Experimental Models of Bacteria-Phytoplankton Interactions and Bacterial Influence on Growth of Toxic Dinoflagellate, *Gymnodinium catenatum*

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Masters in Marine Biotechnology

Submitted in fulfilment of the requirements for the
Degree of Doctorate of Philosophy

National Centre for Marine Conservation and Resource Sustainability

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November 2008

Candidate declaration of originality

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Abstract

Interactions between phytoplankton and their associated marine microbial community are believed to have major effects on the growth dynamics of both bacteria and phytoplankton. Phytoplankton are associated with bacterial communities composed of 10's to 100's of bacterial types with the potential for millions of potentially confounding interactions. This complexity currently hampers research to identify key bacteria and the mechanisms of interaction. To overcome these problems, this work investigates the behaviour and dynamics of simplified experimental models of bacteria-phytoplankton to examine the influence of these microbial interactions on the dynamics of phytoplankton growth. The basis of the experimental system was the toxic dinoflagellate *Gymnodium catenatum,* a well known causative organism of paralytic shellfish poisoning associated with frequent blooms around the globe. Simplified microbial communities in cultures were generated from surface-sterilised resting cysts germinated in the presence of bacteria isolated from non-axenic *G. catenatum* cultures (*Brachybacterium* sp., *Alcanivorax* sp. DG881, *Marinobacter* sp. DG879 and *Roseobacter* sp. DG874) in either uni-bacterial or mixed-bacterial experimental model systems.

Using the simplified experimental model, a range of experimental manipulations of the bacterial community of *G. catenatum* were undertaken to examine specific hypotheses. The first experiment examined the hypothesis that *G. catenatum* has an obligate requirement for bacteria, using antibiotic-resistant and antibiotic- sensitive strains of marine bacteria to provide important negative and positive controls. Addition of antibiotics to cultures with sensitive bacteria resulted in a significant decline in dinoflagellate cell concentration, where as control cultures grown with antibiotic resistant bacteria continued to grow throughout the experiment. Importantly, this experiment demonstrated that removal of bacteria, rather than the action of antibiotic, caused the decline and death of the dinoflagellate culture.

The influence of bacterial community composition on *G. catenatum* batch culture dynamics was examined using uni-bacterial, and mixtures containing the γ-proteobacteria *Alcanivorax* sp. or *Marinobacter* sp. and/or the α-proteobacterium *Roseobacter* sp. Exponential growth rate, death rate, maximum cell concentration and batch culture dynamics were all influenced by the bacterial community composition in the experimental cultures, demonstrating that the bacterial community is a significant factor influencing the growth dynamics of *G. catenatum*. Uni-bacterial *G. catenatum* models showed that dinoflagellate grew significantly faster in the presence of *Alcanivorax* sp. or *Marinobacter* sp. than when grown with the α-proteobacterium *Roseobacter* sp. Pair-wise mixtures and tri-bacterial treatments displayed batch growth patterns intermediate or combined features of the respective uni-bacterial bacterial patterns, suggesting that bacterial community effects on dinoflagellate growth are additive. Epifluorescence microscopy and DAPI staining of cultures showed that uni-bacterial model cultures were not attached or associated with the dinoflagellate cell during logarithmic or stationary phase dinoflagellate growth but an increase in the proportion of bacteria associated with the cells wall was noted in late-stationary to death phase. During death phase, cultures grown with *Roseobacter* sp. showed a significantly higher proportion (18%) of bacteria associated with the dinoflagellate cell, than *Alcanivorax* sp. (7.9%) or *Marinobacter* sp. (11.8 %).

Uni-bacterial *G. catenatum* cultures maintained for more than 6 months exhibited reduced exponential growth rates and low maximum dinoflagellate cell concentrations, and early onset of death phase. Uni-bacterial model cultures using antibiotic-resistant and sensitive-bacteria were used to investigate whether replacement or addition of "new" bacteria (*Brachybacterium* sp. or *Marinobacter* sp.) to cultures could restore or improve dinoflagellate growth. Cultures where the bacterial community was replaced showed improved dinoflagellate growth but long term survival and culture maintenance was not possible, indicating that the dinoflagellate-bacteria relationship in uni-bacterial model cultures may be unstable over long periods.

The *G. catenatum* model system was also used to examine whether dinoflagellate genotype or bacterial community composition was the dominant factor influencing *G. catenatum* growth dynamics. Two clonal parent cultures (GCDE08 and GCHU11) with markedly different batch growth dynamics exponential growth rates and microbial communities were compared with equivalent mixtures of non-clonal progeny established in the presence of microbial communities (8 μm filtrates) from each of the parent cultures. All non-clonal progeny treatments showed similar growth patterns that were different to either parent culture suggesting that genotype is the dominant influence on growth. However, tRFLP analysis showed that, regardless of the bacterial community added at germination, a consistent but different bacterial community was established in all non-clonal progeny cultures. This indicates that the growth dynamics are influenced primarily by the bacterial community composition rather than dinoflagellate genotype, and that the bacterial community may be selected or modified by factors associated with the dinoflagellate genotype.

Acknowledgements

First, I wish to thank my supervisor, Dr Christopher Bolch and his research colleague Dr. David Green, Scottish Association for Marine Science (SAMS), Dunstaffnage Marine Laboratory, UK for developing the current research project. Without their professional guidance and scientific support, this dissertation would not have been possible.

I must also acknowledge the contribution of Dr Christopher Burke, my co-supervisor, whose knowledge in microbiology has been a valuable source while planning experiments.

Thanks to Dr Stephen Tristram, School of Health Sciences, University of Tasmania, for his willingness to share information on developing antibiotic resistant bacteria and further antibiotic applications.

I am indebted to Dr Natalie Moltschaniwskyj, for generously sharing her time and providing statistical guidance all through the project. Thanks also to Dr Rosa Martinez, visiting research fellow, for her valuable comments and suggestions while proof reading manuscripts.

I am grateful to Ms. Cheryl Jones, Australian Maritime College for being a good friend and advisor. Special thanks to her for being a great soul.

I greatly appreciate the assistance provided by Ms. Benita Vincent and Mr. Greg Kent in setting-up initial experiments. A special thanks to Masako Matsumoto for her immense support and help with molecular biology aspects of this work. She has always been there to help with a smile.

Thanks to my colleagues, especially, Sridevi Embar Gopinath, Rebecca Van Gelderen, Rachna Mehta, Nafisa Preethi Sanga, Tanmay Basrur and Vikram Garaniya, for sharing their interests and thus making my stay enjoyable and lively. Thanks to Kiran.D. Ahuja for her support throughout my candidature, specially during the final stages of submission.

Nothing would have been possible without my loving and wonderful husband Bejoy Cyriac. He has been supportive, tolerant and caring all through my journey in achieving my goals. His positive words has always lifted my spirits and rejuvenated my optimism. At this instance, I should also thank our beautiful baby, Tanya Bejoy for keeping me healthy and active during pregnancy and after delivery enabling me to finish this dissertation.

Last, but not the least, I would like to thank my parents, in-laws, brother and all family members for their love and understanding.

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Chapter 1

Introduction

Phytoplankton are the key component of the aquatic food web (Pomeroy 1974; 1979). Excessive growth or sudden population increase in phytoplankton, termed blooms, occur periodically in coastal ecosystems (Alavi *et al.* 2001). These blooms are in general a natural phenomenon that may be influenced by human activities such as discharge of excess nutrients to coastal waters (Hallegraeff *et al.* 2003). In the past two decades reports on phytoplankton blooms have increased in frequency and severity across the globe. Reasons for this increase are thought to be increased eutrophication, climate change and improved scientific monitoring (Smayda 1990; Hallegraeff 1993; Carmichael *et al.* 2001). Harmful algal blooms (HAB) pose a serious threat to waterways, sustainable development of environment, coastal economy, fishery resources and human health. With a world wide expansion in HAB events every year, phytoplankton dynamics are the subject of much scientific research (Anderson 1995).

1.1 Harmful algal blooms and phycotoxins

HAB's are generally classified in two groups; high biomass producers and toxin producers. Blooms of high biomass producers are considered harmful when the algal cell concentrations exceed 10^7 cells L^{-1} (Marzec 2006) and can deplete dissolved oxygen content in the water leading to anoxygenic condition that affect water quality or kill fish. Toxin producers produce toxins that

may contaminate seafood and eventually affect mammals via food chain (Marzec 2006). More than 60,000 human intoxication events are reported world wide every year (Dolah *et al.* 2001). The ecological role of phycotoxins is poorly understood. However, recent evidence indicates that toxins can act as allelochemical agents that increase algal survival in various ecological niches and may provide chemical defence against predators (Graneli & Johansson 2003). **Table 1.1:** summarizes the major five poisoning syndromes, toxin names and causative organisms (Dolah *et al.* 2001)

Table 1.1: Major poisoning syndromes caused by microalgae.

Human health impact	**Toxin name (type)**	**Causative species**
Paralytic shellfish poisoning (PSP)	Saxitoxin (neurotoxin)	Dinophyta- *Alexandrium* spp., *Pyrodinium* spp., *Gymnodinium* spp.
Amnesic shellfish poisoning (ASP)	Domoic acid (neurotoxin)	Diatom- *Pseudo- nitzschia* spp.
Diarrhetic shellfish poisoning (DSP)	Okadoic acid (diarrhetic toxin)	Dinophyta- *Dinophysis* spp.
Neurotoxic shellfish poisoning (NSP)	Brevetoxin (neurotoxin)	Dinophyta – *Karenia brevis*
Ciguatera fish poisoning (CFP)	Ciguatoxin, Maitotoxin (neurotoxin)	Dinophyta – *Gambierdiscus* spp., *Ostreopsis* spp., *Prorocentrum lima*

Dinoflagellates are responsible for many of the common marine phycotoxins (Holmes & Teo 2002). These single celled, eukaryotic protists belong to division Dinophyta and exhibit a range of unique biological phenomena such as bioluminescence, symbiosis or parasitism with invertebrates, and have complex life-cycles (Taylor 1987). Of the more than 4000 identified phytoplankton taxa, only 60 to 80 species are classified harmful. Of these, flagellates comprise 90%, with dinoflagellates (45 to 60 taxa) accounting for 75% of toxic or harmful organisms (Smayda 1989; Anderson 1989; Smayda 1990; Hallegraeff 1993; Sournia 1995).

Paralytic shellfish toxins (PSTs) are potent neurotoxins produced by several dinoflagellate species: *Gymnodinium catenatum*, some *Alexandrium* species, *Pyrodinium bahamense*, and a range of Cyanobacteria. Filter feeding shellfish accumulate these toxins and consumption of the shellfish can poison animals at higher trophic levels in the food chain. PST's include the parent compound saxitoxin (STX) and 20 other chemically related derivatives (Kao 1993; Gallacher *et al.* 1997). In humans, PST intoxication leads to paralytic shellfish poisoning (PSP), a syndrome characterised by gastro intestinal, respiratory and neurological symptoms (Hallegraeff & Sumner 1986). PST's are a major health risk world wide and in particular South-east Asia (Holmes & Teo 2002). The first PSP outbreak in Australia occurred in 1986 when *G. catenatum* appeared in Huon and Derwent estuary, Tasmania (Hallegraeff *et al.* 1988; McMinn *et al.* 1997). Recurring incidents of PSP were reported in 1987 and 1991 incurring heavy losses for the Tasmanian shellfish industry (Hallegraeff 1993).

1.2 Factors affecting HAB dynamics

The bloom dynamics of harmful algal blooms is complex and poorly understood. However with the expansion of HAB outbreaks around the globe, a thorough understanding of the factors influencing bloom dynamics is essential. For a long time a wide range of physical factors: temperature, current, salinity and macro-nutrients were believed to be the primary factors regulating HAB dynamics (Ferrier *et al.* 2002). More recently, the interaction of HAB species with the marine microbial community has been considered a potentially important factor (Doucette *et al.* 1999;

Groben *et al.* 2000; Ferrier *et al.* 2002; Green *et al.* 2004; Pinhassi *et al.* 2004; Grossart *et al.* 2005; Rooney-Varga *et al.* 2005).

A close spatial and temporal association is suggested to exist between bacteria and algae and this relationship may also play a crucial role in important oceanic processes such as carbon cycling and nutrient regeneration (Cole 1982; Lovejoy *et al.* 1998). Bacteria associated with algal cells may be either attached (intracellular or extracellular) or free-living (e.g. Lewis *et al.* 2001; Simon *et al.* 2002), and may have stimulative or inhibitory effects on the growth of algal cell. These interactions are now considered to be of major importance in regulation of both algal and bacterial populations (Simon *et al.* 2002). For example, some bacterial communities appear to promote the formation of algal blooms (Furuki & Kobayashi 1991) while other communities show algicidal effects and/or decompose algal blooms (Fukami *et al.* 1991). Bacteria potentially benefit from utilising phytoplankton exudates (Bell *et al.* 1974; Cole 1982), while phytoplankton cells may use bacterial metabolites such as demineralized nutrients (Golterman 1972), organic growth factors (Ukeles & Bishop 1975, Paerl & Pinckey 1996) or vitamins (Pringsheim 1912; Croft *et al.* 2005). Bacteria may also serve as a major source of CO_2 for algal growth (mainly during CO_2 limitation) (Marshall 1989), or act as parasites or pathogens by penetrating the periplasmic space of algal host cells, resulting in cell lysis and death (Cole 1982; Imai *et al.* 1993).

Bacterial communities associated with phytoplankton cells are typically dominated by the alpha and gamma-proteobacteria (Babinchak *et al.* 1998; Hold *et al.* 2001b; *Green et al.* 2004). Representatives from *Cytophaga-Flavobacter* group and the Planctomycetes are also present (Glockner *et al.* 1999; Hagstrom *et al.* 2000). The bacterial types appear to differ among phytoplankton genera. For example, Hold *et al.* 2001b reported *Roseobacter* sp. as a dominant associate of *Alexandrium* spp. and *Scripsiella trochoidea* cultures. Similarly, *Roseobacter* sp. and *Marinobacter* sp. have been reported in association with *Lingulodinium polyedrum* blooms (Fandino *et al.* 2001). *Roseobacter* sp. has also been identified to dominate *Prorocentrum lima* microflora (Prokic *et al.* 1998). Bacterial flora can vary with different strains of same algal species

as at least 6 bacterial strains specific to 3 culture strains of *Alexandrium* spp. has been reported (Hold *et al.* 2001b)

The role of the bacterial community in phycotoxin production is still open to debate. A bacterial origin for PST was first proposed by Silva (1962, 1982) after particles similar to bacteria was found within dinoflagellate cells. Studies on *Alexandrium tamarense* have shown that presence of bacteria, either intracellular or attached to the dinoflagellate cells may be involved in the production of PST (Silva 1982; Kodama 1990). Several studies have demonstrated that bacteria produce compounds with biological activity similar to PSP compounds (Kodama *et al.* 1988; Franca *et al.* 1996; Gallacher *et al.* 1997), however there is, as yet, no structural data that confirm production of PST by marine bacteria (Martins *et al.* 2003). If bacteria are not directly involved in PST production they may be indirectly involved. For example: they may influence PST content of algal cultures by altering algal host-cell toxicity (Doucette *et al.* 1998; Hold *et al.* 2001a; Uribe & Espejo 2003), undertake biotransformation of the PST derivatives (Smith *et al.* 2001) or by a combination of the above. The exact role-played by bacteria in PST production and toxicity is still unclear, however, there is clear evidence that bacteria may influence HAB population dynamics. This may be via stimulation of toxic algal growth (Sakami *et al.* 1999), promotion or inhibition of sexuality (Adachi *et al.* 1999), the production of algicidal factors (Doucette *et al.* 1999), or the suppression of resting cyst formation (Adachi *et al.* 2002). Little is also known about how the bacterial and algal communities interact at a species level.

1.3 Simplified models of algal bacterial interaction:

Interactions between phytoplankton and the bacterial community are highly complex. Natural phytoplankton communities co-exist with 100's of bacterial types many of which may directly or indirectly influence the algal cell in variety of ways. Even uni-algal cultures may contain 10-40 bacterial types (Green *et al.* 2004) creating opportunity for millions of potential interactions. This wide range of confounding interactions means that identifying key bacterial types and

mechanisms of interaction is almost impossible. To facilitate progress in this field, simplified experimental models of bacterium-dinoflagellate interactions are needed to elucidate the significance and mechanisms of these interactions and their role and importance in HAB dynamics. Initial efforts to generate experimental models have focused on dinoflagellates (Bolch *et al.* 2002, 2004; Green *et al.* 2004). The experimental models developed to date include *Gymnodinium catenatum*, *Lingulodinium polyedra* and *Scrippsiella trochoidea.* These models use surface-sterilised resting stages that are germinated in the presence of single or controlled mixtures of bacteria. The growth response of the established cultures with simplified bacterial communities can then be compared and the community manipulated to determine the dinoflagellates' response to the bacteria. Preliminary experiments with these three models indicate three main growth responses to simplified communities: (a) an algicidal response- members of *Cytophaga/Flexibacter* group caused death at or just after germination (b) slow growth-typically seen with communities composed solely of *Roseobacter* sp. and/or *Rhodobacter* sp. (c) growth similar to or greater than normal mixed bacterial controls when grown with communities composed of *Alcanivorax* sp. or *Marinobacter* sp. (Bolch *et al.* 2002; Vincent 2003; Bolch *et al.* 2004; Green *et al.* 2004).

For the work presented here, *Gymnodinium catenatum* Graham was chosen as the model species for all the experiments. *G. catenatum* is an unarmoured (naked) chain-forming marine dinoflagellate (Graham 1943; Rees & Hallegraeff 1991; Oshima *et al.* 1993) most notable as a producer of paralytic shellfish toxins (PST). Vegetative cells are seen both in temperate and tropical waters (Hallegraeff & Fraga 1998). *G. catenatum* was first associated with PSP incident in Spain in 1976 (Estrada *et al.* 1984). Further, a number of PSP problems have been reported from Morocco (Tagmouti *et al.* 1995), Southern China (Qi *et al.* 1996) the Phillipines (Fukuyo *et al.* 1993), Argentina, Uruguay (Balech 1964; Mendez & Brazeiro 1993) and Western India (Godhe & Karunasagar 1996) (**Fig. 1.1** (a)).

In Australia, blooms of *G. catenatum* were first reported in the mid- 1980's from Southern Tasmania. Benthic cyst surveys suggest that *G. catenatum* was completely absent from Tasmanian waters before 1973 and may have been introduced via ballast water from Japanese wood ship vessels (McMinn *et al.* 1997). In Tasmanian waters, *G. catenatum* blooms are often seen after heavy rainfall with an increased influx of dissolved organic matter (DOM) from land run-off (Hallegraeff *et al.* 1995) (**Fig. 1.1** (b)). *G. catenatum* was chosen for this study because: (a) the life cycle is well known and can be easily manipulated (**Fig. 1.2**); (b) it has a relatively short resting cyst dormancy period (14–21days) allowing relatively rapid cycles of experimentation (Blackburn *et al.* 1989, 2001) (c) a high frequency of HAB events have been caused by *G. catenatum* in the past decades (Hallegraeff 1993; Hallegraeff & Fraga 1998), and (d) the economic impacts has classified the dinoflagellate as a significant species in a short time since it's introduction to Australian waters (Hallegraeff & Sumner 1986; Hallegraeff *et al.* 1989, 1995).

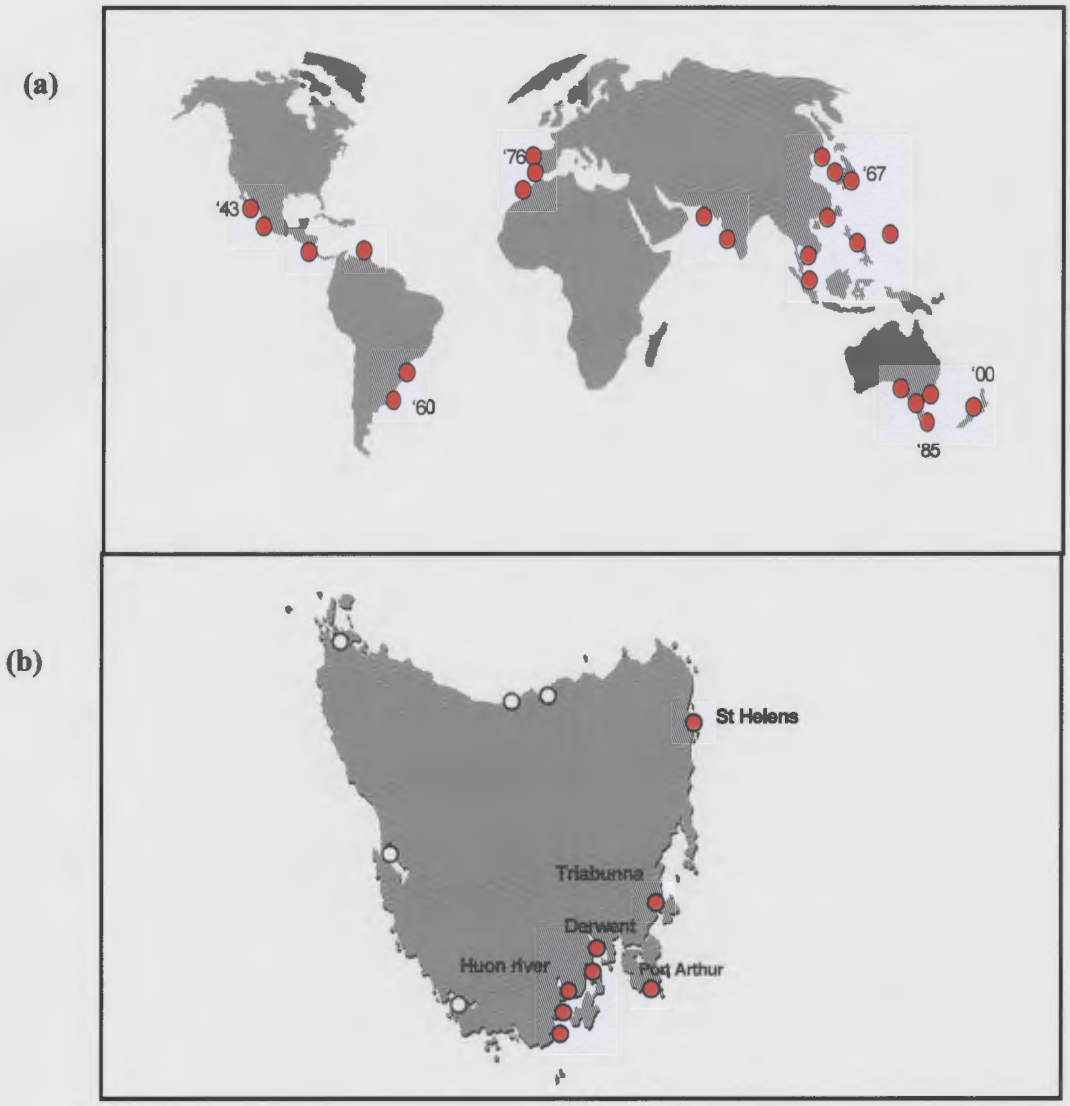

Fig. 1.1: (a) Known global distribution of *G. catenatum* 1985-2002 (modified from Bolch & Reynolds 2002). Numbers indicate year of discovery in selected areas (b) Distribution of *G. catenatum* in Tasmanian waters.

