture and recreational activities, there is a need to assess, understand, and monitor the factors that are influencing the harmful algal blooms. The factors that trigger bloom formation are poorly known for most phytoplankton species, predominantly because most blooms are infrequent and sufficient data on pre-bloom conditions are usually not obtainable (Walch et al., 2001). Given the lack of such significant information, Cembella (1998, p. 650) described the challenges of modeling bloom predictions: “Attempts at dynamic modeling using conventional input parameters (nutrient uptake kinetics, grazing rates, specific growth rates, etc.), and based upon a simple trophic structure paradigm…have been of little utility”. On the whole, progress towards reliable predictions of blooms continues to be made, with factors like self-shading, intraspecific competition for nutrients, bloom maintenance, senescence, and dispersion now being better understood

(Smayda, 1997). Nevertheless, despite their evident importance, the limited knowledge of factors such as microbial interactions, mixotrophy, and allelopathy, cause these complex biological features to not yet being incorporated into models.

This thesis addressed the issue of microbial interactions, and particularly focussed on growth responses of bacterial interaction on phytoplankton. By using experimental model system in conjunction with qPCR assays, this thesis has demonstrated that *Gymnodinium catenatum* express distinctive growth responses depending on the associated bacterial species, and demonstrated that some bacteria are capable of enhancing growth and longevity of laboratory grown algae. Nonetheless, the compounds or pathways that mediate the interactions between *G. catenatum* and the bacteria are still unidentified, but could, as also suggested by Cole (1982), include remineralisation of inorganic nutrients, and/or a production of organic co-factors.

Furthermore, this thesis showed that specific bacteria can play a role in biotoxin production of *G. catenatum*, significantly influencing the toxin profile of the algae through their effect on dinoflagellate physiology. The field studies also suggest that bacteria may have a role in natural populations of *G. catenatum*, with *Marinobacter* sp. DG879 possibly functioning as an indicator-species for *G. catenatum* in Tasmanian waters.

Nevertheless, in terms of the algal-bacterial interactions more research is still required in order to establish mechanisms of interaction and their significance in natural populations of *G. catenatum* and other dinoflagellates. Even considering *G. catenatum* and its interactions with *Marinobacter* sp. DG879 and *Alcanivorax* sp. DG881, there is much to discover in terms of how this relationship is mediated and whether the interactions are important in natural blooms. In order to fully understand their connection, there are several questions that remain to be answered, for example:

- What compounds, signalling processes and/or pathways mediate the interactions between *G. catenatum* and *Alcanivorax* or *Marinobacter*? By using a combination of methods such as liquid chromatography-mass spectrometry (LC-MS), nuclear magnetic resonance spectroscopy (NMR), and polymerase chain reaction (PCR); chemicals can be detected and identified, structures of

organic compounds can be determined, and functional genes can be recognized.

- How are the compounds and signals transduced in both the dinoflagellate and bacterial cells? By tracking stable isotope signatures, in both algal and bacterial cultures, the pathways of compounds such as carbon and nitrogen can be identified.

- What specific physiological effects do the interactions have on the dinoflagellate, the bacterium? If the specific compounds involved in the interactions can be determined, there is a possibility for further manipulation of the experimental culture systems, in turn likely establishing the physiological effects.

- What environmental prerequisites are required for algal-bacterial interactions? By analysing the dissolved organic carbon and available nutrients in the environment their correlation with bacterial and algal abundance can be established

Finding the answers could ultimately allow science to tackle one of the most importance questions we are currently facing in terms of algal/bacterial interactions, i.e. are bacteria the driving force behind algal bloom development and control the extent and the decline of the bloom?

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Appendix 1: Preparation of GSe culture medium for dinoflagellates

(Blackburn, S. I. Bolch C. J. S. Haskard K. A. and Hallegraeff G. M. (2001) Reproductive compatibility among four global populations of the toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae). 40(1): 78-87)

GSe is a variation of G medium (Loeblich, 1975) with the addition of Selenium.

Preparation method

1. Sea water

Sea water is collected from 10 m depth the east of Maria Island on the east coast of Tasmania (lat. 42 deg. 36 min. S, long. 148 deg. 14 min. E). The water is then purified using a filter system consisting of (1) a prefilter (Millipore Rogard) to remove particulate matter, (2) an activated charcoal filter (Cuno-Pacific Aquapure AP117) to eliminate soluble organics, and (3) a 0.45 µm filter (Millipore Durapore) for final purification. The seawater is then transferred to 1L screw-cap Teflon bottles and autoclaved (30 min at 121 ºC).

2. Stock solutions

(1) KNO_3		100 gL^{-1} H_2O
(2) K_2HPO_4		34.8 gL^{-1} H_2O
(3) Vitamins	B_{12}	0.1 mg $100mL^{-1}$ H_2O
	Thiamine HCl	100 mg $100mL^{-1}$ H_2O
	Biotine	0.2 mg $100mL^{-1}$ H_2O
(4) PII Metal Mix	Na_2EDTA	6.0 gL^{-1} H_2O
	$FeCl_3$ x 6 H_2O	0.29 gL^{-1} H_2O
	H_3BO_4	6.85 gL^{-1} H_2O
	$MnCl_2$ x 4 H_2O	0.86 gL^{-1} H_2O
	$ZnCl_2$	0.06 gL^{-1} H_2O
	$CoCl_2$ x 6 H_2O	0.026 gL^{-1} H_2O
(5) Selenium	H_2SeO_3	1.29 mgL^{-1} H_2O
(6) Soil Extract	(See below)	

3. GSe concentrated nutrient solution (excluding Soil extract)

A nutrient solution is made up in distilled water; for a 100mL mix:

10 mL Nitrate (KNO_3) stock

5 mL Phosphate (K_2HPO_4) stock

5 mL Vitamin stock

25 mL PII Metal Mix

5 mL Selenium (H_2SeO_3) stock

50 mL distilled water

The mixed solution is filter-sterilized under aseptic conditions using a 0.22 µm disposable filter unit.