The lifecycle of *G. catenatum* follows a pattern typical of most dinoflagellates. Population growth is by vegetative (asexual) reproduction with *G. catenatum* forming chains up to 32 cells and occasionally 64 cells in rapidly dividing cultures (Blackburn *et al.* 1989). Sexual reproduction appears to be triggered by nutrient limitation which then stimulates gamete formation (Blackburn *et al.* 1989). The gametes fuse to form a motile diploid zygote that loses motility to form a resistant benthic-resting cyst (hypnozygote). Production of resting cysts is a common feature of

dinoflagellates and can have a major influence on bloom initiation, survival and decline (Bolch *et al.* 2002). Resting cysts can survive in sediments for years until the favourable environmental conditions trigger germination to re-establish a vegetative population (Blackburn *et al.* 1989) (**Fig. 1.2**; **Fig. 1.3**).

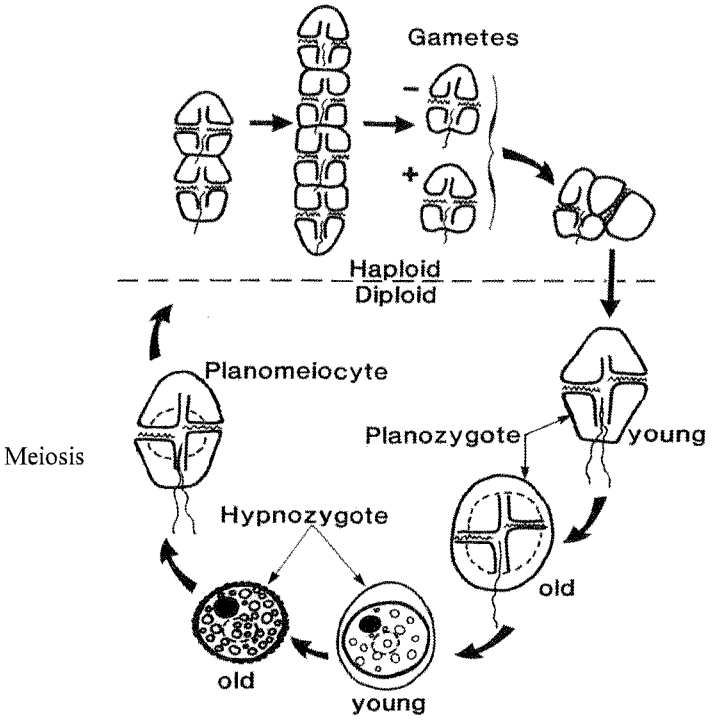

Fig. 1.2: Life cycle of *G. catenatum* (Blackburn *et al.* 1989)

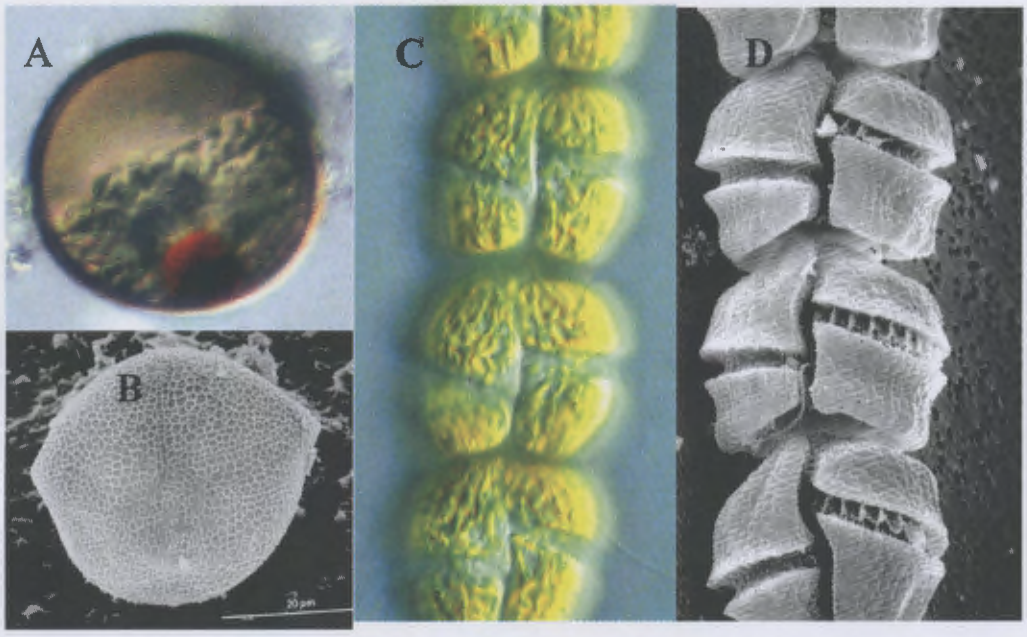

Fig. 1.3: (A) & (B) LM and SEM image of *G. catenatum* resting cyst. (C) & (D) LM and SEM image of vegetative cells of *G. catenatum* showing chains. (images from Bolch 1999)

The bacterial flora associated with *G. catenatum* has been comprehensively documented by Green *et al.* (2004) who used 16SrDNA sequence analysis to identify a wide range of culturable bacteria associated with laboratory cultures. This work laid the foundation for subsequent studies aiming to understand the influence of bacteria on *G. catenatum* growth, physiology and toxicity. The bacterial community associated with *G. catenatum* cultures is dominated by alpha and gamma proteobacteria (70%), with Bacteriodetes (26%) and Actinobacteria (3%) as minor components. The dominant alpha-proteobacteria are primarily from the *Roseobacter* and *Rhodobacter* clades, whereas the dominant gamma-proteobacteria are *Marinobacter* sp. and *Alcanivorax* sp., both capable of utilising hydrocarbon as a sole carbon source (Green *et al.* 2004). The role of the hydrocarbon-degrading bacteria on dinoflagellate growth was further investigated by Vincent (2003). This work demonstrated that (a) *G. catenatum* growth in uni-bacterial *G. catenatum* cultures is stimulated by *Marinobacter* sp. and *Alcanivorax* sp. but not by a range of other bacteria and (b) bacterial communities may exhibit species or group specific-effects on dinoflagellate growth (Vincent 2003).

1.4 Thesis aims and outline

The current study aims to use the simplified experimental dinoflagellate-bacteria models to investigate the importance and influence of the associated bacterial community on the growth of *G. catenatum.*

Specifically this thesis aims to:

1. Determine whether *G. catenatum* has an obligate requirement for marine bacteria.
2. Determine whether the bacterial community composition has a significant effect on dinoflagellate growth dynamics.
3. Examine the spatial relationship between specific stimulatory bacteria and the dinoflagellate cells.
4. Determine whether the dinoflagellate genotype or the bacterial community is the dominant factor influencing growth of *Gymnodinium catenatum.*

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Chapter 2

Obligate requirement of the dinoflagellate *Gymnodinium catenatum* for marine bacteria

2.1 INTRODUCTION

The growth of marine phytoplankton cells is generally considered to be controlled by a combination of physical factors such as temperature and light (Thompson 1999), the availability and uptake of major (C, N, P, and Si) and minor nutrients (e.g. Iron and other trace metals) (Morel & Hudson 1985) and the effects of predation (grazing) on the population (Turner *et al.* 1998). However, phytoplankton cells are also subject to a potentially vast array of chemical and biological interactions with microbes such as viruses (Castberg *et al.* 2001), bacteria (Doucette *et al.* 1999) or other protozoans (Stone 1990), that may have a significant or controlling influence on phytoplankton populations. These microbial interactions are increasingly considered a major factor influencing phytoplankton growth and species succession (e.g. Doucette *et al.* 1998)

The potential for phytoplankton to alter the bacterial community is well known. The term "phycosphere" was first coined by Bell & Mitchell (1972) to describe a zone around phytoplankton cells where microbial activity is substantially altered by 1) an increased surface area to which

bacteria can attach (Bidle & Fletcher 1995); 2) exuding dissolved organic compounds and complex polysaccharides that bacteria can utilise for growth (e.g Janse *et al.* 2000); or 3) anti-bacterial chemicals that may select for or against particular bacterial groups (e.g. Sandsladen *et al.* 2003). It is now increasingly clear that bacterial activity can also influence the phytoplankton community by: 1) production of growth promoters that favour particular algal species (e.g. Furuki & Koboyashi 1991); 2) production of inhibitory or algicidal substances (e.g. Doucette *et al.* 1999; Kodani *et al.* 2002; Skerratt *et al.* 2002), or 3) interfering with or promoting sexual reproduction (Adachi *et al.* 2003).

Stimulation of phytoplankton growth by marine bacterial communities has been reported repeatedly over the past few decades (e.g. Ukeles & Bishop 1975; Mouget *et al.* 1995; Sakami *et al.* 1999; Ferrier *et al.* 2002; Bolch *et al.* 2002), yet surprisingly little is known of the specificity of stimulation, or the mechanisms and compounds responsible for stimulation. For example, marine bacteria have long been known as a source of the essential vitamin B_{12} (cyanocobalamin) for many phytoplankton species (Haines & Guillard 1974), yet the specific molecular basis underlying this interaction has only recently been elucidated (Croft *et al.* 2005).

Dinoflagellate cells harbour complex bacterial communities composed of 10's to 100's of bacterial genotypes (e.g., Hold *et al.* 2001; Alavi *et al.* 2001; Green *et al.* 2004), therefore a potentially vast array of alga-bacterium or bacterium-bacterium interactions may influence a dinoflagellate cell. For example, a specific bacterium has been shown to protect the dinoflagellate *Karenia brevis* from lysis by a normally algicidal bacterium (Mayali & Doucette 2002). This complexity and confounding activity currently impedes attempts to identify key bacterial types, define their effect/function, or elucidate the mechanisms of interaction.

To investigate specific responses and mechanisms of interaction this chapter uses simplified experimental models of bacterium-dinoflagellate interactions using the toxic model species, *Gymnodinium catenatum. G. catenatum* cultures in the laboratory are typically dominated

by Alphaproteobacteria (40-70%), the Gammaproteobacteria (5-15%), and the Bacteroidetes (*Cytophaga/Flexibacter*, CFB*)* (5-15%) (Green *et al.* 2004). Using the simplified models, previous work has tested a wide range of cultured *G. catenatum*-associated bacteria for their capability to support growth of *G. catenatum* in uni-bacterial dinoflagellate culture. This work has suggested the presence of a bacterial community may be essential for growth of *G. catenatum* laboratory cultures (Bolch *et al.* 2002; Vincent 2003; Bolch *et al.* 2004).

Many studies over the last several decades have reported the growth dynamics of phytoplankton in the presence or absence of bacteria (e.g. Singh *et al.* 1982; John & Flynn 2000; Hold *et al.* 2001). These studies have often come to contradictory conclusions about the obligate requirement of the bacterial community for phytoplankton growth. Previous studies with *G. catenatum* in our laboratory have shown poor germination of surface sterile cysts in the absence of bacteria and that long term culture maintenance was impossible (Vincent 2003; Bolch *et al.* 2004), suggesting that *G. catenatum* requires marine bacteria for growth and that the requirement may be obligatory.

This chapter uses the *G. catenatum*-bacteria models to establish whether *G. catenatum* has an obligate requirement for bacteria. Antibiotic-resistant and antibiotic-sensitive strains of growth-stimulating bacteria were used in combination with antibiotic treatment to investigate the obligate requirement of *Gymnodinium catenatum* for marine bacteria directly after germination and in well established laboratory cultures.

2.2 MATERIALS AND METHODS

2.2.1 Dinoflagellate culture and cyst production

Compatible mating strains of *Gymnodinium catenatum,* GCHU11 and GCDE08 were grown at 19°C (+/- 2.5°C) in 150 mL Erlenmeyer flasks in GSe medium (Blackburn *et al.* 1989)

under white fluorescent light of 90±10 μmoles m^{-2} s^{-1} with 12L:12D photoperiod. Late-logarithmic phase GCHU11 and GCDE08 cultures were crossed as described by Blackburn *et al.* (2001). Briefly, 1 mL of each strain was transferred to 55 mm polystyrene petri-dishes containing 10 mL of sterile nitrogen- and phosphate-deficient GSe medium, the dishes sealed with Parafilm™ and incubated for 3-5 weeks under 90±10 μmoles m^{-2} s^{-1} until sufficient resting cysts were evident in crosses.

2.2.2 Surface-sterilisation of cysts

Resting cysts were isolated from crosses using a hand-drawn glass micropipette, washed by micropipette transfer through a series of two to three, 55 mm petri dishes containing sterile GSe medium, and transferred to 1.5 mL sterile centrifuge tubes in 0.5 mL of sterile GSe medium. Cysts were surface-sterilised by addition of 50 μl of 6% H_2O_2 (Orion Laboratories Pty Ltd, Australia) to achieve a final concentration of 0.55% (v/v) of H_2O_2. Tubes were gently mixed, wrapped in aluminium foil, and left for 45 mins at room temperature. Tubes were then centrifuged for 30 s at 14000 rpm, and all but 50 μl of the supernatant removed. Residual H_2O_2 was removed by resuspension of the cysts in 500 μl of sterile GSe medium followed by centrifugation as above; this step was repeated and the cysts resuspended in sterile GSe medium (Bolch *et al.* 2004). All sterile cyst suspensions were checked for sterility by spread plating 100 μL of the cyst suspension onto modified Zobell's Marine Agar (ZM1) (Green *et al.* 2004) and the plates incubated in the dark for 3 days at 24°C. Cyst suspensions showing evidence of bacterial contamination were discarded, the treatments terminated and excluded from the experiment.

2.2.3 Bacterial culture

During preliminary trials aimed at generating uni-bacterial cultures of *G. catenatum* with either *Alcanivorax* sp. DG881 or *Marinobacter* sp. DG879, a bacterial contaminant was found to

support *G. catenatum* growth in unibacterial cultures. The colonies were yellow in colour while *Alcanivorax* sp. (Hara *et al.* 2003) and *Marinobacter* sp. (Green *et al.* 2006) generally form transparent and cream colour colonies **(Fig. 2.1)**. Through isolation and sequencing of 16SrDNA, the bacterial isolate was identified as a *Brachybacterium* species. Further studies in our laboratory determined that *Brachybacterium* sp. could utilize hydrocarbons similar to *Marinobacter* sp. and *Alcanivorax* sp. (Green *et al.* 2004). For the current study we examined the growth stimulating ability of *Brachybacterium* sp. which to our knowledge has not been reported in association with any phytoplankton species until now.

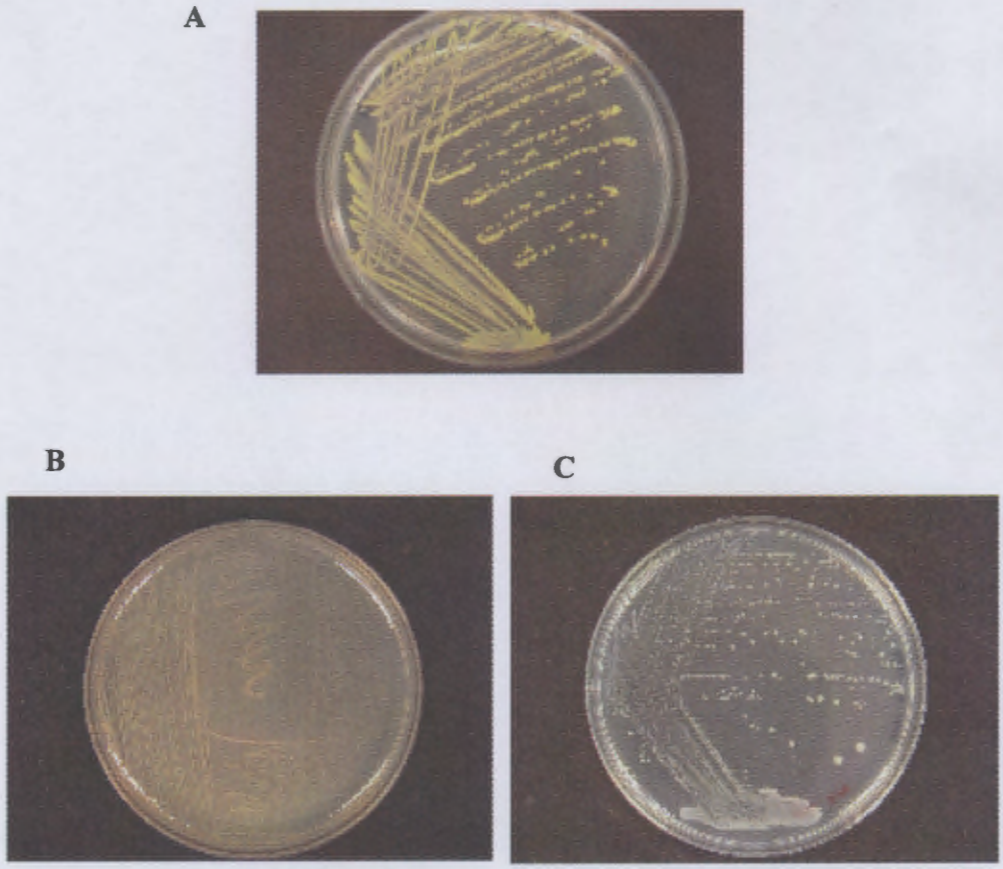

Fig. 2.1: Plates show (A) *Brachybacterium* sp. (B) *Alcanivorax* sp. DG881 (C) *Marinobacter* sp. DG879 growth on Zobell's marine agar

Cultures of the *Marinobacter* sp. DG879 and *Brachybacterium* sp. were maintained on either ZM1 (Appendix 2), prepared in 75% filtered seawater (26 ppt), or the same medium prepared at 1/10 concentration of nutrients (referred to here as ZM/10). ZM1 medium contained 5 gL^{-1} of bacterial peptone, 1g L^{-1} of yeast extract and was solidified with 15 gL^{-1} of Difco-Bacto^{TM} agar. Both media were supplemented with autoclave sterilised 5 mL, 100 x marine supplements containing filter-sterilised trace elements and vitamins (Green *et al.* 2004). For culturing *Brachybacterium* sp., 1% sodium acetate was added as a carbon source similar to *Alcanivorax* sp. (Green *et al.* 2004).

2.2.4 Generation of antibiotic strains

To generate antibiotic resistant strains of *Marinobacter* sp. DG879 and *Brachybacterium* sp., the antibiotic sensitivity profile of both bacteria was determined using Kirby-Bauer disc diffusion tests based on National Clinical Committee Laboratory Standards (Bauer *et al.* 1966). The following antibiotic test discs were used: Erythromycin (60 μg), Novobiocin (30 μg), Cephazolin (30 μg), Pencillin (10 U), Streptomycin (25 μg) and Tobramycin (30 μg) (Oxoid inc., Australia). Bacterial inoculum was prepared by suspending bacterial cells from fresh overnight ZM1 broth (without agar) cultures in 3.5% saline to a turbidity equivalent to 0.5 McFarland standard. The agar surface was swabbed with the bacterial cell suspension to achieve an even "lawn" of bacteria. Antibiotic discs were aseptically transferred, one on each agar plate, and plates were incubated at 25°C for 72 hours. The zone of inhibition (diameter) seen around antibiotic discs was measured and bacterial isolates were classified as sensitive (S), intermediate (I) or resistant (R) ($S \geq 20$ mm; I= 20 mm; $R \leq 19$ mm (Fuller *et al.* 2007) (**Table 2.1**). *Marinobacter* sp. showed sensitivity to all the six antibiotics while *Brachybacterium* sp. showed sensitivity to all except tobramycin (intermediate) (**Table 2.1**). Two antibiotics to which each bacterial strain was sensitive were selected to induce resistance; streptomycin & tobramycin for *Marinobacter* sp., erythromycin & cephazolin for *Brachybacterium* sp.

Table 2.1: Sensitivity results for various antibiotics tested.

Antibiotics	*Brachybacterium* sp. (zone diameter in mm)	*Marinobacter* sp. (zone diameter in mm)
Erythromycin	30	31
Novobiocin	52	39
Cephazolin	37	53
Pencillin	42	55
Streptomycin	22	29
Tobramycin	20 *	31

* Zone of inhibition (20 mm) showed *Brachybacterium* sp. neither sensitive nor resistant to Tobramycin.

Resistance to the selected antibiotics was induced in bacterial isolates by repeated plating with antibiotic discs based on Kirby-Bauer disc diffusion method (Bauer *et al.* 1966) as discussed earlier. A lawn of antibiotic sensitive *Marinobacter* sp. or *Brachybacterium* sp. cell suspensions prepared as previously described was spread on to ZM1 plates. After 2- 3 mins of drying, tobramycin or streptomycin discs were transferred aseptically to plates with *Marinobacter* sp. and cephazolin or erythromycin discs to *Brachybacterium* sp. Plates were incubated at 25°C for five to six days. With increased incubation time spontaneous mutants were seen in the zone of inhibition near the antibiotic discs. Putative mutants were isolated with a sterile loop, spread plated and exposed again to antibiotic discs. Usng this procedure, the bacterial strains developed resistance to the respective antibiotics over passage through 5-6 transfers. Resistance was confirmed when there was no visible zone of inhibition seen around the disc, or bacterial growth up to the disc edge (**Fig. 2.2**).

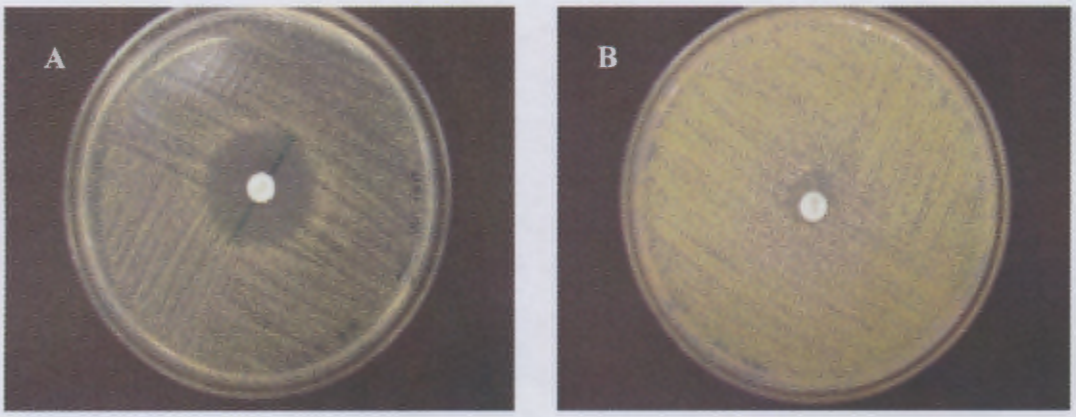

Fig. 2.2: Generation of antibiotic resistant strains based on Kirby-Bauer disc diffusion method. Plates show *Brachybacterium* sp. with cephazolin discs. (A) Clear zone of inhibition around the disc; (B) Bacterial growth to the edge of disc.

2.2.5 *G. catenatum* cultures grown with antibiotic sensitive or resistant bacteria.

Antibiotic sensitive or resistant bacterial strains for use in germination and growth experiments were grown in ZM1 broth medium (without agar), with shaking, for two days at 20°C. One mL of culture was transferred to 1.5 mL centrifuge tubes and the bacteria pelleted by centrifugation for 2 min at 13000 rpm. The supernatant was then removed and the cells resuspended in 800 µl of sterile GSe medium. This was repeated three times to remove traces of ZM1 bacterial medium. The bacterial cells were then resuspended in one mL of GSe medium, transferred to five mL of GSe medium in 20 mL McCartney bottles, and incubated at 25°C for 1 day to allow the bacteria to acclimatise to algal growth medium. The bacterial cell concentration was estimated by triplicate direct cell count using an improved Neubauer haemocytometer (Brand, Germany).

For the experimental set-up, 25-30 sterilised cysts were aseptically aliquoted into 36 mm petri dishes and the prepared antibiotic sensitive or resistant bacterial cultures added to the sterile resting cysts and/or controls as described in **Table 2.2** (refer to **Fig. 2.3** for illustration). All treatments, positive and negative control were carried out in triplicate. All dishes were sealed with Parafilm^{TM} and incubated at 19°C +/- 2.5°C at a light intensity of 90±10 µmoles m^{-2} s^{-1} with a 12L:12D photoperiod. Treatments and controls containing resting cysts were assessed every 3-4 days, for germination using a Lieca S9 stereomicroscope under dark light illumination for 30 days after germination was first observed in each treatment or control. Germination (%) was determined based on number of empty cysts counted in replicate dishes. As a relative measure of initial post-germination growth, motile dinoflagellate cells were counted under the microscope to determine number of moving cells per germinated cyst.

Table 2.2: Description of treatments and controls used to establish *G. catenatum* cultures. All treatments and controls (except the media sterility control) consisted of triplicate 36 mm petri dishes, containing 25-30 surface-sterilised *G. catenatum* resting cysts in 1.9 mL of GSe medium with the addition of the treatments described below. All treatments and the positive control were subsequently used to establish 100 mL flask cultures for later experiments.

Treatment	**Sterile *G. catenatum* resting cysts plus**
Sterility control	200 µL of sterile GSe (no resting cysts)
Positive control	200 µL of 8 µm filtrate from mid-log phase cultures of GCDE08 and GCHU11.
Negative control	Sterile GSe medium
Treatment 1	*Brachybacterium* sp. sensitive to cephazolin and erythromycin added to a final concentration of 10^5 CFU mL^{-1}
Treatment 2	Erythomycin resistant *Brachybacterium* sp. added to a final concentration of 10^5 CFU mL^{-1}
Treatment 3	Cephazolin resistant *Brachybacterium* sp. added to a final concentration of 10^5 CFU mL^{-1}
Treatment 4	*Marinobacter* sp. DG879 sensitive to tobramycin and streptomycin added to a final concentration of 10^5 CFU mL^{-1}
Treatment 5	Tobramycin resistant *Marinobacter* sp. DG879 added to a final concentration of 10^5 CFU mL^{-1}
Treatment 6	Streptomycin resistant *Marinobacter* sp. DG879 added to a final concentration of 10^5 CFU mL^{-1}
Treatment 7	*Brachybacterium* sp. (sensitive) and *Marinobacter* sp. DG879 (sensitive) added to a final concentration of 10^5 CFU mL^{-1}
Treatment 8	Erythromycin resistant *Brachybacterium* sp. and streptomycin resistant *Marinobacter* sp. DG879 added to a final concentration of 10^5 CFU mL^{-1} each.
Treatment 9	Cephazolin resistant *Brachybacterium* sp. and tobramycin resistant *Marinobacter* sp. DG879 added to a final concentration of 10^5 CFU mL^{-1} each.
Treatment 10	Erythromycin resistant *Brachybacterium* sp. and tobramycin resistant *Marinobacter* sp. DG879 added to a final concentration of 10^5 CFU mL^{-1} each.

After 30 days, treatments and the positive control were transferred to sterile 150 mL Erlenmeyer flasks containing 100 mL of sterile GSe medium. These cultures were grown under same conditions until sufficiently high cell concentration was available for additional growth experiments. Negative control (no bacteria added) failed to survive beyond 30 days (as expected) and were not included in further growth studies. The established 100 mL cultures were transferred to fresh sterile 150 mL Erlenmeyer flasks with 100 mL of GSe medium.

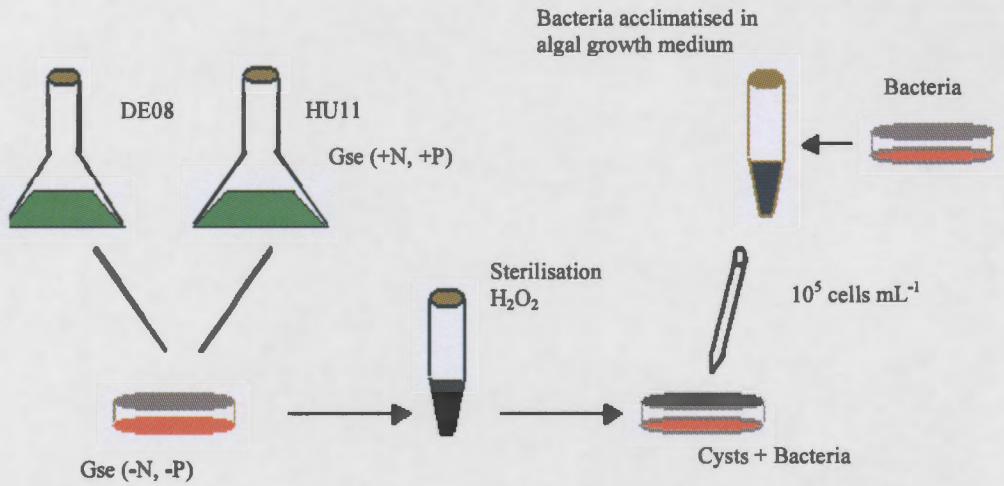

Fig. 2.3: Diagram summarizing the approach for establishing model cultures of *G. catenatum* from surface-sterilised resting cysts.

2.2.6 Minimum inhibitory concentration (MIC) for experiments

Preparation of antibiotic solution: Streptomycin, Erythromycin, Cephazolin and Tobramycin (MP Biomedicals, Australia) in powdered form were weighed and dissolved in sterile filtered seawater following manufacturer's instructions. Stock solutions were prepared using the formula.

Weight of antibiotic = V x C x (1000/P)

where V= volume in mL required, C= final concentration of solution, P= potency of the antibiotic powder (provided by manufacturer) (Andrews 2001).

The minimum inhibitory concentration (MIC) of each antibiotic was determined using a microdilution susceptibility test in 12 well polystyrene microtitre plates (Corning Incorporated, Corning, NY, USA). Antibiotic sensitive and resistant *Marinobacter* sp. or *Brachybacterium* sp. were tested to determine the effective range of antibiotic concentration **(Table 2.3)**. Twelve different antibiotic concentrations ($\mu g\ mL^{-1}$) were tested for each antibiotic. Briefly, 1 mL of ZM1 broth media was aliquoted in to the wells of microtitre plates and a series of 12, 50% (1 into 2) dilutions was created with the antibiotic solution (erythromycin 0.02-50; cephazolin 0.05-120; tobramycin 0.01-20; streptomycin 0.03-75) ($\mu g\ mL^{-1}$). Sensitive or resistant 72 hrs plate cultures of *Marinobacter* sp. or *Brachybacterium* sp. were mixed thoroughly with 12 mL of GSe growth media and incubated overnight at 25°C. Bacterial concentration was estimated and diluted to 10^7 CFU mL^{-1} for each bacterial isolate by triplicate direct cell count using an improved Neubauer haemocytometer (Brand, Germany). One mL of the bacterial suspension was then added to each well in the microplate, and the plates incubated for 48 to 72 hrs at 25°C. The MIC is defined as the lowest concentration of antimicrobial agent at which no growth or turbidity could be detected visually (Andrews 2001). Wells that showed complete bacterial inhibition or no turbidity were also

confirmed by spread plating on Zobell's marine agar. Absence of bacterial growth on plates confirmed the MIC (**Fig. 2.4**).

Table 2.3: Minimum inhibitory concentrations of erythromycin and cephazolin (*Brachybacterium* sp.), and tobramycin and streptomycin (*Marinobacter* sp.). Bacterial isolates grown in ZM1 plates were tested for effective antibiotic concentrations that would remove antibiotic sensitive bacteria and not resistant bacteria.

Antibiotic added (μg mL^{-1})/ Bacterial isolate	**1st well**	**2nd well**	**3rd well**	**4th well**	**5th well**	**6th well**	**7th well**	**8th well**	**9th well**	**10th well**	**11th well**	**12th well**
Erythromycin	5	**2.5**	1.25	0.625	0.312	0.156	0.078	0.039	0.019	0.009	0.004	0.002
Erythromycin sensitive *Brachybacterium* sp.	No growth	**No growth**	Less turbid	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
Erythromycin resistant *Brachybacterium* sp.	Growth	**Growth**	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
Cephazolin	12	6	**3**	1.5	0.75	0.375	0.187	0.093	0.046	0.023	0.011	0.005
Cephazolin sensitive *Brachybacterium* sp.	No growth	No growth	**No growth**	Less turbid	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
Cephazolin resistant *Brachybacterium* sp.	No growth	Growth	**Growth**	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
Tobramycin	2	**1**	0.5	0.25	0.125	0.062	0.031	0.015	0.007	0.003	0.002	0.001
Tobramycin sensitive *Marinobacter* sp.	No growth	**No growth**	Less turbid	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
Tobramycin resistant *Marinobacter* sp.	No growth	**Growth**	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
Streptomycin	7.5	**3.75**	1.875	0.937	0.468	0.234	0.117	0.058	0.029	0.014	0.007	0.003
Streptomycin sensitive *Marinobacter* sp.	No growth	**No growth**	Less turbid	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
Streptomycin resistant *Marinobacter* sp.	Growth	**Growth**	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth

* No growth = no visible turbidity and no bacterial growth on Zobell's marine agar plates.

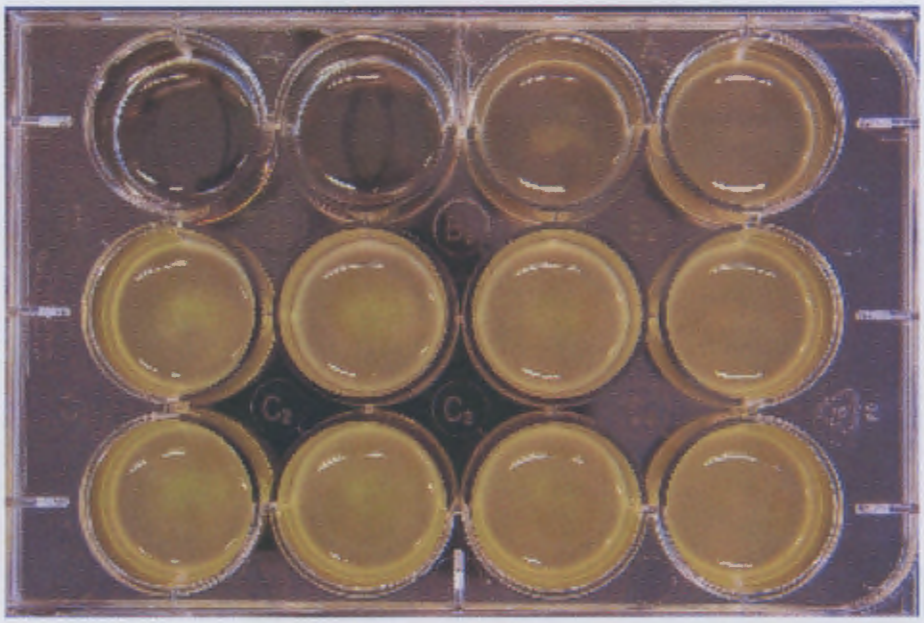

Fig. 2.4: Example of 12 well microtitre plate used for MIC trials. Turbidity or faint yellow colour indicates bacterial growth. The example shown is *Brachybacterium* sp. sensitive to erythromycin, treated with erythromycin (0.002–5 μgmL^{-1}); Well A2 (second in row 1) was considered MIC (2.5 μgmL^{-1}) of erythromycin.

2.2.7 MIC determination for *G. catenatum* cultures

The MIC experiments were used to determine the range of antibiotic concentrations to be applied to *G. catenatum* cultures. A similar MIC experiment was performed using *G. catenatum* cultures at four different antibiotic concentrations (**Table 2.4**). One mL of GSe media was aliquoted to each well. One mL of antibiotic solution was pipetted to the first well and a series of 12, 50% (1 into 2) dilutions created. Sub-samples of *G. catenatum* culture (1 mL) grown with single and pairwise combinations of antibiotic-sensitive or antibiotic-resistant *Brachybacterium* sp. and *Marinobacter* sp. was added to each well and the microtitre plates were incubated at 19°C +/- 2.5°C at a light intensity of 90±10 $\mu moles\ m^{-2}\ s^{-1}$ with a 12L: 12D photoperiod. For uni-bacterial *G. catenatum* cultures, the MIC was considered to be the lowest concentration of antibiotic that inhibited the growth of antibiotic-sensitive strains but not the antibiotic-resistant bacteria. Spread

plating on to ZM1 plates was used to confirm the antibiotic effect on bacteria. In *G. catenatum* cultures grown with pairwise combination of sensitive or resistant bacteria, MIC was defined as the lowest antibiotic concentration that inhibited growth of the targeted (sensitive) bacterial type but not the resistant bacterial type. For example; in *G. catenatum* cultures grown with cephazolin-resistant *Brachybacterium* sp. and streptomycin resistant *Marinobacter* sp., the MIC of cephazolin was the concentration that killed *Marinobacter* sp. only (**Table 2.5**).

Confirmation that only the targeted antibiotic-sensitive bacterial type had been removed was tested by 16SrDNA sequencing of randomly selected colonies following antibiotic treatment. Culture samples taken on day 6 after antibiotic addition were plated onto ZM1 plates and 5-10 random colonies selected for sequencing (section 2.3.4).

Table 2.4: Minimum inhibitory concentration of erythromycin and cephazolin (*Brachybacterium* sp.), and tobramycin and streptomycin (*Marinobacter* sp.). *G. catenatum* cultures grown with single bacterial type was tested for effective antibiotic concentration that would remove antibiotic sensitive bacteria but not the resistant bacteria.

Antibiotic added (μg mL^{-1}) / Bacterial isolate	**1st well**	**2nd well**	**3rd well**	**4th well**
Erythromycin	5	**2.5**	1.25	0.625
Erythromycin sensitive *Brachybacterium* sp.	No growth	**No growth**	Growth	Growth
Erythromycin resistant *Brachybacterium* sp.	Growth	**Growth**	Growth	Growth
Cephazolin	**6**	3	1.5	0.75
Cephazolin sensitive *Brachybacterium* sp.	**No growth**	Growth	Growth	Growth
Cephazolin resistant *Brachybacterium* sp.	**Growth**	Growth	Growth	Growth
Tobramycin	2	**1**	0.5	0.25
Tobramycin sensitive *Marinobacter* sp.	No growth	**No growth**	Growth	Growth
Tobramycin resistant *Marinobacter* sp.	No growth	**Growth**	Growth	Growth
Streptomycin	7.5	3.75	1.87	0.93
Streptomycin sensitive *Marinobacter* sp.	**No growth**	Growth	Growth	Growth
Streptomycin resistant *Marinobacter* sp.	**Growth**	Growth	Growth	Growth

Table 2.5: Minimum inhibitory concentration of erythromycin, cephazolin (*Brachybacterium* sp.) and tobramycin, streptomycin (*Marinobacter* sp.). *G. catenatum* cultures grown with pair-wise combination of bacteria were tested for effective concentration that would remove the antibiotic sensitive bacterial isolate in the mixture and not the resistant bacteria.

Treatments	**Antibiotic ($\mu g\ mL^{-1}$)**	**1st well**	**2nd well**	**3rd well**	**4th well**	**5th well**
Sensitive *Brachybacterium* sp. + sentive *Marinobacter* sp.	Cephazolin	6	**3**	**1.5**	0.75	0.375
Brachybacterium sp. and *Marinobacter* sp.		No Growth	No Growth	**No Growth**	Growth	Growth
Erythromycin resistant *Brachybacterium* sp.+ streptomycin resistant *Marinobacter* sp.	Erythromcyin	10	5	**2.5**	1.25	0.625
Brachybacterium sp.		No Growth	Growth	**Growth**	Growth	Growth
Marinobacter sp.		No Growth	No Growth	**No Growth**	Growth	Growth
Cephazolin resistant *Brachybacterium* sp.+ tobramycin resistant *Marinobacter* sp.	Cephazolin	6	3	**1.5**	0.75	0.375
Brachybacterium sp.		No growth	Growth	**Growth**	Growth	Growth
Marinobacter sp.		No growth	No growth	**No Growth**	Growth	Growth
Erythromycin resistant *Brachybacterium* sp.+ tobramycin resistant *Marinobacter* sp.	Tobramycin	4	2	**1**	0.5	0.25
Brachybacterium sp.		No growth	No growth	**No growth**	Growth	Growth
Marinobacter sp.		No growth	Growth	**Growth**	Growth	Growth
Cephazolin resistant *Brachybacterium* sp.+ streptomycin resistant *Marinobacter* sp.	Streptomycin	7.5	3.75	**1.87**	0.937	0.468
Brachybacterium sp.		No growth	No growth	**No growth**	Growth	Growth
Marinobacter sp.		No growth	Growth	**Growth**	Growth	Growth

2.2.8 Antibiotic application to *G. catenatum* cultures-microtitre plate cultures.

Prior to applying antibiotics to 100 mL *G. catenatum* cultures, antibiotic treatments were performed using 12-well microtitre plate cultures of *G. catenatum*. Briefly, 3 mL of GSe and 1 mL of *G. catenatum* cultures grown with single (sensitive/resistant) bacteria, or pairwise combinations was transferred aseptically to triplicate wells. One mL of antibiotic solution was aliquoted to the wells (see **Table 2.6** for antibiotic concentrations used). The antibiotics are not stable for longer than 6 days at 19°C, hence a second dose of antibiotic was added on day 7. Microtitre plates were sealed with Parafilm^{TM} and incubated at 19°C +/- 2.5°C at a light intensity of 90±10 μmoles m^{-2} s^{-1} with a 12L: 12D photoperiod. Triplicate subsamples were withdrawn daily for 7 days to estimate dinoflagellate cell concentration using a Sedgwick-Rafter counting chamber (Guillard 1973), and bacterial cell concentration (CFU mL^{-1}) by serial dilution plating on to ZM1 (Buck & Cleverdon 1960).

2.2.9 Antibiotic application to *G. catenatum* cultures – 100 mL Erlenmeyer flask cultures.

Triplicate *G. catenatum* cultures in 150 mL Erlenmeyer flasks were grown to mid-logarithmic phase in the presence of antibiotic sensitive or resistant *Brachybacterium* sp. or *Marinobacter* sp. (see **Table 2.6**) and treated with MIC concentrations of antibiotics at day 5 after subculturing. Repeated doses of antibiotics were added on day 10 and 15. Bacterial cell and dinoflagellate cell concentrations were estimated daily for 21 days from triplicate sub-samples using serial dilution plating on to ZM1 agar plates and Sedgwick- Rafter chamber counts respectively (as described earlier).

To confirm that antibiotic treatment had removed only the targeted sensitive bacteria in mixed bacterial cultures, culture samples on day 10 were plated onto ZM1 and 5-10 random colonies was selected for 16SrDNA sequence analysis.

2.2.10 DNA extraction, PCR and DNA sequencing

Sub-samples of *G. catenatum*-bacterium cultures were passed through an 8 μm nuclepore filter (Millipore, USA) to remove dinoflagellate cell biomass and 120 μl of the filtrate spread onto ZM1 agar and incubated at 25°C for 24-48 hrs. Randomly selected colonies (5–10) were re-inoculated on to fresh ZM1 agar and incubated until sufficient colony biomass was available for DNA extraction.

Bacterial genomic DNA extraction from bacterial colonies was based on a cetyltrimethylammonium bromide purifictaion (CTAB) method (Ausubel *et al.* 1999) (see Appendix 4). Polymerase chain reaction (PCR) was carried out using a MJ Research PTC-200 Thermal Cycler (MJ Research, USA). Reactions were carried out in a 50 μl reaction volumes containing 0.2 mM of primer 27F (AGAGTTTGATCMTGGCTCAG) and 1492R (ACGGCTACC - TTGTTACGACTT) (Weisburg *et al. 1*991), 2.5 U of Taq polymerase (BioTaq, Bioline, UK), 3 mM $MgCl_2$, 200 mM of each dNTP and Bioline ammonium buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris HCl (pH 8.8), 0.1% Tween-20). PCR cycling included an initial denaturation at 96°C for 5 min., followed by 30 cycles of: denaturation at 95 °C for 15 s, annealing at 49°C for 30 s, extension at 72°C for 1 min; and a final polishing step at 72°C for 5 mins. Completed reactions were held at 15°C until removed and stored at 4°C until later analysis.