4. Soil extract solution

Soil is collected from a natural, sandy bushland, uncultivated environment were no fungicides, insecticides or garden fertilizers have been present.

(1) Dry soil is sifted once through a coarse sieve and twice through a finer (1mm mesh) sieve

(2) 1 kg of soil is mixed into 2 litres of distilled water.

(3) Soil mix is the autoclaved for 60 minutes at 121ºC and cooled overnight.

(4) Filter the soil mix through absorbent cotton wool packed into the stem of a glass filter funnel.

(5) Centrifuge fluid at 5000 rpm for 20 minutes in 250 mL polyethylene centrifuge tubes and after, collect the brown supernatant.

(6) Filter the liquid again through absorbent cotton wool.

(7) Dispense the supernatant into 100 mL glass bottles (50 mL aliquots)

(8) Autoclave the bottles for 15 minutes at 121 ºC

(9) After cooling, wrap the caps with Parafilm to prevent contaminations.

(10) Store soil extract at 4 ºC.

5. Distilled water/MilliQ

Distilled water was autoclaved in 2L Schott bottles and allowed to cool before it was used to prepare media.

6. To prepare final media

In sterile Schott bottles, the following was added aseptically

800 mL Sterile sea water (1)

200 mL Sterile water/ MilliQ (5)

20 mL GSe concentrated nutrient solution (3)

5 mL Soil Extract (4)

Modifications

A modification of GSe was used in the cyst production experiments. This modification was GSe $^{-N-P}$ media, in which Nitrate and Phosphate were removed from the GSe concentrated nutrient solution and replaced by sterile MilliQ water.

Appendix 2: Preparation of ZoBell marine agar for bacteria

(Zobell, C. E. (1941). Studies on marine bacteria. I. The cultural requirements of heterotrophic aerobes. *Journal of Marine Research* 4, 42–75)

To prepare 1 L

1000 mL MilliQ water

33 g Sea salts (for 28‰ salinity)

5 g Bacto Peptone

1 g Yeast Extract

15 g Bacto-Agar

5 mL 100x Marine Supplement (see below)

Dissolve all ingredients in the MilliQ water, and for ease of handling, split the mixture into two 1 litre Schott bottles. Autoclave for 30 minutes at 121ºC and pour the plates when the agar has cooled to 55 ºC.

100 x Marine Supplement

1 mL 10 x Trace elements (see below)

10 mL 2 mg/L Na_2SeO_3

84 mL MilliQ water

Prepare and autoclave the above stock. Store at 4ºC.

10 x Trace Elements

4.36 g Na_2EDTA

3.15 g $FeCl_3$ x 6 H_2O

0.022 g $ZnSO_4$ x 7 H_2O

0.01 g $CoCl_2$ x 6 H_2O

0.18 g $MnCl_2$ x 4 H_2O

0.0063 g Na_2MoO_4 x 2 H_2O

Combine all above and filter sterilize. Store at 4ºC.

Appendix 3: Gymnodinium catenatum *and bacterial strain list*

Culture strain #	CS-code	Tasmanian Origin	Strain history/ clonal	Isolation date	Isolator	Culture media	Toxicology
GCDE 05	CS-301/05	Derwent River	n/a	8/1 1987	S. Blackburn	GSe	Toxic
GCDE 06	CS-301/06	Derwent River	Chain of 10 cells	8/1 1987	S. Blackburn	GSe	Toxic
GCDE 08	CS-301/08	Derwent River	n/a	15/6 1987	S. Blackburn	GSe	Toxic
GCDE 09	CS-301/09	Derwent River	Chain of 4 cells	n/a	S. Blackburn	GSe	Toxic
GCHU 02	CS-302/02	Huon River	Chain of 8 cells	6/6 1986	S. Blackburn	GSe	Toxic
GCHU 11	CS-302/11	Huon River	4-cell chain, produced from wild resting cyst	20/6 1988	S. Blackburn	GSe	Toxic
GCHU 20	CS-302/20	Huon River	n/a	4/4 1990	S. Blackburn	GSe	Toxic
GCHA 01	CS-304/01	Hastings Bay	Chain, product of wild resting cyst	29/6 1990	C. Bolch/ S. Blackburn	GSe	Toxic
GCVS 04	CS- 799	Verona Sands	Single chain	1/1 2002	C. Bolch	GSe	Presumed toxic
GCLV 01	CS-800	Louisville	Single chain	1/1 2002	C. Bolch	GSe	Presumed toxic

Bacterial strains

Culture strain #	Bacterial species	Isolated from	Isolator	Growth media	Effect on *G. catenatum* growth
DG 874	*Roseobacter*-like	*G. catenatum* DE 08	David Green	ZM 1	+
DG 879	*Marinobacter* sp.	*G. catenatum* DE 08	David Green	ZM 1	+
DG881	*Alcanivorax* sp.	*G. catenatum* DE 08	David Green	ZM 1	+
N/A	*Brachybacterium* sp.	*G. catenatum* DE06	Elisabeth Albinsson	ZM 1 + Na-acetate	unknown
N/A	Janibacter sp.	*G. catenatum* DE06	Elisabeth Albinsson	ZM 1	unknown

+ = always giving positive growth of *Gymnodinium catenatum* germinants.