The 16SrDNA products amplified were separated by submerged horizontal gel electrophoresis through 1.5% agarose/TBE gel. The PCR products were compared with Hyperladder I, (Bioline, UK) as a size standard. Gels were examined for expected product size and purity under UV light and photographed using a UVP DigiDoc-It imaging system (UVP corporation, CA, USA)

Successful PCR products were purified using Montage-PCR ultrafilters (Millipore, USA) based on manufacturer protocols and the DNA resuspended in MilliQ water (Millipore, USA). The

DNA concentration was estimated using a Turner TBS380 DNA fluorometer (Turner Designs, USA) according to standard protocols. Samples including purified template and primer (27F) were prepared and transported to the Australian Genomic Research Facility (AGRF), Queensland, Australia for dye-terminator DNA sequencing and electrophoresis. AGRF used a ABI 3730xl capillary DNA sequencer (PE Applied Biosystems, CA, USA) to elecrophorese the sequencing reactions. The resulting sequence electropherograms were viewed using Chromas lite 2.01 (Technelysium Pty Ltd, Australia) and sequences corrected by manual inspection.

Nucleotide sequence alignment and comparison was performed using BLAST (Basis Local Assignment Tool), accessed via to GenBank database (National Centre for Biotechnology Information, NCBI, (http://www.ncbi.nlm.nih.gov/BLAST/). Similar Genbank DNA sequences were aligned with the 16SrDNA sequences obtained using CLUSTALW (http:// www.ebi.ac.uk/clustalw/), a multiple alignment program for DNA.

2.2.11 Statistical analysis

Significant differences among treatments and controls for cyst germination and cells per germinated cyst (at day 30) were compared using one-way ANOVA (with Tukey's LSD post-hoc tests). To compare *G. catenatum* response to removal of co-existing bacteria, a paired sample t-test for dependent groups was performed. Algal cell numbers on day 0 and day 7 or 21 were compared for statistical significance. All statistical analysis were performed using statistical analysis software SPSS ver. 11.5 (LEAD technologies, Chicago, USA).

2.3 RESULTS

2.3.1 Resting cyst germination and growth of *G. catenatum* to day 30.

Germination of resting cysts in the presence of single bacteria or pair- wise combination of sensitive or resistant *Brachybacterium* sp. and *Marinobacter* sp. ranged from 24% to 39%. The negative control (sterile cysts with no bacteria) showed the poorest (10%) germination. The majority of germinated cells died within the 30 day observation period and long-term culture of these treatments was not possible. In contrast, germination rates in treatments with sensitive and/or resistant bacteria were similar to the positive control ($f = 1.230$; $df = 12, 26$; $P > 0.112$) and all treatments survived beyond 30 days allowing long term culture maintenance (**Fig. 2.5**).

The number of motile dinoflagellate cells per germinated cyst at day 30 did not vary significantly among the treatments and positive control ($f = 5.239$; $df = 12, 26$; $P > 0.996$), however, the negative control (no bacteria) had significantly fewer moving cells at day 30 ($P = 0.013$) (**Fig. 2.6**)

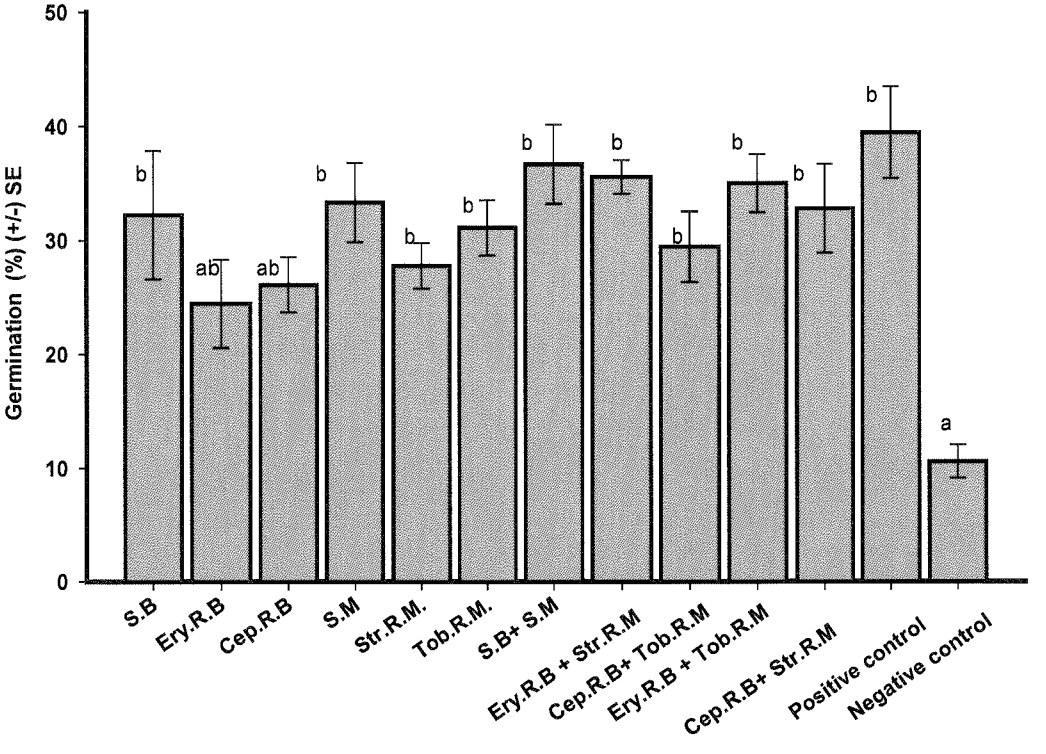

Fig. 2.5: Germination (%) (± standard error) in different treatments, positive and negative control. B= *Brachybacterium* sp.; M= *Marinobacter* sp. DG879; Ery= erythromycin; Cep= cephazolin; Tob= tobramycin; Str= streptomycin; S= antibiotic sensitive; R= resistant. Superscripts indicate significant differences (P= < 0.05)

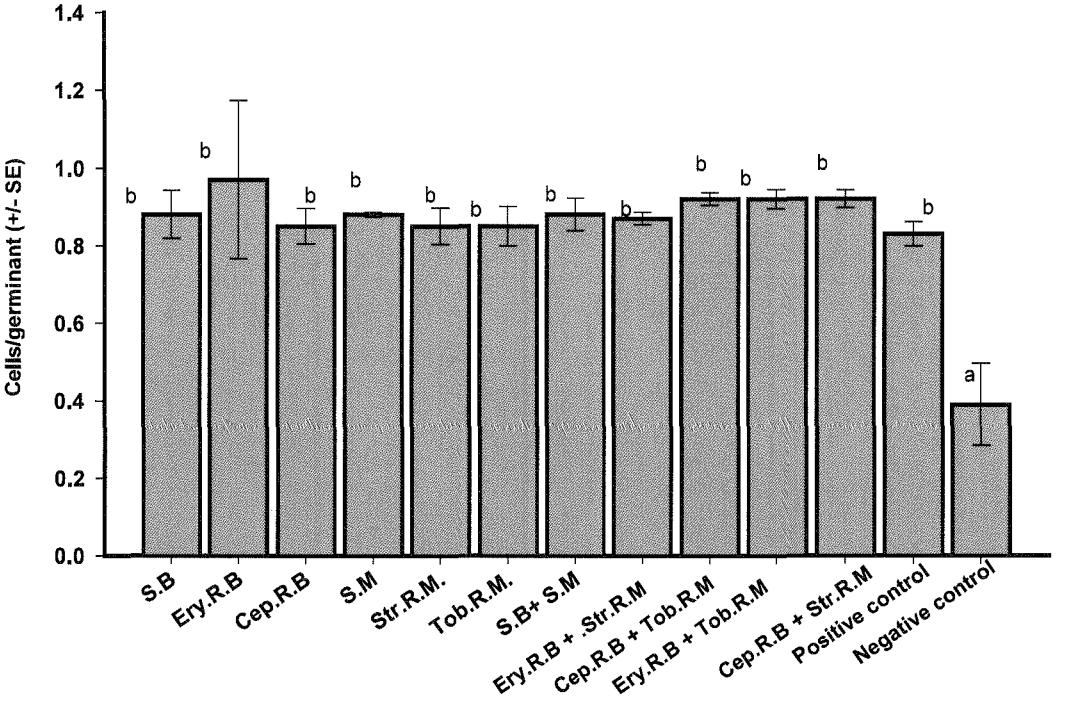

Fig. 2.6: Moving cells per germinant (± standard error) in different treatments and controls at day 30. B= *Brachybacterium* sp.; M= *Marinobacter* sp. DG879; Ery= erythromycin; Cep= cephazolin; Tob= tobramycin; Str= streptomycin; S= antibiotic sensitive; R= resistant. Superscripts indicate significant differences (P= < 0.05)

2.3.2 Minimum inhibitory concentrations (MIC)

The MIC of antibiotics effective to remove antibiotic sensitive bacteria (but not the resistant isolate) was determined as 2.5 μg mL^{-1} of erythromycin; 6 & 1.5 μg mL^{-1} of cephazolin (*Brachybacterium* sp.) and 1 μg mL^{-1} of tobramycin and 7.5 & 1.87 μg mL^{-1} of streptomycin (*Marinobacter* sp.) (**Table 2.6**). The antibiotic concentrations added to cultures of *G. catenatum* grown with pair-wise combinations of resistant bacteria effectively eliminated the targeted sensitive bacteria without affecting the other resistant bacterial isolate in the culture (**Table 2.6**). For example, in the *G. catenatum* culture grown in the presence of erythromycin-resistant *Brachybacterium* sp. and streptomycin-resistant *Marinobacter* sp., 2.5 μg mL^{-1} of erythromycin eliminated only *Marinobacter* sp. leaving *Brachybacterium* sp. to support *G. catenatum* growth (**Table 2.6**).

Table 2.6: Final concentration of various antibiotics used in the experiment based on MIC assay.

	***G. catenatum* grown with**	**Antibiotic used**	**Final antibiotic concentration used ($\mu g\ mL^{-1}$)**
1	Erythromycin sensitive/resistant *Brachybacterium* sp.	Erythromycin	2.5
2	Cephazolin sensitive/resistant *Brachybacterium* sp.	Cephazolin	6
3	Tobramycin sensitive/resistant *Marinobacter* sp.	Tobramycin	1
4	Streptomycin sensitive/resistant *Marinobacter* sp.	Streptomycin	7.5
5	*Brachybacterium* sp.(sensitive) + *Marinobacter* sp.(sensitive)	Cephazolin	1.5
6	Erythromycin resistant *Brachybacterium* sp. + streptomycin resistant *Marinobacter* sp.	Erythromycin	2.5
7	Cephazolin resistant *Brachybacterium* sp. + tobramycin resistant *Marinobacter* sp.	Cephazolin	1.5
8	Erythromycin resistant *Brachybacterium* sp. + tobramycin resistant *Marinobacter* sp.	Tobramycin	1
9	Cephazolin resistant *Brachybacterium* sp. + streptomycin resistant *Marinobacter* sp.	Streptomycin	1.87

2.3.3 Antibiotic application to *G. catenatum* cultures - microtitre plate cultures.

Addition of antibiotics to *G. catenatum* microtitre plate cultures grown with antibiotic-sensitive bacteria resulted in a dramatic and immediate reduction in bacterial concentration (**Fig. 2.7** B & D; **Fig. 2.8** B & D) over days 1-5, followed by decline of dinoflagellate cell concentration beginning from around day 2-3. The antibiotics added were effective to remove the sensitive bacteria completely by day 4-5. In every case, reduction in the bacterial cell numbers (**Fig. 2.7** B & D) was followed by significant decrease in algal cell numbers, resulting in at least a 10 fold reduction by day 7 ($t = 24.87$; $df = 11$; $P = 0.000$) (**Fig. 2.7** A & C). In contrast growth of cultures with antibiotic resistant bacteria were unaffected by the antibiotic application (**Fig. 2.7** B & D; **Fig. 2.8** B & D). Bacterial cells survived the treatment; continued exponential growth and significantly higher algal cell numbers were seen on day 7 compared to day 0 ($t = -9.777$; $df = 11$; $P = 0.000$) (**Fig. 2.7** A & C; **Fig. 2.8** A & C).

G. catenatum cultures grown with pair-wise combinations of sensitive bacteria showed a rapid reduction in bacterial cell numbers after antibiotic treatment and bacteria were completely removed by day 5 (**Fig. 2.9** B & D). Reduction in bacterial cell concentration was followed by a decline in *G. catenatum* cell concentration from day 2 and a 10 fold significant reduction in cell concentration was evident by day 7 ($t = 10.533$; $df = 2$; $P = 0.009$).

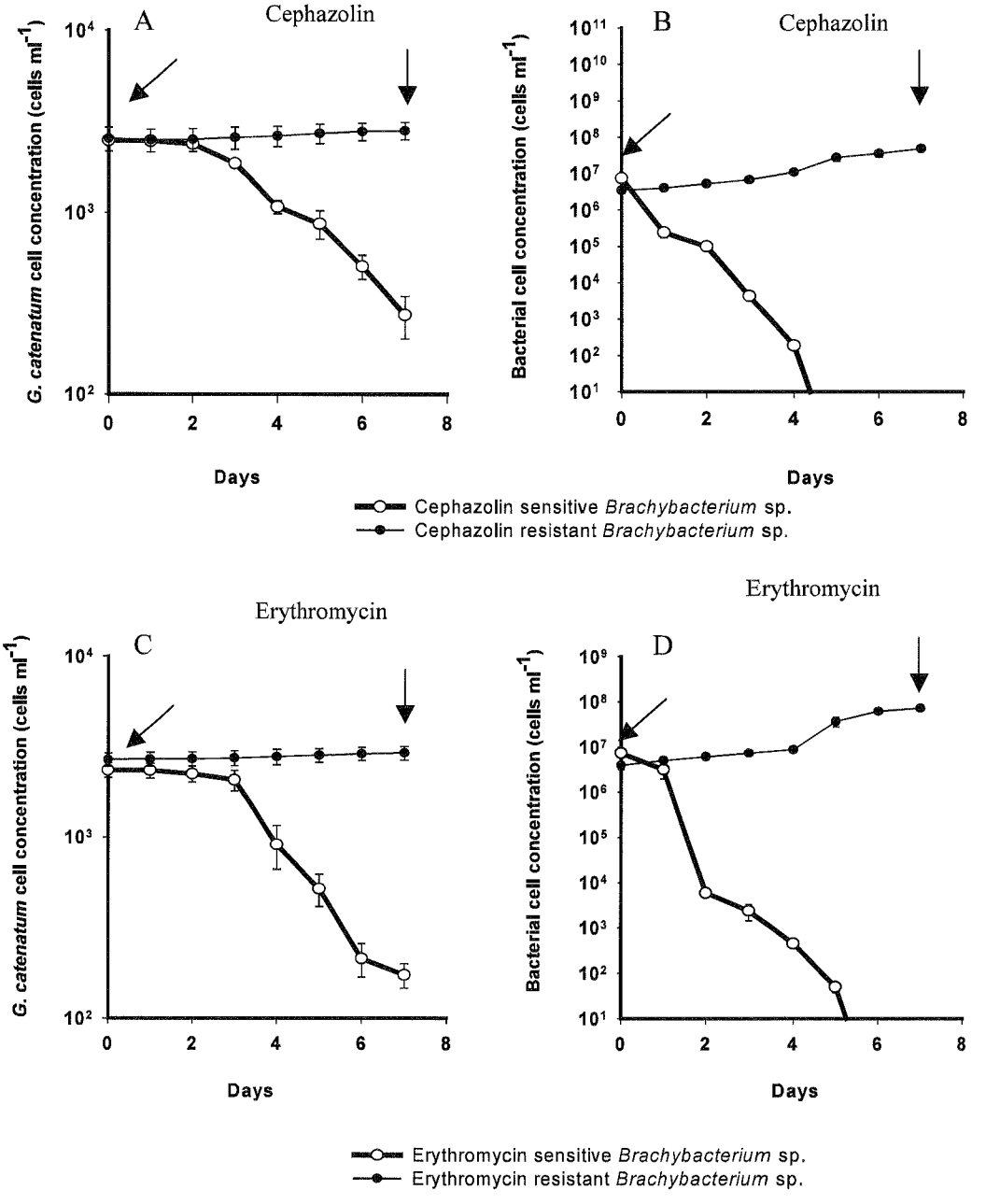

Fig. 2.7: Effect of cephazolin or erythromycin application on microtitre plate *G. catenatum* cultures grown with antibiotic-sensitive or antibiotic-resistant *Brachybacterium* sp. (± standard error). (A & C) *G. catenatum* cell concentration (cells mL^{-1}); (B & D) bacterial cell concentration (cells mL^{-1}). Arrows indicate day of antibiotic addition.

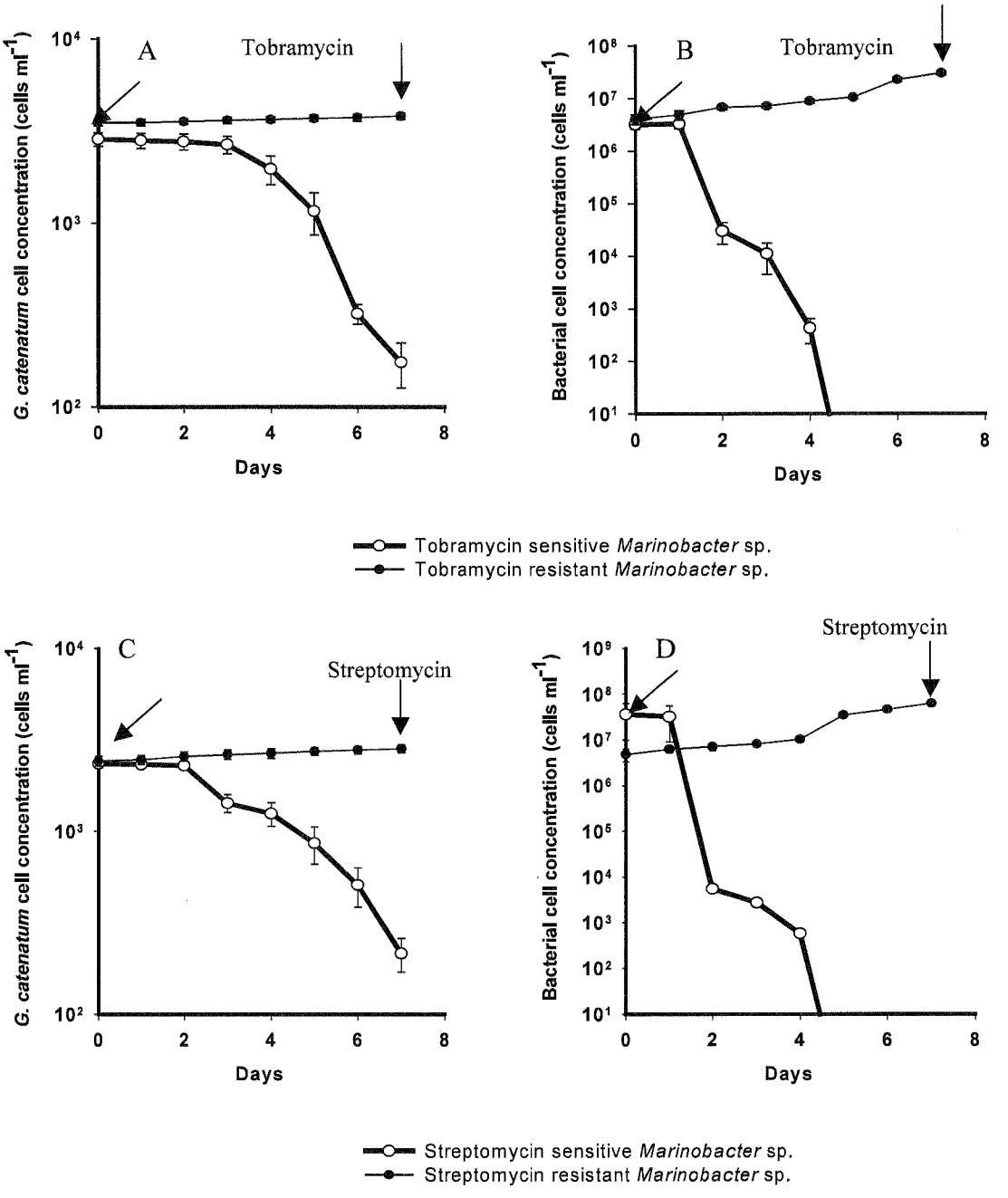

Fig. 2.8: Effect of tobramycin or streptomycin application on microtitre plate *G. catenatum* cultures grown with antibiotic-sensitive or antibiotic-resistant *Marinobacter* sp. DG879 (± standard error). (A & C) *G. catenatum* cell concentration (cells mL^{-1}); (B & D) bacterial cell concentration (cells mL^{-1}); Arrows indicate day of antibiotic addition.

Antibiotic application to *G. catenatum* grown with pair-wise combinations of sensitive bacteria, removed both bacterial types by day 5 (**Fig. 2.9** B & D) followed by dramatic reduction in algal cell numbers (**Fig. 2.9** A & C). In contrast, *G. catenatum* cultures grown with pair-wise combination of antibiotic-resistant bacterial types upon antibiotic treatment to remove one of the bacterial type showed a initial reduction in bacterial cell numbers until day 2 (**Fig. 2.9** B & D) which was also reflected in algal cell concentration (**Fig. 2.9** A & C). As the bacterial cell numbers increased from day 2–4, the algal mean cell concentration also improved. However, the increase in algal cell numbers at day 7 was not significantly ($t = -1.084$; $df = 11$; $P = 0.301$) higher from day 0 (**Fig. 2.9** A & C).

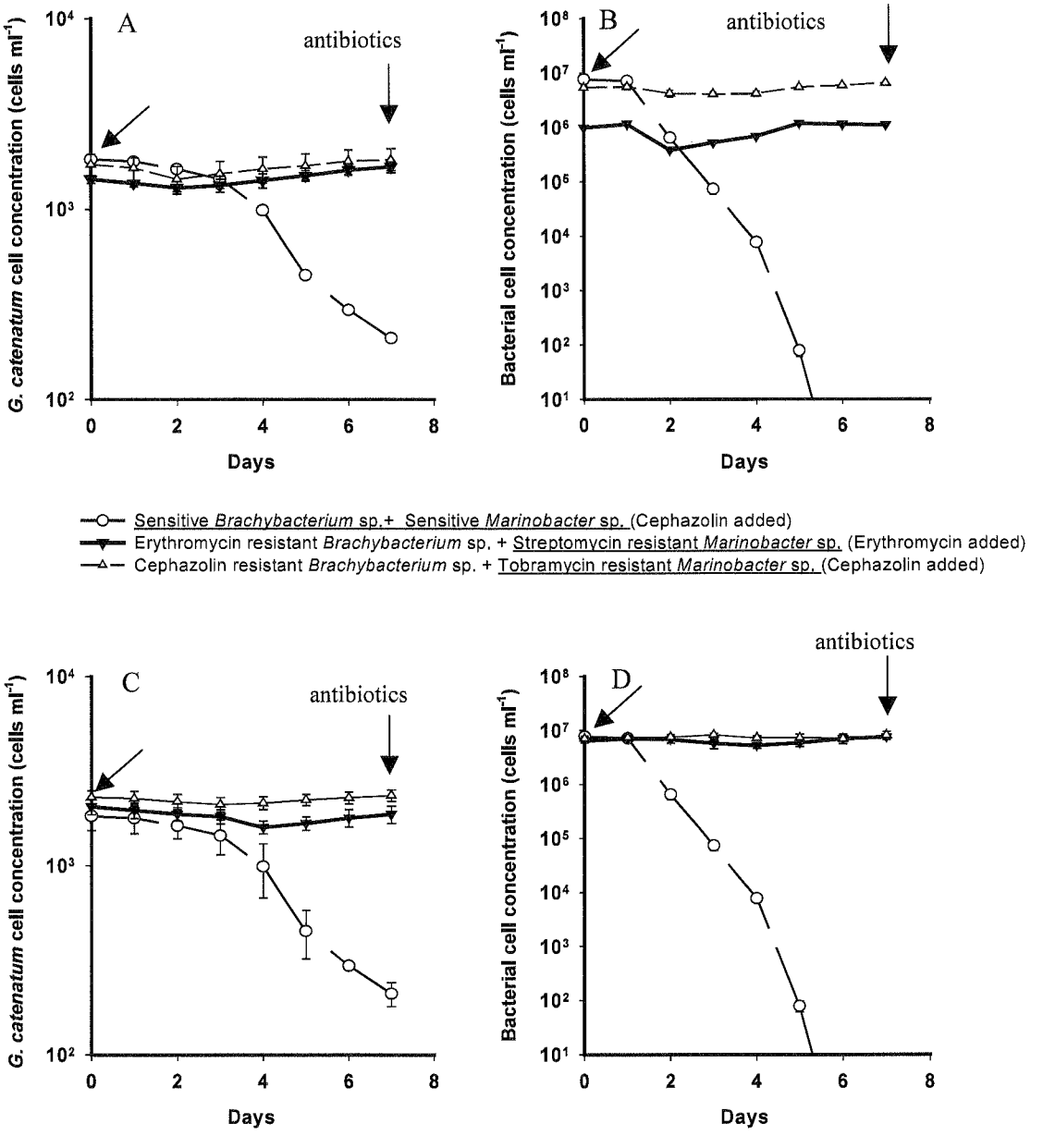

Fig. 2.9: Effect of antibiotic addition on *G. catenatum* microtitre cultures grown with pair-wise combinations of antibiotic-sensitive or antibiotic-resistant *Brachybacterium* sp. (± standard error). (A & C) *G. catenatum* cell concentration (cells mL^{-1}); (B & D) bacterial cell concentration (cells mL^{-1}); Underline indicates the isolate removed by the antibiotic. Arrows indicate day of antibiotic addition.

2.3.4 Antibiotic application to *G. catenatum* cultures – 100 mL Erlenmeyer flasks.

The higher volume trials allowed tracking algal growth or decline for 21 days. Results obtained from antibiotic addition experiments performed in 100 mL flasks were similar (**Fig. 2.10**) to experiments performed in microtitre plates. *G. catenatum* cultures grown with single sensitive bacterial isolate upon antibiotic application showed a decline in bacterial cell density that was followed by 10 fold, significant reduction, in algal cell numbers by day 21 (t= 11.55; df= 11; *P*= 0.000). In contrast, antibiotics added to *G. catenatum* grown with single resistant bacteria, had little or no effect on bacterial growth and algal cell numbers continued to increase until the experiment was terminated (day 21). Test for significance revealed significantly higher *G. catenatum* concentration on day 21 compared to day 0, (t= - 8.86; df= 11; *P*= 0.000) **(Fig. 2.10; Fig. 2.11)**.

In cultures grown with pair-wise combination of sensitive bacteria, antibiotic treatment removed both the antibiotic-sensitive bacteria, however the treatment did not have a significant effect on algal cell numbers compared to positive control on day 21 (t= 1.655; df= 5; *P*= 0.159). Similarly in *G. catenatum* cultures grown with pair-wise combinations of resistant bacteria, removal of one of the bacterial type did not show significant effect on algal cell numbers at the end of trial (t= 1.407; df= 11; *P*= 0.187) (**Fig. 2.12**).

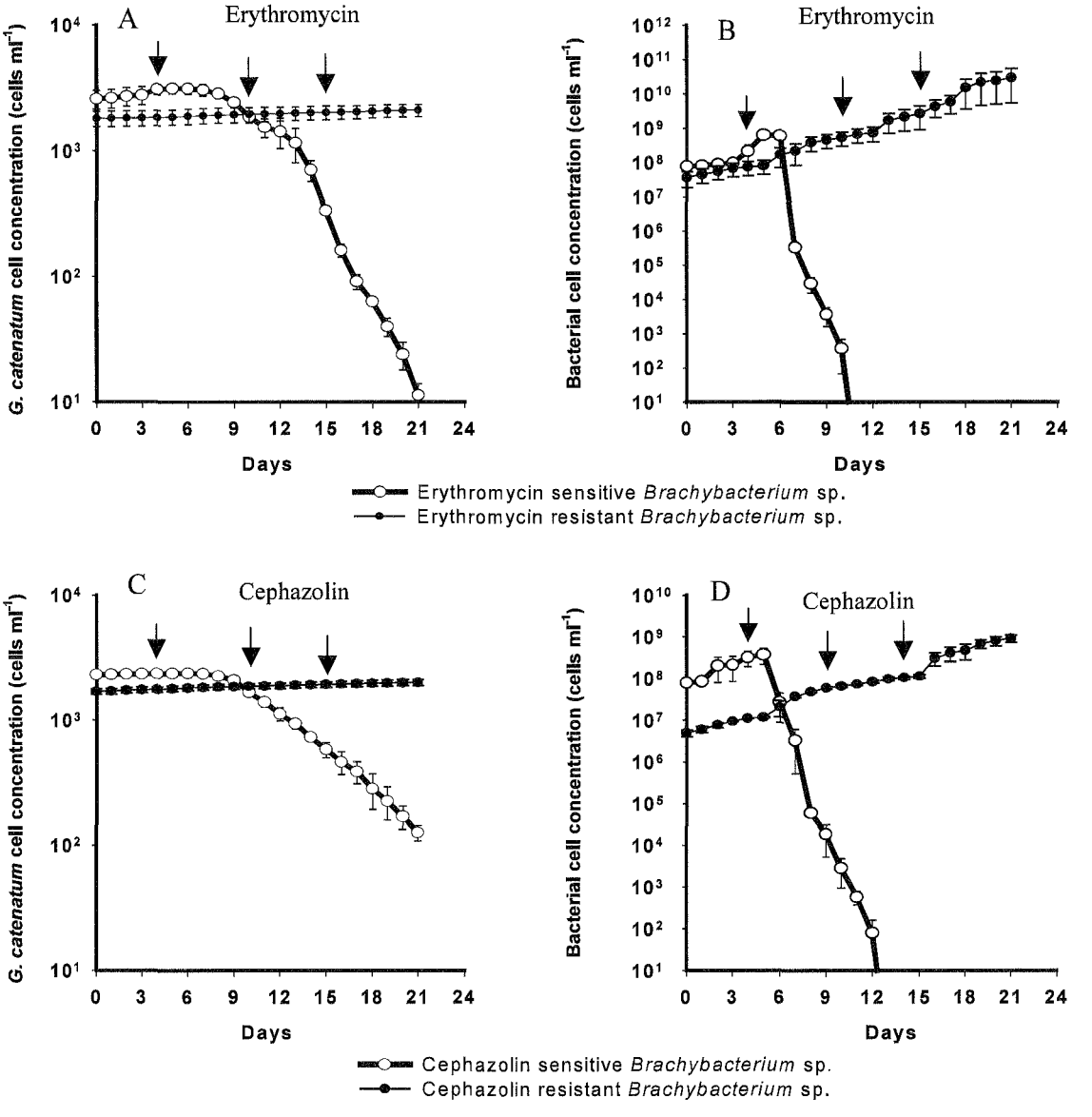

Fig. 2.10: Effect of antibiotic application to *G. catenatum* 100 mL log phase flask cultures grown with antibiotic-sensitive or antibiotic-resistant *Brachybacterium* sp. (± standard error). (A & C) *G. catenatum* cell concentration (cells mL^{-1}); (B & D) bacterial cell concentration (cells mL^{-1}). Arrows indicate day of antibiotic addition.

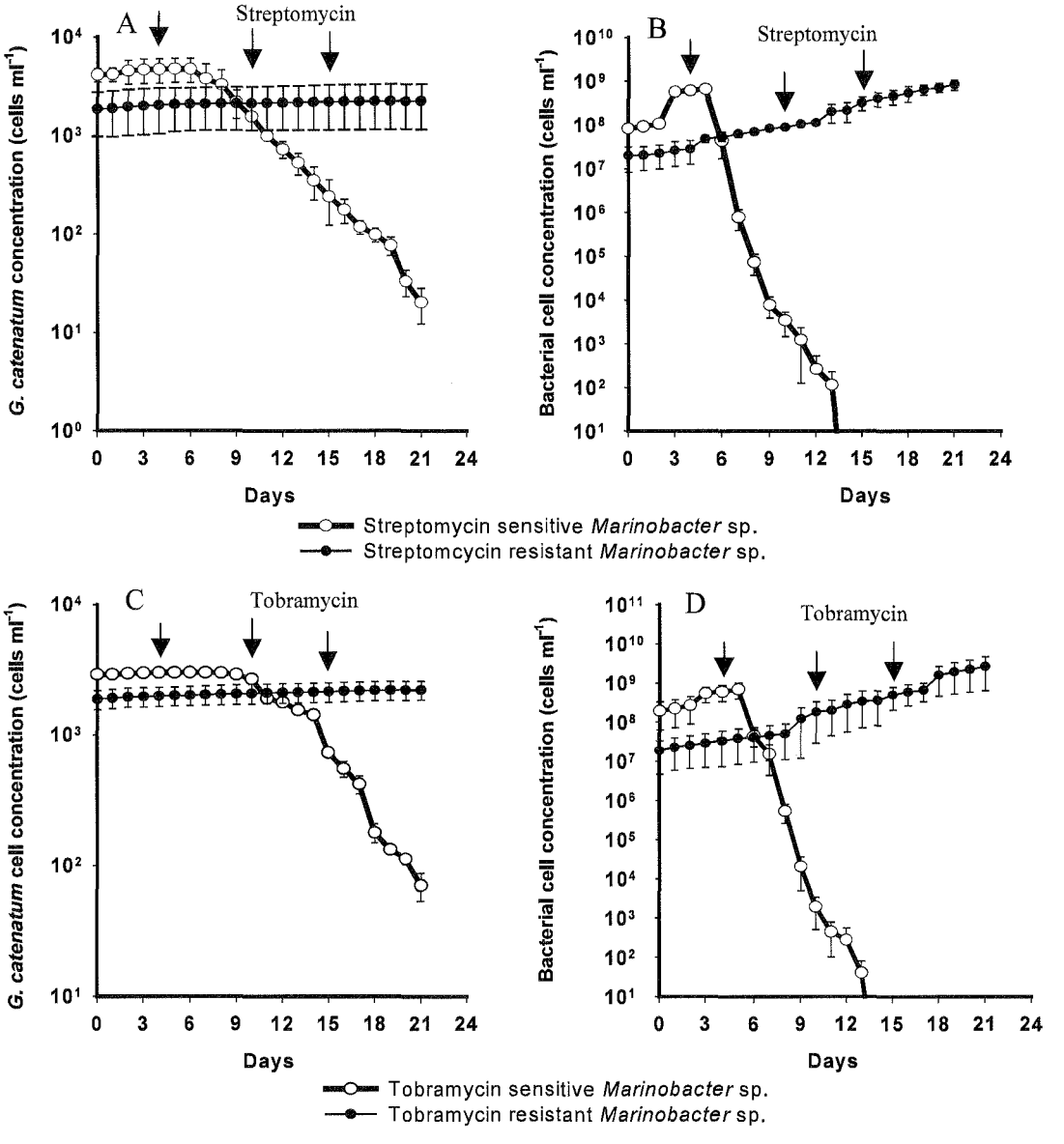

Fig. 2.11: Effect of antibiotic addition to *G. catenatum* 100 mL log phase flask cultures grown with antibiotic-sensitive or antibiotic-resistant *Marinobacter* sp. DG879 (± standard error). (A & C) *G. catenatum* cell concentration (cells mL^{-1}); (B & D) bacterial cell concentration (cells mL^{-1}). Arrows indicate day of antibiotic addition.

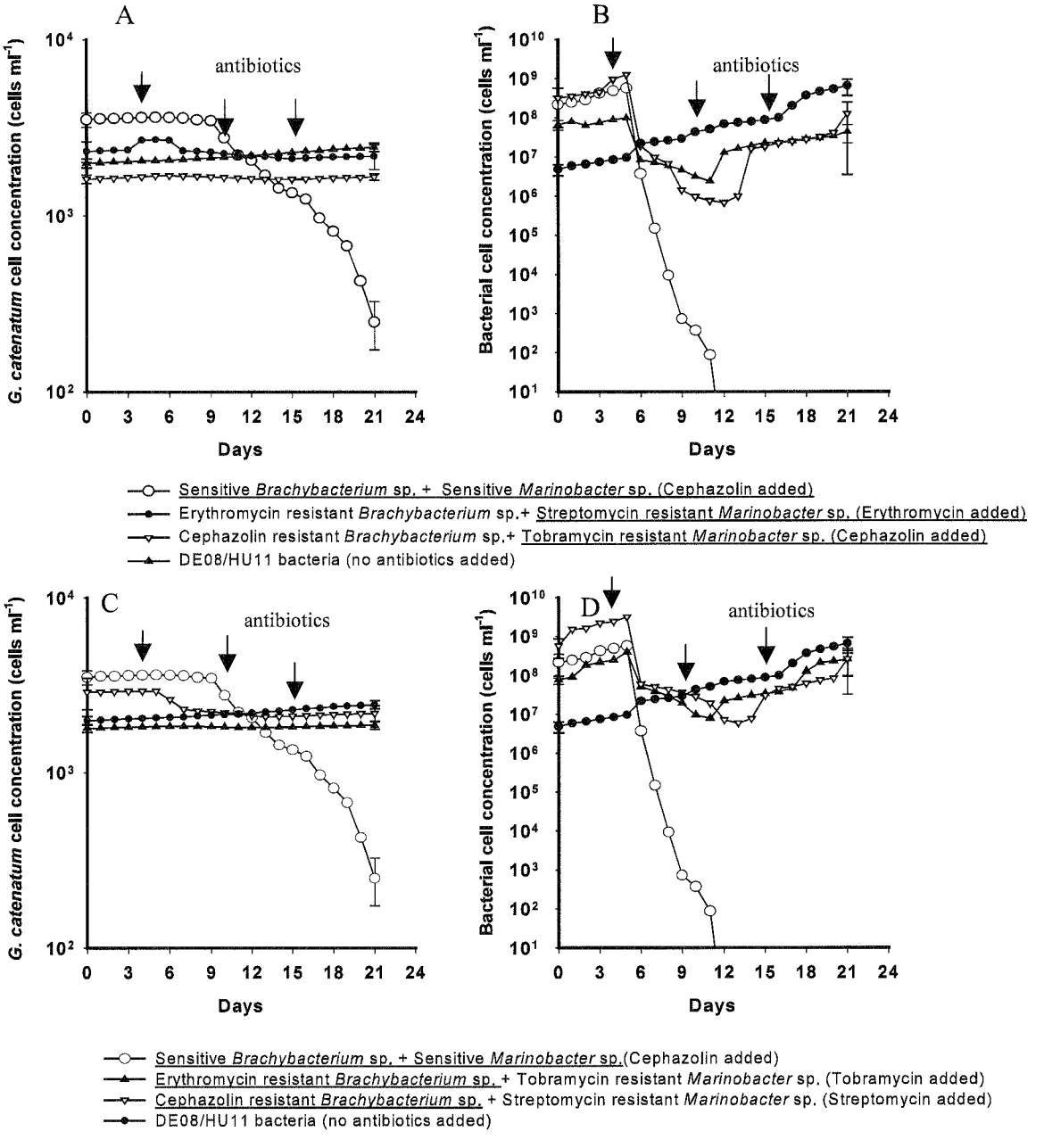

Fig. 2.12: Effect of antibiotic addition to 100 mL *G. catenatum* log phase flask cultures grown with pair-wise combination *Brachybacterium* sp. and *Marinobacter* sp. DG879. (A & C) *G. catenatum* cell concentration (cells mL^{-1}); (B & D) bacterial cell concentration (cells mL^{-1}). Arrows indicate day of antibiotic addition. Standard error bars shown only for statistically compared days (0 & 21) as overlapping bars obscure trends of growth curve. Underlining indicates the isolate removed by the antibiotic.

2.3.5 16SrDNA sequence analysis:

DNA sequence analysis of colonies of the bacteria remaining after antibiotic treatment in pair-wise combination of resistant bacterial types consistently recovered only the resistant bacterial type in each case. For cultures in which *Marinobacter* sp. was removed with erythromycin (treatment 6; **Table 2.6**), the DNA sequence recovered from colonies shared 99% sequence identity with *Brachybacterium* sp. (PSGB10, RODSPM16, GN0406-11.4ps.b, SKJH-25, PB10) and matched the sequence of the *Brachybacterium* sp. used in the experiment (**Fig. 2.13**). Cephazolin treatment to remove *Marinobacter* sp. showed (treatment 7, **Table 2.6**), the DNA sequence recovered from the remaining bacterial colonies to share 100% sequence identity with *Brachybacterium* sp. (PSGB10, RODSPM16, GN0406-11.4ps.b, PB10). Similarly cultures to which tobramycin or streptomycin (treatment 8, 9; **Table 2.6**) was added to remove *Brachybacterium* sp., the DNA sequence recovered showed 100% sequence identity with *Marinobacter* sp. DG879 (AY258107).

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Brachybacterium sp.     GTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC 60
PSGB10                  GTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC 60
RODSPM16                GTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC 60
GN0406-11.4ps.b         GTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC 60
SKJH-25                 GTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC 60
PB10                    GTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC 60
                        ************************************************************

Brachybacterium sp.     GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGT 120
PSGB10                  GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGT 120
RODSPM16                GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGT 120
GN0406-11.4ps.b         GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGT 120
SKJH-25                 GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGT 120
PB10                    GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGT 120
                        ************************************************************

Brachybacterium sp.     GGGGGATGACGGCCTTCGGGTTGTAAACCCCTTTCAGTAGGGAAGAAGCGAGAGTGACGG 180
PSGB10                  GGGGGATGACGGCCTTCGGGTTGTAAACCCCTTTCAGTAGGGAAGAAGCGAGAGTGACGG 180
RODSPM16                GGGGGATGACGGCCTTCGGGTTGTAAACCCCTTTCAGTAGGGAAGAAGCGAGAGTGACGG 180
GN0406-11.4ps.b         GGGGGATGACGGCCTTCGGGTTGTAAACCCCTTTCAGTAGGGAAGAAGCGAGAGTGACGG 180
SKJH-25                 GGGGGATGACGGCCTTCGGGTTGTAAACCCCTTTCAGTAGGGAAGAAGCGAGAGTGACGG 180
PB10                    GGGGGATGACGGCCTTCGGGTTGTAAACCCCTTTCAGTAGGGAAGAAGCGAGAGTGACGG 180
                        ************************************************************

Brachybacterium sp.     TACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGC 240
PSGB10                  TACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGC 240
RODSPM16                TACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGC 240
GN0406-11.4ps.b         TACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGC 240
SKJH-25                 TACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGC 240
PB10                    TACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGC 240
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Brachybacterium sp.   AAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGTGGCTTGTCGCGTCTGCCGT 300
PSGB10                AAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGTGGCTTGTCGCGTCTGCCGT 300
RODSPM16              AAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGTGGCTTGTCGCGTCTGCCGT 300
GN0406-11.4ps.b       AAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGTGGCTTGTCGCGTCTGCCGT 300
SKJH-25               AAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGTGGCTTGTCGCGTCTGCCGT 300
PB10                  AAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGTGGCTTGTCGCGTCTGCCGT 300
                      ****************************************************** 

Brachybacterium sp.   GAAAACCCGAGGCTCAACCTCGGGCGTGCGGTGGGTACGGGCAGGCTAGAGTGTGGTAGG 360
PSGB10                GAAAACCCGAGGCTCAACCTCGGGCGTGCGGTGGGTACGGGCAGGCTAGAGTGTGGTAGG 360
RODSPM16              GAAAACCCGAGGCTCAACCTCGGGCGTGCGGTGGGTACGGGCAGGCTAGAGTGTGGTAGG 360
GN0406-11.4ps.b       GAAAACCCGAGGCTCAACCTCGGGCGTGCGGTGGGTACGGGCAGGCTAGAGTGTGGTAGG 360
SKJH-25               GAAAACCCGAGGCTCAACCTCGGGCGTGCGGTGGGTACGGGCAGGCTAGAGTGTGGTAGG 360
PB10                  GAAAACCCGAGGCTCAACCTCGGGCGTGCGGTGGGTACGGGCAGGCTAGAGTGTGGTAGG 360
                      ************************************************************ 

Brachybacterium sp.   GGAGACTGGAACTCCTGGTGTAGCGGTGAAATGCGCAGATATCAG-AAGAACACCGATGG 419
PSGB10                GGAGACTGGAACTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAAGAACACCGATGG 420
RODSPM16              GGAGACTGGAACTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAAGAACACCGATGG 420
GN0406-11.4ps.b       GGAGACTGGAACTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAAGAACACCGATGG 420
SKJH-25               GGAGACTGGAACTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAAGAACACCGATGG 420
PB10                  GGAGACTGGAACTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAAGAACACCGATGG 420
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Fig. 2.13: ClustalW multiple sequence alignment of *Brachybacterium* sp. remaining in cultures after treatment with erythromycin to remove *Marinobacter* sp. Sequence is compared with 5 *Brachybacterium* spp. (99% similarity) obtained from GenBank database (BLAST).

2.4 DISCUSSION

The experiments presented here constitute the first conclusive evidence that *G. catenatum* has an obligate requirement for marine bacteria for long term survival. Antibiotic application to *G. catenatum* cultures grown with antibiotic sensitive bacteria showed rapid reduction of bacterial cell density followed by a gradual decline in *G. catenatum* cell numbers. In contrast *G. catenatum* grown with antibiotic resistant bacterial strains were unaffected by antibiotics providing clear evidence that the dinoflagellate cell decline is not a direct result of the antibiotics, but is due to the decline or removal of the bacteria. Previous cyst germination studies included a negative control in which sterile *G. catenatum* cysts were germinated in bacteria free medium. Cysts germinated without bacteria did not survive or grow and long term maintenance of cultures was not possible, suggesting the need for marine bacteria for algal growth (Bolch *et al.* 2002; Vincent 2003; Bolch *et al.* 2004). Bacteria-free controls in the present study also showed poor germination and growth and long term maintenance was not possible.

Generating and maintaining axenic or bacteria-free cultures of dinoflagellates is difficult but widely reported in the literature (Boczar *et al.* 1988; Dantzer & Levin 1997; Doucette & Powell 1998; Alavi *et al.* 2001; Wang *et al.* 2004). Although these studies report achieving axenic status, it is unclear whether the cultures remained bacteria free throughout the study as bacterial detection measures are often limited to culture media and rarely reported in detail. For example, Dantzer & Levin (1997) reported bacteria-free status in *A. tamerense* cultures using marine agar. As 95% of marine bacterial strains are currently considered uncultivable (Schut *et al.* 1993), then unculturable bacteria may well have been present. Doucette & Powell (1998) used both epifluorescence microscopy and marine agar to ensure absence of bacteria in *A. lusitanicum* cultures but did not report the frequency of monitoring, therefore the bacteria-free status throughout the experiment is questionable. In the current and previous studies (Bolch *et al.* 2002; Vincent 2003; Bolch *et al.* 2004) cultivable bacteria were not detected on ZM1 agar in the bacteria-free controls. However, up to 40-50% bacteria in *G. catenatum* cultures may be unculturable (Green *et al.* 2008, unpublished

data), therefore unculturable bacteria may still have been present in the bacteria-free controls. Even if present, the consistent lack of growth in negative controls and antibiotic-sensitive treatments indicate that they were unable to support the growth of the dinoflagellate. Future studies could include 16S rDNA PCR using universal primers on DNA extracted from negative control cultures and sterile cyst germinations to confirm whether these treatments remain bacteria-free.

It appears that not all dinoflagellates require bacteria for growth as some studies have reported that removal of bacteria by antibiotics has little or no effect on the growth of *A. catenella* and *A. tamarense* (Uribe & Espejo 2003; Ho *et al.* 2006). However, both these studies have not ruled out the presence of unculturable bacteria within the dinoflagellate cells that may have survived antibiotic treatment. In contrast, the present work and Geier (2003) have shown that removal of bacteria kills *G. catenatum* cells. Lush (1999) reported reduced *A. minutum* cell densities during continuous exposure to antibiotics, with cultures failing to grow beyond 20 days from the antibiotic addition. Intermittent antibiotic exposures with fixed periods of recovery allowed cultures to grow, but at a much slower rate than normal.

Unlike previous studies, the work presented here has sufficient experimental controls to determine whether the death of the dinoflagellate cells is due to the antibiotics or alternatively due to the removal of the bacterial community. Continued dinoflagellate growth in the antibiotic resistant controls demonstrate quite clearly that direct antibiotic toxicity is not the cause of the dinoflagellate cell decline. While this work demonstrates the requirement for *G. catenatum*, observations by other workers suggest that only certain dinoflagellates may have an obligate requirement for bacteria to support growth both in laboratory cultures and natural blooms.

The simplified *G. catenatum*-bacteria model cultures used here allows the removal of vast majority of bacterial community, minimising the likelihood of other antibiotic-resistant bacteria surviving the antibiotic treatment. While other unculturable bacteria may be present in the experimental model cultures used here, the failure of the antibiotic-sensitive cultures clearly

indicates that (if present) they are not capable of supporting the growth of *Gymnodinium catenatum.* This supports the concept that the bacterial requirement of *Gymnodinium catenatum* may be limited to relatively few bacteria rather than being a general feature of many marine bacteria.

Experiments with pair-wise mixtures of bacteria where one bacterial type was removed by antibiotics indicate that algal cell growth is partly dependent on bacterial concentration or growth. The reduction of bacterial concentration in the few days following antibiotic addition is mirrored by a temporary cessation of dinoflagellate growth (**Fig. 2.9**; **Fig. 2.12**). One explanation for this pattern is that growth promotion is mediated by a factor produced only by actively growing bacterial cells and that the factor is utilized by the dinoflagellate. Under this scenario, a reduction of bacterial growth would rapidly reduce the production and concentration of growth stimulating factor and in turn affect growth of the dinoflagellate. It is also possible that the growth stimulating factors are unstable or degraded over a period of days. The subsequent recovery of algal growth indicates that bacterial and algal growth dynamics are strongly coupled in the experimental model cultures. Mouget *et al.* 1995 reported similar interactions between *Scenedesmus bicellularis* and *Pseudomans diminuta* where the stimulation of algal growth occurred only during bacterial growth phase.

Bacteria may stimulate phytoplankton growth by excreting growth-promoting secondary metabolites such as vitamins and other growth promoting compounds. For example Vitamin B_{12} or cobalamin deficient algae have been proved to be benefited through a symbiotic relationship with bacteria (Croft *et al.* 2005). Another possibility is that Iron (Fe), a major nutrient for marine phytoplankton essential for NO_3^- utilization, cellular activities and chlorophyll biosynthesis, is involved in the growth stimulation. Iron-chelators (siderophores) produced by marine bacteria have been shown to increase the solubility of Iron in water, thereby enhancing availability to algal cells (Liebson *et al.* 1995). The growth medium (GSe) used in the present study contains all known essential nutrients, trace-metals and vitamins for algal growth, including Ferric (Fe^{3+}) ions, but rate of uptake by the dinoflagellate may be mediated or controlled by binding to organic chelating molecules.

Algal cells release hydrocarbons and other organic matter in the form of exudates or leakage from broken cells to bacteria (Azam 1998). Several reports suggest that hydrocarbons may affect rate of algal photosynthesis (Gordon & Prouse 1973; Nuzzi 1973; Lacaze 1974). In a similar study Karydis & Fogg (1980) suggested hydrocarbons affect chlorophyll a and protein content of algal cells. Further studies reported higher concentrations of hydrocarbons to significantly inhibit growth of microalgae while lower concentrations had less or no-effect on growth (Dunstan *et al.* 1975; Fabregas *et al.* 1984; Siron *et al.* 1991). *G. catenatum* cells are rich in hydrocarbons, fatty acids (Hallegraeff *et al.* 1991). *Alcanivorax borkumensis* DG881 and *Marinobacter* sp. DG879 are capable of utilizing hydrocarbons (this study, Vincent 2003) and it is possible that these bacterial isolates may have supported growth by removing or utilizing hydrocarbons produced by *G. catenatum* cells. The finding that *Brachybacterium* sp. has growth stimulating activity is interesting. *Brachybacterium* sp. has not been reported in association with phytoplankton cells, and is only known in marine systems from a phylotype associated with arctic and antartic ice (Brinkmeyer *et al.* 2003). However, *Brachybacterium* sp. has been reported to utilize hydrocarbons as a sole carbon source (Yan 2006). Hence, it is equally possible that *Brachybacterium* sp. may have also enhanced algal growth utilizing hydrocarbons from *G. catenatum* cells. Safonova *et al.* (1999) reported similar algal growth promoting activity by alcanotrophic bacteria in black oil. This study identified bacteria to restore and stimulate growth of algal cells exposed to black oil.

In conclusion, this work demonstrates unequivocally that *G. catenatum* has an obligate requirement for the presence of specific growth-stimulating marine bacteria. This requirement appears necessary directly after cyst germination and extends throughout the vegetative growth phase in culture-based experiments.

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Chapter 3

Breakdown of bacteria-dinoflagellate interaction in *G. catenatum* cultures.

3.1 INTRODUCTION

In the phycosphere, bacteria form an integral part of the dinoflagellate environment and may be considered symbionts or mutualist partners. Such mutual interactions between bacteria and algae have been extensively reviewed as a major factor influencing algal growth (Golterman 1972; Paerl & Pinckney 1996; Doucette *et al.* 1998; Croft *et al.* 2005). Bacteria may benefit from an association with phytoplankton by using exudates while algal growth may be supported by bacterial products such as vitamins and other growth factors. While there is some understanding of the bacterial factors controlling phytoplankton growth, little is known about cell death or mortality in algal cultures (Sheldrake 1974).

Unexplained and rapid cessation of growth (or "crashes") are a common feature of dinoflagellates both in bloom populations and laboratory cultures, yet such incidents are rarely explored and the causes remain a mystery (Fogg & Thake 1987; Usup & Azanza 1988; Heiskanen 1993). In general a reduction in phytoplankton population size in the natural environment is due to grazing and sedimentation, bacterial or viral infections (Walsh 1983; Veldhuis *et al.* 2001). However, in uni-bacterial phytoplankton cultures maintained in a controlled environment with sufficient nutrients, temperature and light, these explanations do not apply.

In this study, after several months of sub-culturing uni-bacterial cultures of *G. catenatum* generated with *Brachybacterium* sp. or *Marinobacter* sp. (preliminary trial studies) showed reduced

growth rates, reduced maximum dinoflagellate cell concentrations, and sudden reductions in biomass even at relatively low cell densities. One possible explanation for this phenomenon is that the bacterial-dinoflagellate relationship that maintains dinoflagellate growth was degrading over time. To test this hypothesis a controlled experiment was conducted to replace the "failing bacteria" with "fresh bacteria". By using combinations of antibiotic sensitive and resistant strains of *Brachybacterium* sp. and *Marinobacter* sp., antibiotic sensitive strains in the failing cultures could be supplemented or replaced with "fresh" resistant strains and subsequent growth compared to cultures retaining "failing bacteria".

3.2 MATERIALS AND METHODS

3.2.1 Preparation of bacterial filtrate from "failing" and "fresh" *G. catenatum* cultures

G. catenatum "failing" (old) cultures (32 weeks) established in the presence of *Brachybacterium* sp. or *Marinobacter* sp., during initial trial experiments and "fresh" (new) cultures (16 weeks) grown with antibiotic-sensitive or antibiotic-resistant, *Brachybacterium* sp. and *Marinobacter* sp. (chapter 2) were maintained at 19°C +/- 2.5°C at a light intensity of 90±10 μmoles m^{-2} s^{-1} with a 12L: 12D photoperiod. Replicate log-phase "failing" and "fresh" cultures were passed through an 8 μm nucleopore filter (Millipore, USA) to separate the bacterial biomass.

3.2.2 Minimum inhibitory concentration (MIC) for experiments

Minimum inhibitory concentration (MIC) of antibiotics for the experiment was performed in a 12-well microtitre plate (Corning Incorporated, Corning, NY, USA). Antibiotic solutions of erythromycin and tobramycin (MP Biomedicals, Australia) were prepared as described in chapter 2 (section 2.3). A MIC experiment was performed to determine the antibiotic concentration effective to kill the antibiotic sensitive bacteria but not the resistant bacteria (as in chapter 2). From this range-finding experiment, 2.5 μg mL^{-1} of erythromycin and 1 μg mL^{-1} of tobramycin proved

effective to retain resistant *Brachybacterium* sp. or resistant *Marinobacter* sp. in the cultures while killing the respective antibiotic-sensitive bacterial types.

3.2.3 Bacterial replacement trial in micro-well plates

Replicate (4), unhealthy, 32 week old *G. catenatum* cultures grown with *Brachybacterium* sp. or *Marinobacter* sp. were selected. One mL of the 32 week *G. catenatum* culture was added to all wells. Bacterial filtrates (8 μm), antibiotics and GSe were added to the wells as described in **Table 3.1**; **Table 3.2**. The plates were sealed with Parafilm^{TM} and incubated at 19°C +/- 2.5°C at a light intensity of 90±10 μmoles m^{-2} s^{-1} with a 12L:12D photoperiod. *G. catenatum* cell concentration was estimated daily for 7 days from triplicate sub-samples using a Sedgwick-Rafter counting chamber (Guillard 1973). Bacterial cell concentration was estimated daily for 10 days from triplicate sub-samples by serial dilution-plating onto ZM-1 agar (Buck & Cleverdon 1960). A second dose of antibiotic was added on day 7 as antibiotics are not stable beyond 6 days at 19°C.

3.2.4 Bacterial replacement experiments in 100 mL Erlenmeyer flasks

After 10 days, surviving cultures from the microtitre plate experiment were transferred to sterile 150 mL Erlenmeyer flasks containing 75 mL of GSe medium. Repeated doses of antibiotics (2.5 μg mL^{-1} of erythromycin and 1 μg mL^{-1} of tobramycin) were administered aseptically on day 4 and 10 after transfer. Dinoflagellate and bacterial cell concentration was estimated daily for 15 days as described earlier.

3.2.5 Statistical analysis

Statistical analysis software, SPSS ver. 11.5 (LEAD technologies, Chicago, USA) was used for all analysis. Growth rate was calculated according to the methods of Guillard (1973) and

expressed as instantaneous growth rate ($days^{-1}$). ANOVA with planned-contrasts were used to test significant differences in growth rates between treatments to investigate specific hypotheses (see **Table 3.1**; **Table 3.2** for a description of the planned contrasts).

Table 3.1: Bacterial replacement experiment with *Brachybacterium* sp. performed in 12-well microtitre plates.

Treatments	1mL of 32 week old *G. catenatum* culture grown with *Brachybacterium* sp. +	Aim of Treatment	Expected outcome & ANOVA with planned contrasts
A	1 mL of 8 µm filtrate from new *G. catenatum* cultures grown with erythromycin resistant *Brachybacterium* sp. (10^5 CFU mL^{-1}) + 1 mL of erythromycin + 1 mL of GSe.	Replace old bacterial flora with new erythromycin resistant bacterial flora	New resistant bacteria (A) supports growth different to that of old bacterial flora (E) *Planned contrast - A versus E* New resistant bacteria (A) supports dinoflagellate growth rate different to that of supplemented bacterial treatment (C) *Planned contrast – A versus C*
B	1 mL of 8 µm filtrate from new *G. catenatum* cultures grown with erythromycin sensitive *Brachybacterium* sp. (10^5 CFU mL^{-1}) + 1 mL of erythromycin + 1 mL of GSe.	New bacteria killed by erythromycin	Negative growth, decline in algal cell concentration.
C	1 mL of 8 µm filtrate from new *G. catenatum* cultures grown with erythromycin sensitive *Brachybacterium* sp. (10^5 CFU mL^{-1}) + 2 mL of GSe (no antibiotics added)	Old bacteria retained and supplemented by new erythromycin sensitive bacteria	Supplemented bacterial treatment (C) supports dinoflagellate growth rate different to that of new resistant bacteria (A) *Planned contrast – C versus A* Supplemented bacterial treatment (C) supports dinoflagellate growth rate different to that of old bacterial flora (E) *Planned contrast – C versus E*
D	1 mL of 8 µm filtrate from old *G. catenatum* cultures grown with erythromycin sensitive *Brachybacterium* sp. (10^5 CFU mL^{-1}) + 1 mL of erythromycin + 1 mL of GSe	Old bacteria killed by erythromycin	Negative growth, decline in algal cell concentration, compared to cultures retaining the old bacteria (E) *Planned contrast – D versus E*
E	1 mL of 8 µm filtrate from old *G. catenatum* cultures grown with erythromycin sensitive *Brachybacterium* sp. (10^5 CFU mL^{-1}) + 2 mL of GSe (no antibiotics added)	Retain old bacteria	Old bacteria continue to support growth (E) less than that of new bacteria replacement (A) *Planned contrast – E versus A* Old bacterial flora (E) supports growth different to that of the supplemented bacterial treatment (C) *Planned contrast – E versus C*

Table 3.2: Bacterial replacement experiment with *Marinobacter* sp. performed in 12-well microtitre plates.

Treatments	1mL of 32 week old *G. catenatum* culture grown with *Marinobacter* sp. +	Aim of Treatment	Expected outcome & ANOVA with planned contrasts
A	1 mL of 8 µm filtrate from new *G. catenatum* cultures grown with tobramycin resistant *Marinobacter* sp. (10^5 CFU mL^{-1}) + 1 mL of tobramycin + 1 mL of GSe.	Replace old bacterial flora with new tobramycin resistant bacterial flora	New resistant bacteria (A) supports growth different to that of old bacterial flora (E) *Planned contrast - A versus E* New resistant bacteria (A) supports dinoflagellate growth rate different to that of supplemented bacterial treatment (C) *Planned contrast – A versus C*
B	1 mL of 8 µm filtrate from new *G. catenatum* cultures grown with tobramycin sensitive *Marinobacter* sp. (10^5 CFU mL^{-1}) + 1 mL of tobramycin + 1 mL of GSe.	New bacteria killed by tobramycin	Negative growth, decline in algal cell concentration.
C	1 mL of 8 µm filtrate from new *G. catenatum* cultures grown with tobramycin sensitive *Marinobacter* sp. (10^5 CFU mL^{-1}) + 2 mL of GSe (no antibiotics added)	Old bacteria retained and supplemented by new tobramycin sensitive bacteria	Supplemented bacterial treatment (C) supports dinoflagellate growth rate different to that of new resistant bacteria (A) *Planned contrast – C versus A* Supplemented bacterial treatment (C) supports dinoflagellate growth rate different to that of old bacterial flora (E) *Planned contrast – C versus E*
D	1 mL of 8 µm filtrate from old *G. catenatum* cultures grown with tobramycin sensitive *Marinobacter* sp. (10^5 CFU mL^{-1}) + 1 mL of tobramycin + 1 mL of GSe	Old bacteria killed by tobramycin	Negative growth, decline in algal cell concentration, compared to cultures retaining the old bacteria (E) *Planned contrast – D versus E*
E	1 mL of 8 µm filtrate from old *G. catenatum* cultures grown with tobramycin sensitive *Marinobacter* sp. (10^5 CFU mL^{-1}) + 2 mL of GSe (no antibiotics added)	Retain old bacteria	Old bacteria continue to support growth (E) less than that of new bacteria replacement (A) *Planned contrast – E versus A* Old bacterial flora (E) supports growth different to that of the supplemented bacterial treatment (C) *Planned contrast – E versus C*

3.3 RESULTS

3.3.1 *Brachybacterium* sp. or *Marinobacter* sp. replacement experiment in microtitre plates.

Failing bacteria in old *G. catenatum* cultures when replaced with fresh antibiotic resistant bacteria from new *G. catenatum* cultures enhanced algal growth; a 47%- 90% increase in cell numbers was evident by day 7. Similarly fresh antibiotic sensitive bacteria when added to old *G. catenatum* cultures also showed increase in algal cell numbers by day 7 (**Fig. 3.1**).

Old bacteria added to *G. catenatum* cultures, regardless of antibiotic addition or no antibiotics showed rapid reduction in bacterial cell numbers followed by 39-59 % decline in algal cell numbers by day 7 (**Fig. 3.1**).

Planned comparison analysis performed to compare exponential growth rates of various treatments are detailed in **Table 3.3**. Outcomes of both *Brachybacterium* sp. and *Marinobacter* sp. experiments were consistent and included in one table.

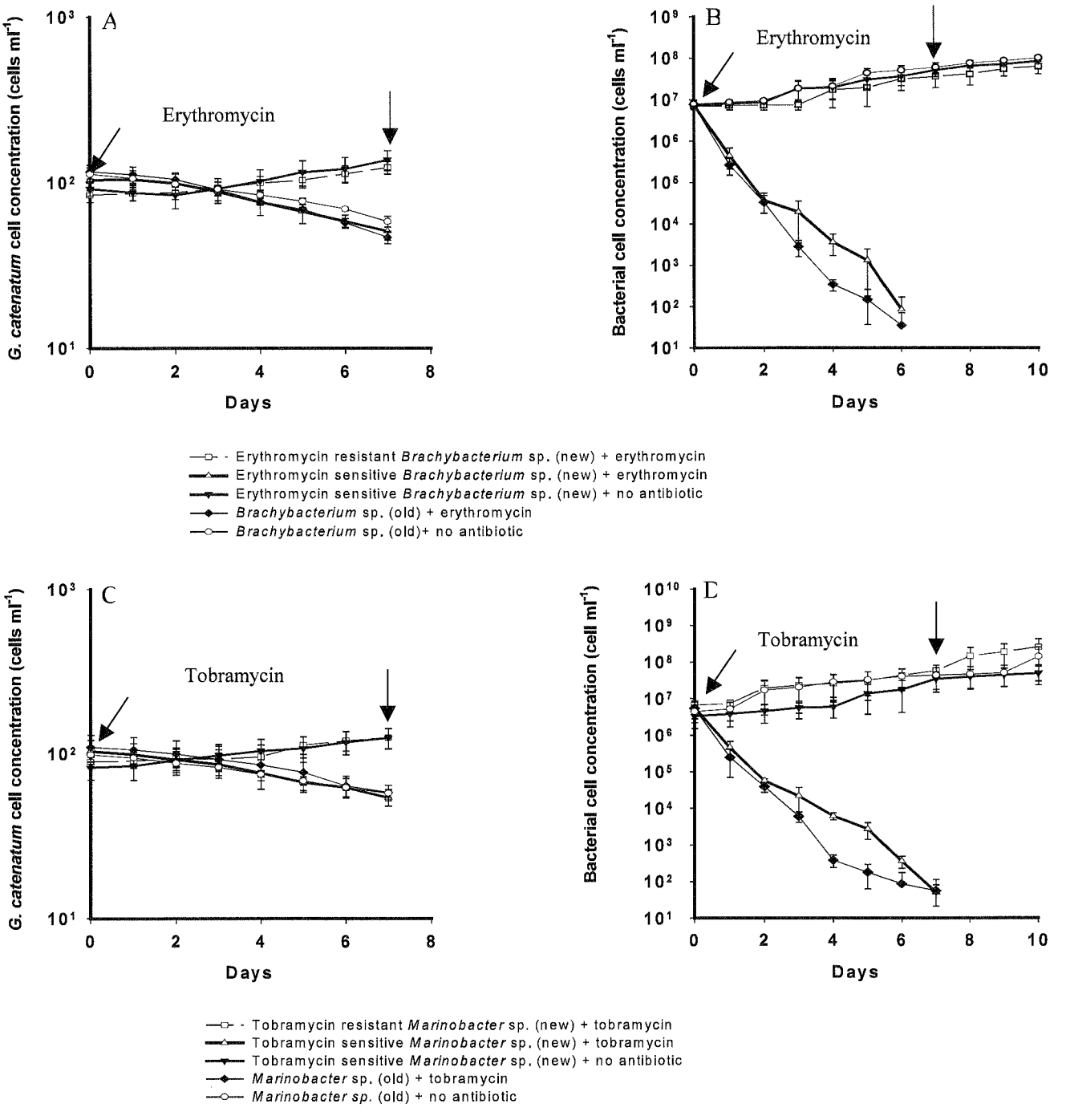

Fig. 3.1: Effect of replacement or addition of new *Brachybacterium* sp. or *Marinobacter* sp. DG879 to *G. catenatum* cultures showing poor growth (A) & (B) *G. catenatum* and bacterial cell concentration (± standard error) in *Brachybacterium* sp. replacement trial. (C) & (D) *G. catenatum* and bacterial cell concentration (± standard error) in *Marinobacter* sp. replacement experiment. Arrows indicate day of antibiotic addition. Microtitre plate experiments.

Table 3.3: Description of experimental outcomes and interpretation from the ANOVA with planned comparisons.

Planned Contrast	Result and significance	Interpretation
A versus E	Replacement new bacteria treatments (A) showed a significantly higher dinoflagellate growth rates compared to the treatments retaining old bacteria (E). (*Brachybacterium* sp.; t= 5.623; df= 4; *P*= 0.000) **(Fig. 3.2)** (*Marinobacter* sp.; t= 6.214; df= 4; *P*= 0.000) **(Fig. 3.3)**	Cultures where new bacteria replaced old bacteria (A) were capable of improving the dinoflagellate growth rate compared to cultures retaining only the old bacteria (E)
A versus C	Both treatments showed positive dinoflagellate growth rates, however, growth rate with replacement new bacteria (A) were not significantly different from those containing old bacteria supplemented with new bacteria (C). (*Brachybacterium* sp.; t= -0.019 df= 4; *P*= 0.985) **Fig. 3.2)** (*Marinobacter* sp.; t= -0.788; df= 4; *P*= 0.443) **(Fig. 3.3)**	Adding new bacteria to cultures containing old bacteria (C) showed the same positive dinoflagellate growth rate as treatments where old bacteria were completely replaced by new bacteria (A)
C versus E	Dinoflagellate growth rates in cultures containing old bacteria supplemented with new bacteria (C) were significantly higher than cultures retaining old bacteria only (E) (*Brachybacterium* sp.; t= 5.642; df= 4; *P*= 0.000) **(Fig. 3.2)** (*Marinobacter* sp. ; t= 7.002; df= 4; *P*= 0.000) **(Fig. 3.3)**	Adding new bacteria to cultures containing old bacteria (C) showed a positive growth rate significantly higher than cultures retaining only the old bacterial flora (E)
D versus E	Both treatments failed to grow and showed negative dinoflagellate growth rates. (*Brachybacterium* sp.; t= - 0.758; df= 4; *P*= 0.460) **(Fig. 3.2)** (*Marinobacter* sp. ; t= -0.516; df= 4; *P*= 0.613) **(Fig. 3.3)**	Removing (D) or retaining the old bacterial flora (E) resulted in the same negative growth rate of the algal cells.

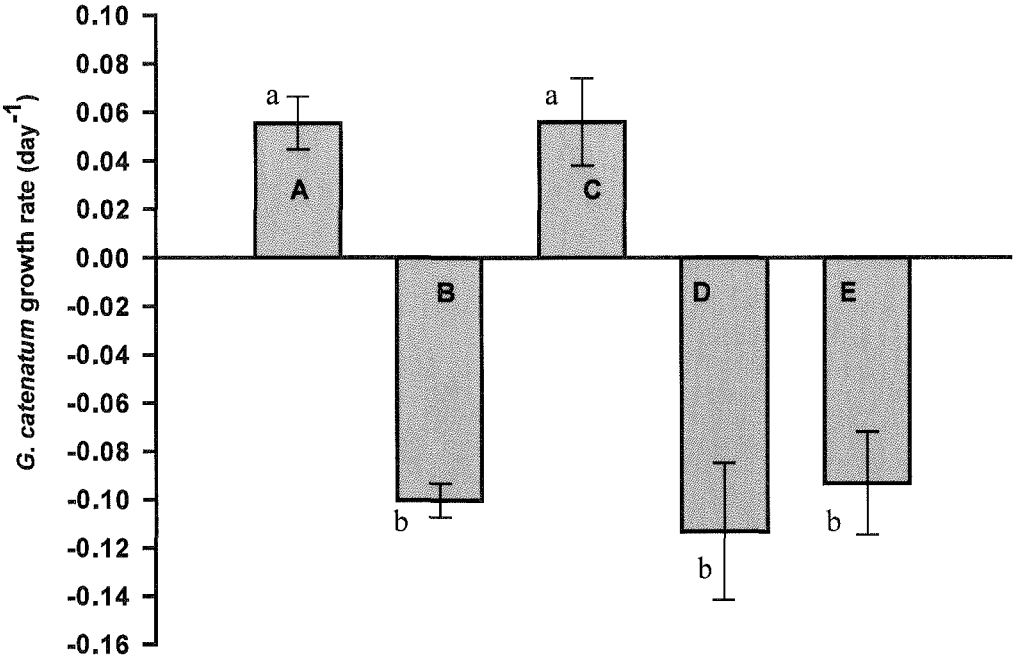

Fig. 3.2: Exponential growth rates (day^{-1}) of microtitre plate *G. catenatum* cultures after replacement or addition of *Brachybacterium* sp.

A = Old *Brachybacterium* sp. replaced with new antibiotic resistant *Brachybacterium* sp.

B = Old and new *Brachybacterium* sp. removed by erythromycin treatment.

C = Old *Brachybacterium* sp. retained and supplemented by new antibiotic sensitive *Brachybacterium* sp.

D = Old *Brachybacterium* sp. killed with erythromycin.

E = Old *Brachybacterium* sp. retained.

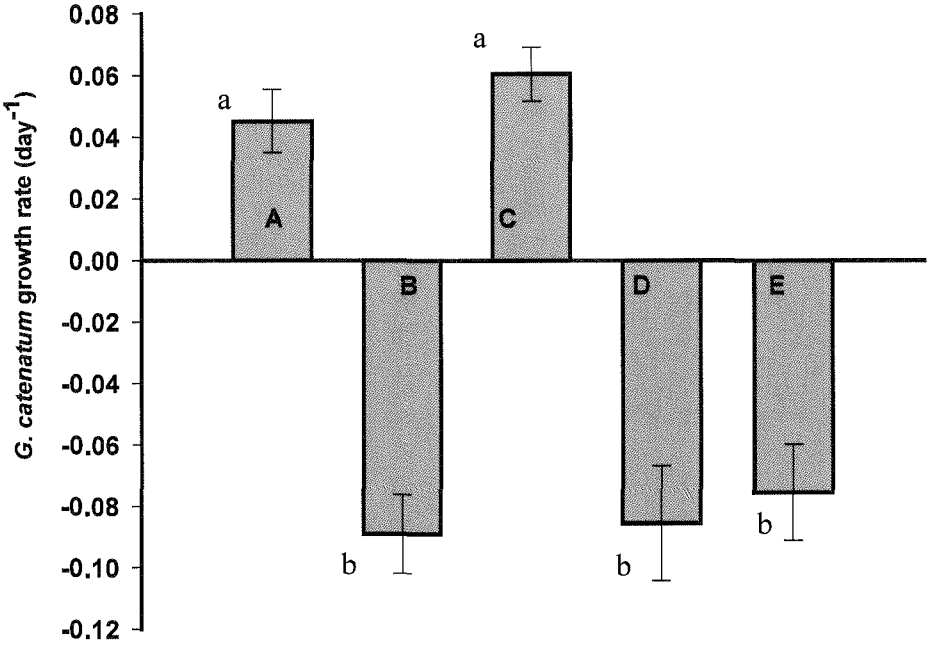

Fig. 3.3: Exponential growth rates (day^{-1}) of microtitre plate *G. catenatum* cultures after replacement or addition of *Marinobacter* sp.

A = Old *Marinobacter* sp. replaced with new antibiotic resistant *Marinobacter* sp.

B = Old and new *Marinobacter* sp. removed by erythromycin treatment.

C = Old *Marinobacter* sp. retained and supplemented by new antibiotic sensitive *Marinobacter* sp.

D = Old *Marinobacter* sp. killed with erythromycin.

E = Old *Marinobacter* sp. retained.

3.3.2 Flask surviving bacterial replacement treatments.

Microtitre plate *G. catenatum* cultures that increased in algal cell numbers as a result of replacing old bacterial flora or addition of new bacteria were transferred to 100 mL Erlenmeyer flasks. All cultures could not be maintained beyond 15 days (**Fig. 3.4**). During the initial 6-8 days *G. catenatum* cells exhibited 27% - 33% increase in algal cell concentration to around days 5-8, but then showed 40% - 50 % reduction in algal cell numbers by day 15.

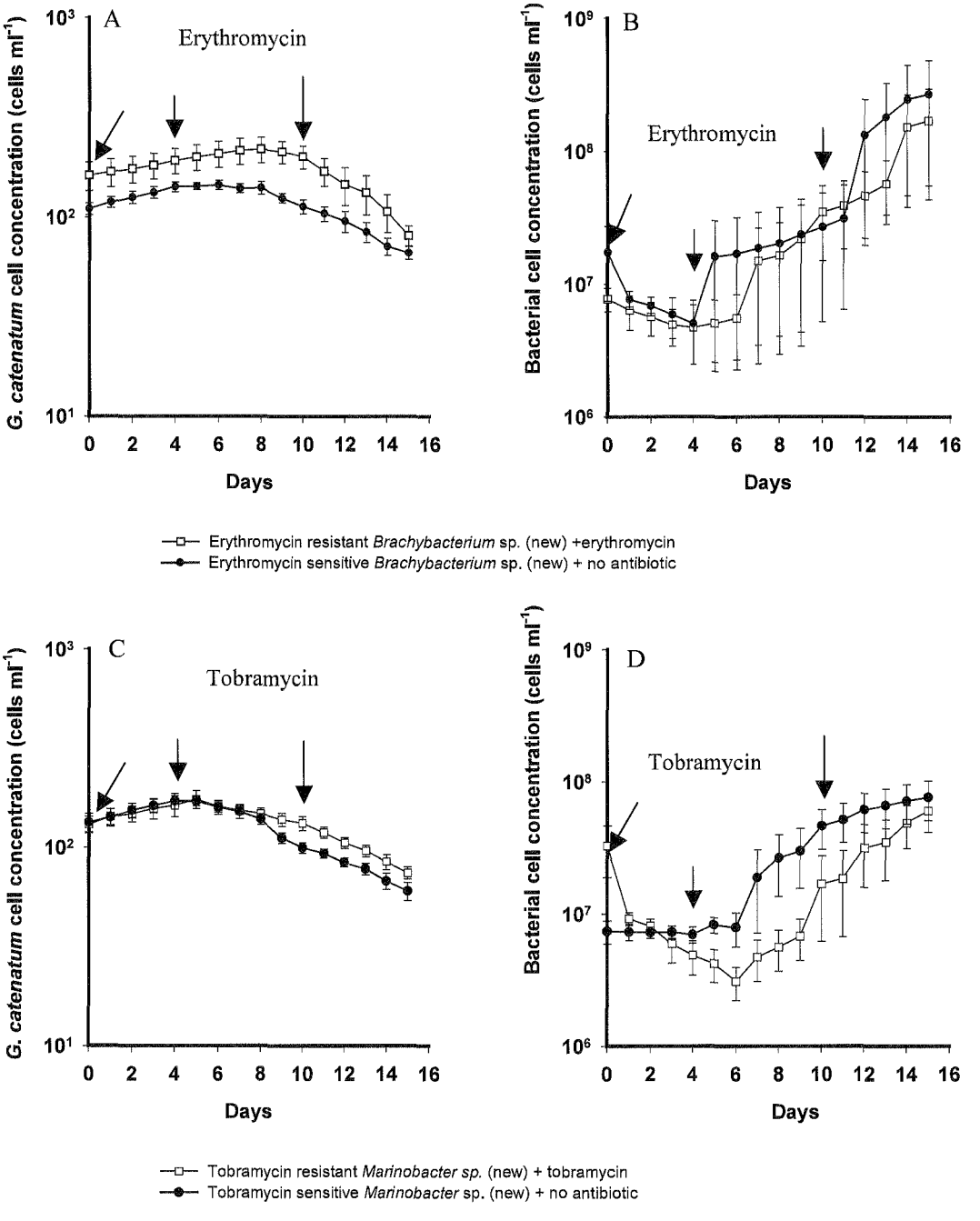

Fig. 3.4: Effect of replacement or addition of bacterial flora in flask cultures of *G. catenatum.* (A) & (B) *G. catentum* and bacterial cell concentration (± standard error) in *Brachybacterium* sp. replacement experiment. (C) & (D) *G. catenatum* and bacterial cell concentration (± standard error) in *Marinobacter* sp. replacement experiment. Arrows indicate day of antibiotic addition.

3.4 DISCUSSION

The outcome of the experiments and analyses conducted here are consistent with the hypothesis that a gradual loss of growth stimulating capacity of the bacteria is the reason for the poor growth in uni-bacterial *G. catenatum* cultures in our laboratory. Bacterial strains from newly established uni-bacterial *G. catenatum* cultures appeared to stimulate algal growth in the microtitre plates for up to 7 days. However, surviving cultures scaled up to 100 mL flasks eventually declined after a further 5-9 days regardless of addition or replacement of new bacteria. This suggests bacteria in old algal cultures may have lost their ability to promote algal growth, and that, in this experiment, the growth stimulating ability, or interaction with *G. catenatum* cells could not be completely recovered.

Microbial genes are highly dynamic and evolving whereby new genes may be acquired and pre-existing genes lost by mutation (Lawrence 1999; Lawrence & Roth 1999). Bacterial mutation is also higher in aged cultures altering physiological and other general activities of bacteria (Finkel *et al.* 2000). The production of bacterial growth factors by the bacteria used here, may be controlled by only a few microbial genes and it is possible that accumulated mutations may have resulted in the down-regulation of genes responsible for producing algal growth factors, leading to the gradual decline of the uni-bacterial *G. catenatum* cultures over the >30 weeks of serial sub-culture. While this loss of production of growth factors is evident in uni-bacterial *G. catenatum* cultures, it appears to not present a problem in cultures containing a mixed bacterial community that can be maintained indefinitely (> 20 yrs). The strains used to establish the model cultures used here (GCHU11 and GCDE08) were isolated in the mid-1980's (Blackburn *et al.* 1989) and continue to exhibit normal growth dynamics in uni-algal cultures with a mixed baterial flora. It is possible that a range of required growth factors are produced by a range of bacteria present in cultures, and that the physiological effects of a lack of these factors are manifested over relatively long timescales, similar to vitamin deficiency (Grossart 1999).

Loss of cell biomass in natural phytoplankton populations is generally due to grazing, sinking and algal cell lysis. At the same time phytoplankton cells also lyse due to autocatalytic cell death pathway triggered by, physiological stress, nutrient depletion, bacterial or viral infections causing severe mortalities in algal cultures in the laboratory and natural environment (Bratbak *et al.* 1993; Brussard *et al.* 1995; Berges & Falkowski 1998; Kirchman 1999; Vardi *et al.* 1999; Segovia *et al.* 2003). The extent to which algal population may recover after a crash still remains unclear and further recovery may also vary with different algal species (Veldhius *et al.* 2001). In the uni-bacterial cultures used here, it is unknown whether a viral infection was involved in the algal cell death, however, replacing the bacteria restored growth initially suggesting that mortality was not entirely due to another factor like a viral infection or automortality by the dinoflagellate cells.

Mutualism theory suggests co-operation may erode into parasitism in complex interaction. When interactions offer weak benefit to one partner there is a possibility for a shift in mutualism (Sachs & Simms 2006). Such a loss in mutualism may have lead to competition for micronutrients or inorganic compounds in this study. Algae may have evolved a inferior partner allowing bacteria to benefit solely from this association in course of time. This possibility could have been explored provided *G. catenatum* growth was restored with 'fresh bacteria'.

The failure of algal cells to survive in 100 mL flasks may be due to the long term exposure to antibiotics (**Fig. 3.4**). The cells were subjected to 3 doses of antibiotics over the 15 days and perhaps the high concentration of antibiotics was directly toxic to the dinoflagellate cells. However, cultures supplemented with "fresh" bacteria without antibiotic treatment also failed to grow in 100 mL flasks indicating that antibiotic exposure was unlikely to be the cause of the decline. All treatments with resistant bacteria or no-antibiotics showed normal bacterial growth, regardless of the response of the dinoflagellate. Clearly the algal metabolites required by the bacteria were in

sufficient supply for bacterial growth, yet the bacteria were unable to continue to support growth of the dinoflagellate.

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Chapter 4

Growth of *G. catenatum* in the presence of defined marine bacterial communities.

4.1 INTRODUCTION

The association between bacteria and phytoplankton in marine ecosystem are complex and are believed to influence the growth of each other (Fukami *et al.* 1991). However, little is known of how these communities interact at the species composition level (Rooney-Varga *et al.* 2005). Bacterial activity plays a predominant role in oceanic processes such as biogeochemical cycling, but may also influence phytoplankton growth, reproduction, cyst formation and mortality (Doucette *et al.* 1998). Interactions may range from highly specific, like symbiotic interactions (Croft *et al.* 2005) through to parasitic or non-specific interactions such as competition, commensalism and mutualism (Grossart 1999).

There are a number of ways in which phytoplankton and bacterial cells may interact. Phytoplankton may stimulate bacterial growth by supplying much of the organic matter for bacterial utilization and in adverse environmental conditions compete for nutrients, or produce antibiotics limiting bacterial growth (Cole 1982). Similarly, bacteria are known to promote algal

growth by production of stimulatory products like vitamins, iron and essential nutrients, and may also exhibit a detrimental effect on algal growth by excretion of toxic and algal lytic compounds (Cole 1982). Studies have also reported the potential for bacteria to influence phytoplankton stoichiometry by generating a switch in the nature of nutrients, limiting algal growth, and thus regulating HAB formation and decline (Elser *et al.* 1988; Danger *et al.* 2007).

Species-specific interactions between algae and phytoplankton have been reviewed extensively, suggesting that bacteria may play a major role in phycotoxin production and bloom regulation (Boczar *et al.* 1988; Fukami *et al.* 1996; Dantzer & Levin 1997; Kim *et al.* 1998; Yoshinaga *et al.* 1998; Doucette & Powell 1998; Hold *et al.* 2001). The broad aim of the present work is to examine the influence of bacteria-dinoflagellate interactions on dinoflagellate growth, and eventually elucidate the mechanisms and compounds that may mediate these interactions.

In the phycosphere, 10's to 100's of bacterial genotypes are associated with phytoplankton cells and hence a wide range of complex interactions may be expected (Hold *et al.* 2001; Alavi *et al.* 2001; Green *et al.* 2004). Laboratory cultures of *G. catenatum* harbour a relatively consistent but complex bacterial community typically dominated by alpha-Proteobacteria (*Roseobacter/Rhodobacter* clades), the gamma-Proteobacteria and the Bacteroidetes (*Cytophaga/Flexibacter*, CFB) (Green *et al.* 2004). Previous work with the simplified experimental models of *G. catenatum* used here suggested that only a limited range of bacterial types can support the growth of *G. catenatum* in uni-bacterial dinoflagellate cultures. (Vincent 2003; Bolch *et al.* 2004; Green *et al.* unpublished data)

This chapter examines dinoflagellate and bacterial growth dynamics in *G. catenatum* cultures grown with simplified bacterial communities composed of *Marinobacter* sp., *Alcanivorax* sp., and/or *Roseobacter* sp. over a complete batch culture cycle. The subsequent batch culture dynamics and exponential growth rate of the dinoflagellate varied significantly with different composition and complexity of the bacterial community. This indicates, bacterial community may

be a potentially significant factor influencing the growth dynamics of *Gymnodinium catenatum* blooms.

4.2 MATERIALS AND METHODS

G. catenatum culture maintenance, cyst production, surface sterilisation of cysts was based on methods described in chapter 2 (refer to sections 2.2.1 and 2.2.2).

4.2.1 Uni-bacterial and controlled-community *G. catenatum* cultures

G. catenatum-associated bacteria, *Alcanivorax* cf. *borkumensis* DG881, *Marinobacter* sp. DG879 and *Roseobacter* sp. DG874 from our culture library were maintained in ZM1 agar similar to methods discussed in chapter 2 (refer to section 2.2.3). Further preparation of bacterial cultures for germination and growth experiments was also based on methods described in chapter 2 (refer to section 2.2.5). For culturing *Alcanivorax* sp. DG881, Zobell's medium was supplemented with 1% sodium acetate as a carbon source (Green *et al.* 2004).

Sterilised resting cysts were aseptically aliquoted into 36 mm petri dishes and the prepared bacterial cultures added to the sterile resting cysts and/or controls as briefly described in **Table 4.1**. All treatments and positive and negative control were carried out in triplicate. Each dish contained 30-40 surface-sterile resting cysts in 1.9 mL of sterilized GSe medium. All bacterial inoculations contained an estimated final concentration of 10^5 CFU mL^{-1} of each bacterial strain. All dishes were sealed with parafilm^{TM} and incubated at 19°C +/- 2.5°C at a light intensity of 90±10 µmoles m^{-2} s^{-1} with a 12L:12D photoperiod. All treatments and controls containing resting cysts were assessed under Lieca S9 stereomicroscope every 3-4 days for 30 days after initial germination was observed. Germination (%) was determined based on number of empty cysts counted in replicate dishes. Motile dinoflagellate cells were also counted under the microscope to determine number of moving cells per germinated cyst.

Table 4.1: Description of treatments and controls used in germination experiment. All treatments and controls (except the media sterility control) consisted of duplicate 36 mm petri dishes, containing 30-40 surface-sterilised *G. catenatum* resting cysts in 1.9 mL of GSe medium with the addition of the treatments described below. All treatments and the positive control were subsequently used to establish 100 mL flask cultures for later experiments.

Treatment	**Sterile *G. catenatum* resting cysts plus**
Sterility control	200 µL of sterile GSe (no resting cysts)
Positive control	200 µL of 8 µm filtrate from mid-log phase cultures of GCDE08 and GCHU11.
Negative control	Sterile GSe medium
Treatment 1	*Alcanivorax* sp. DG881 added to a final concentration of 10^5 CFU mL^{-1}
Treatment 2	*Marinobacter* sp. DG879 added to a final concentration of 10^5 CFU mL^{-1}
Treatment 3	*Roseobacter* sp. DG874 added at a final concentration of 10^5 CFU mL^{-1}
Treatment 4	*Alcanivorax* sp. DG881 and *Marinobacter* sp. DG879 added at a final concentration of 10^5 CFU mL^{-1}each
Treatment 5	*Alcanivorax* sp. DG881 and *Roseobacter* sp. DG 874 added at a final concentration of 10^5 CFU mL^{-1} each
Treatment 6	*Marinobacter* sp. DG879 and *Roseobacter* sp. DG874 added at a final concentration of 10^5 CFU mL^{-1} each
Treatment 7	*Alcanivorax* sp. DG881, *Marinobacter* sp. DG879 and *Roseobacter* sp. DG874 added at a final concentration of 10^5 CFU mL^{-1} each

After 30 days, two replicates from each treatment and the positive control were transferred to sterile 150 mL Erlenmeyer flasks containing 100 mL of sterile GSe medium. These cultures were grown at 19°C +/- 2.5°C at a light intensity of 90±10 μmoles m^{-2} s^{-1} with a 12L: 12D photoperiod until sufficient cell concentration was available for additional growth experiments. Negative control (no bacteria added) failed to survive beyond 30 days and was not included in further growth studies. The established 100 mL cultures were transferred to fresh sterile 150 mL Erlenmeyer flasks containing 100 mL of GSe medium. Dinoflagellate cell concentration was estimated every 4 days from triplicate sub-samples by *in-vivo* fluorometry (Kiefer 1973) and cell counts using a Sedgwick-Rafter counting chamber (Guillard 1973). Bacterial cell concentration (CFU mL^{-1}) in treatments and controls were estimated from triplicate sub-samples by serial dilution-plating (Buck & Cleverdon 1960) onto ZM1 agar.

4.2.2 Statistical analysis

Growth and death rates were calculated according to the methods of Guillard (1973) and expressed as instantaneous growth rate ($days^{-1}$). Differences among duplicate treatments and controls for cyst germination, cells per germinated cyst (at day 30), dinoflagellate exponential growth rate and death rate, and maximum cell concentration (cells mL^{-1}) were compared using one-way ANOVA. Significant differences among treatments were determined by Tukey's LSD post-hoc tests. All analyses were carried out using the statistical analysis software SPSS ver. 11.5 (LEAD technologies, Chicago, USA).

4.3 RESULTS

4.3.1 Germination and initial growth of *G. catenatum*

Germination of resting cysts in the presence of different bacteria or bacterial communities ranged from 15% to 53% and differed significantly across treatments and controls (**Fig. 4.1**). Sterile cysts with no bacterial addition (negative control) generally showed poor germination (15%) and the majority of cells died within the 30 day observation period; long-term culture of these treatments was not possible. In contrast, all treatments where sterile cysts were geminated in the presence of specific bacteria or bacterial communities showed significantly higher germination rates than the negative control ($f= 6.687$; $df= 8, 18$; $P= 0.000$), survived beyond 30 days and could be sub-cultured long-term (> 30 weeks).

Cysts germinated in the presence of a mixed bacterial community from the parent crossing strains (positive control) showed germination rates typical of non-sterilised cysts (54%) from earlier studies (Bolch *et al.* 2002; Vincent 2003). All bacterial treatments exhibited germination rates similar to the positive control ($P> 0.066$), with the exception of treatments containing only *Roseobacter* sp. ($P= 0.013$) and the negative control ($P= 0.001$) (**Fig. 4.1**).

As a relative measure of initial post-germination growth, the number of moving cells per germinated resting cyst was recorded over the first 30 days (**Fig. 4.2**). No significant differences were observed at day 30 among the treatments and positive control ($f= 4.422$; $df= 8, 18$; $P> 0.982$), however, the negative control showed significantly fewer moving cells at day 30 ($P= 0.007$).

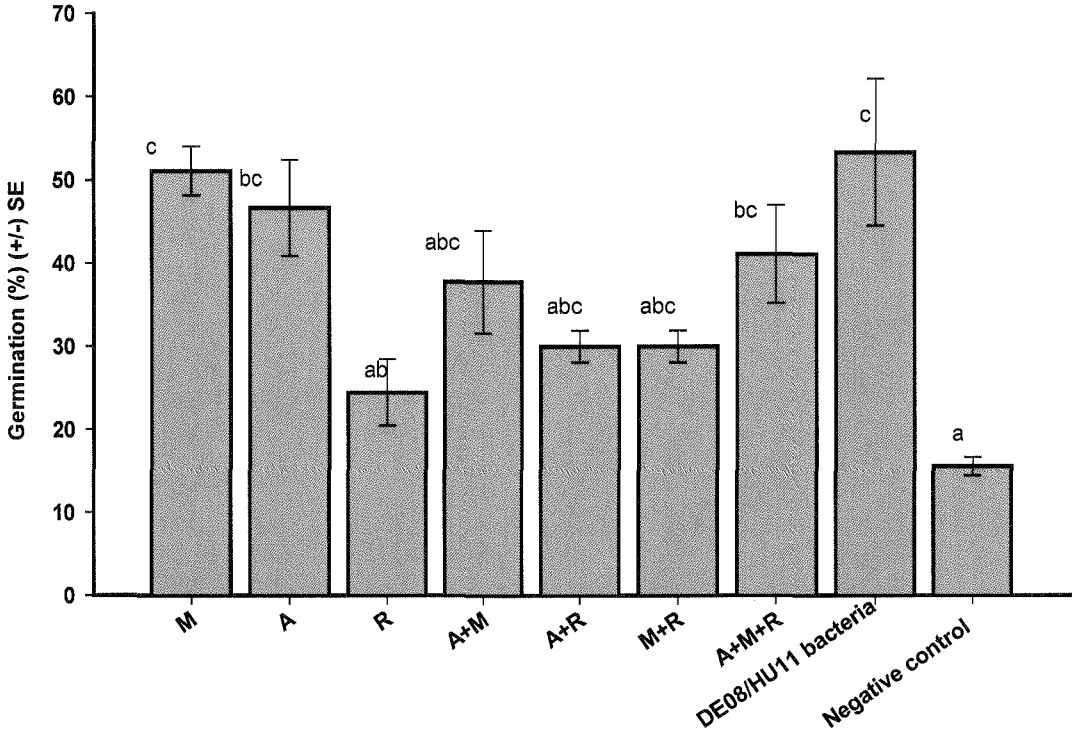

Fig. 4.1: Germination (%) (± standard error) in different treatments, positive and negative control. M= *Marinobacter* sp. DG879; A= *Alcanivorax* sp. DG881; R= *Roseobacter* sp. DG874; A+M+R= all three bacteria mixed. Superscripts indicate significant differences ($P < 0.05$).

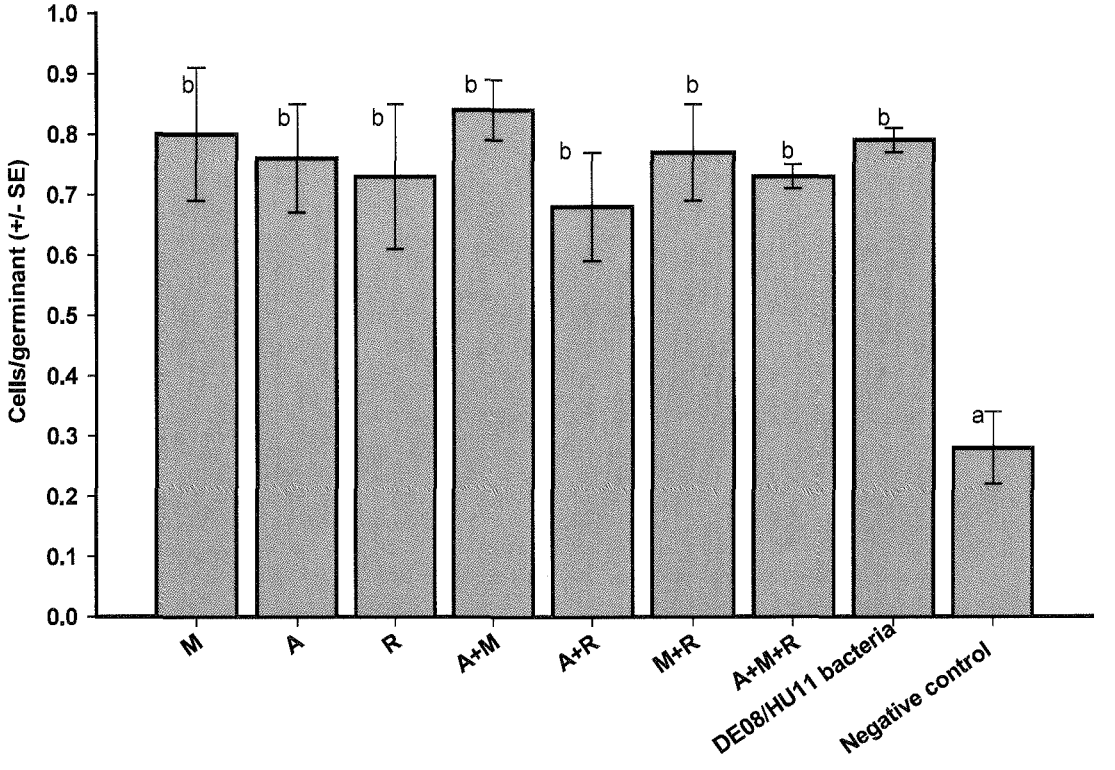

Fig. 4.2: Moving cells per germinant (± standard error) in different treatments, positive and negative control. M= *Marinobacter* sp. DG879; A= *Alcanivorax* sp. DG881; R= *Roseobacter* sp. DG874; A+M+R= all three bacteria mixed. Superscripts indicate significant differences ($P < 0.05$).

4.3.2 Growth dynamics of *G. catenatum* in batch culture

All cultures, other than the negative control, were cultured successfully to 100 mL volumes in 150 mL Erlenmeyer flasks over a period of 8 weeks. Growth curves derived from *in-vivo* fluorescence data (Appendix 5) and cell count data were found to be very similar. As fluorescence estimates of cell concentration are potentially unreliable estimates of cell concentration outside logarithmic-phase (Falkowski & Kiefer 1985; Cullen *et al.* 1988), only the cell count data are used here. Correlation analysis revealed good correlation between algal cell numbers and relative fluorescence in different treatments (R^2= 0.9065) and parent strains GCHU11 (R^2= 0.9708); GCDE08 (R^2= 0.8215) during exponential phase (**Fig. 4.3**).

Marked differences in dinoflagellate batch culture dynamics were evident between *G. catenatum* cultures grown in the presence of different simple bacterial communities composed of 1 to 3 bacteria. The onset and duration of batch culture phase for all cultures are summarized in **Table 4.2**. No distinct lag-phase was evident in treatments or controls, however, the exponential growth phase was considerably longer and the stationary phase shorter in treatments containing *Roseobacter* sp. alone, or in combination with the other two bacteria.

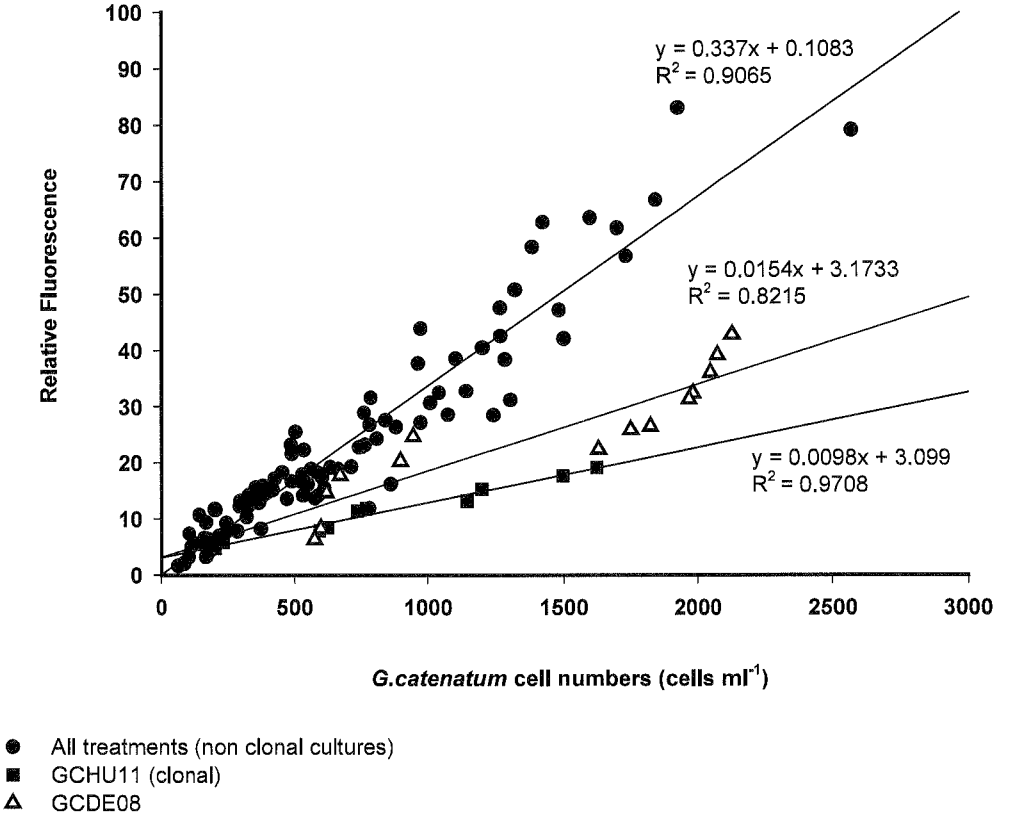

Fig. 4.3: Relationship between algal cell numbers (cells mL^{-1}) and relative fluorescence in exponential growth phase of all treatments and parent GCHU11 and GCDE08 cultures. The solid lines represent fitted regression equation.

Table 4.2: Comparison of the duration and length of growth phases in *G. catenatum* cultures grown in the presence of simple uni-bacterial, mixed bacterial communities and parent GCHU11 and GCDE08 *G. catenatum* cultures.

Treatments	Growth phase (days)			
	Exponential	**Declining**	**Stationary**	**Death**
Alcanivorax sp.	0-12 (12)	12-28 (16)	28-36 (8)	36-68
Marinobacter sp.	0-12 (12)	12-28 (16)	12-28 (16)	40-68
Roseobacter sp.	0- 40 (40)	40-44 (4)	44-52 (8)	52-68
Alcanivorax sp. +*Marinobacter* sp.	0-12 (12)	12-16 (4)	16-40 (24)	40-68
Alcanivorax sp. +*Roseobacter* sp.	0-16 (16)	16-28 (12)	28-44 (16)	44-68
Marinobacter sp. +*Roseobacter* sp.	0-24 (24)	24-36 (12)	36-44 (8)	44-68
Alcanivorax sp. +*Marinobacter* sp. +*Roseobacter* sp.	0-20 (20)	0 (0)	20-28 (8)	28-68
Mixed DE08/HU11 bacteria community	0-16 (16)	16-24 (8)	24-36 (12)	36-68
GCHU11	0-16 (16)	0 (0)	16-28 (12)	28-68
GCDE08	0-12 (12)	0 (0)	12-44 (32)	44-68

Figures in brackets indicate number of days

Cultures grown with single bacterial addition exhibited consistent growth among replicates but distinct dinoflagellate batch culture growth curves for each bacterial type (**Fig. 4.4**). Cultures grown with *Marinobacter* sp. or *Alcanivorax* sp. exhibited exponential growth rates (day 0-12) that were significantly higher than positive control containing mixed culture bacteria (f= 23.99; df= 9, 10; *P*= 0.000, 0.033) and cultures containing only *Roseobacter* sp. (*P*= 0.000, 0.008) (). Cultures grown with *Roseobacter* sp. showed the slowest exponential growth rate and did not reach stationary phase till day 40-44 (**Fig. 4.4** (c); **Fig. 4.5**).

Cultures with *Marinobacter* sp. or *Alcanivorax* sp. reached maximum dinoflagellate cell concentrations of 2.0-2.5 x 10^3 cells mL^{-1}, while cultures grown with *Roseobacter* sp. reached concentrations, 1.5 x10^3 cells mL^{-1}. However, these differences were not significant (f= 6.916; df= 9, 10; *P*= 0.528; 0.371) (**Fig. 4.6**). Cultures grown with *Roseobacter* sp. exhibited a significantly more rapid decline during death phase than cultures grown with *Marinobacter* sp. (f= 19.301; df= 9, 10; *P*= 0.001) or *Alcanivorax* sp. (f= 19.301; df= 9, 10; *P*= 0.000) (**Fig. 4.7**).

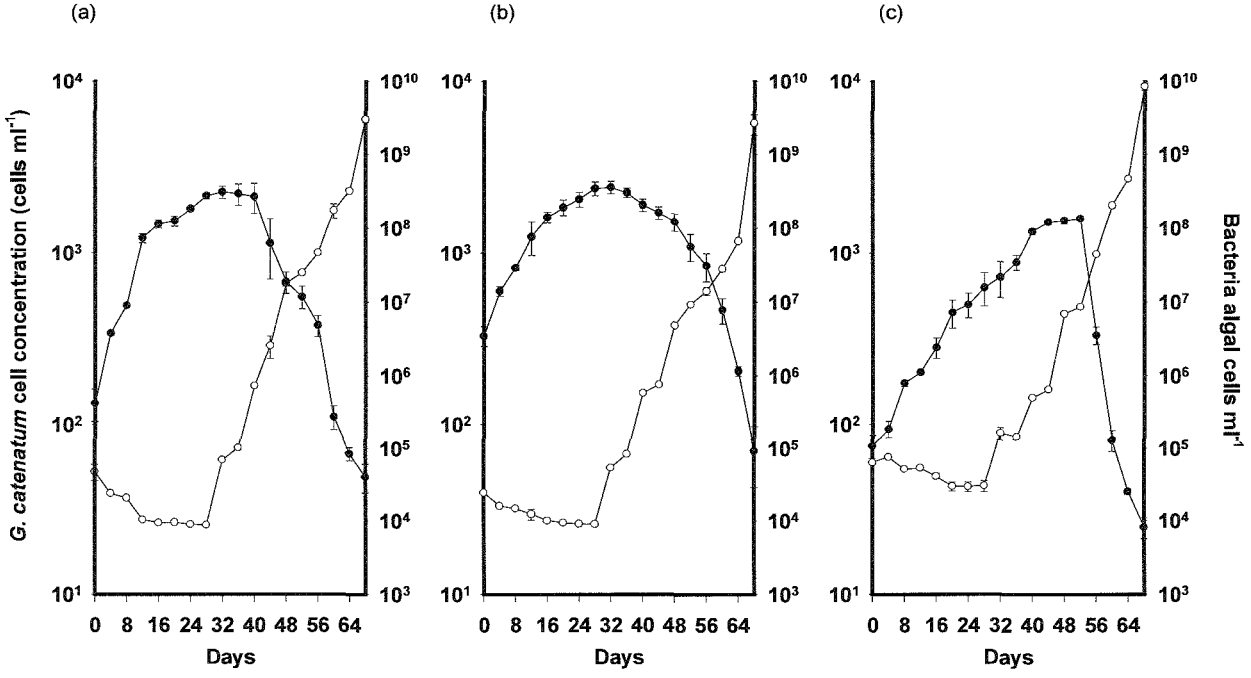

Fig. 4.4: Batch growth curves from replicate cultures of *G. catenatum* grown with single marine bacteria (a) *Marinobacter* sp. DG879; (b) *Alcanivorax* sp. DG881; (c) *Roseobacter* sp. DG874 (± standard error). Curves with closed circles indicate *G. catenatum* cell concentration (cells mL^{-1}) and curves with open circles indicate bacteria/ algal cell concentration (cells mL^{-1}).

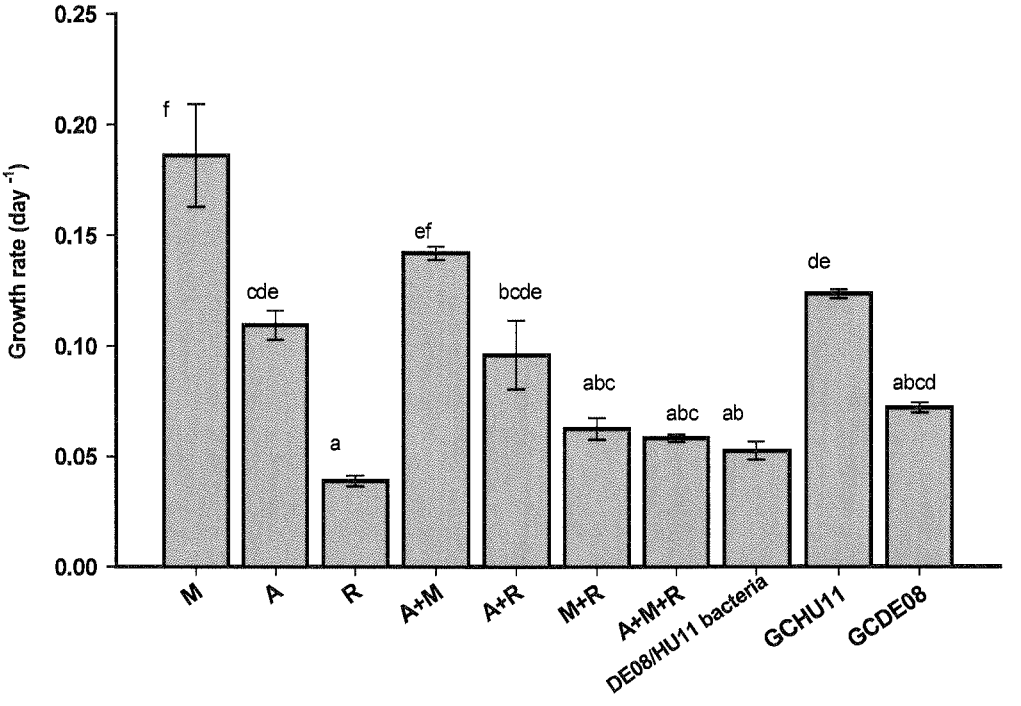

Fig. 4.5: Exponential growth rates (day^{-1}) of replicate *G. catenatum* cultures grown with single bacteria, pair-wise combination of bacteria, cultures with synthetic communities of 3 bacteria, mixed bacterial communities from non-axenic parent cultures, parent GCHU11 and GCDE08 cultures. M= *Marinobacter* sp. DG879; A= *Alcanivorax* sp. DG881; R= *Roseobacter* sp. DG874; A+M+R= all three bacteria mixed. Superscripts indicate significant differences (P= < 0.05)

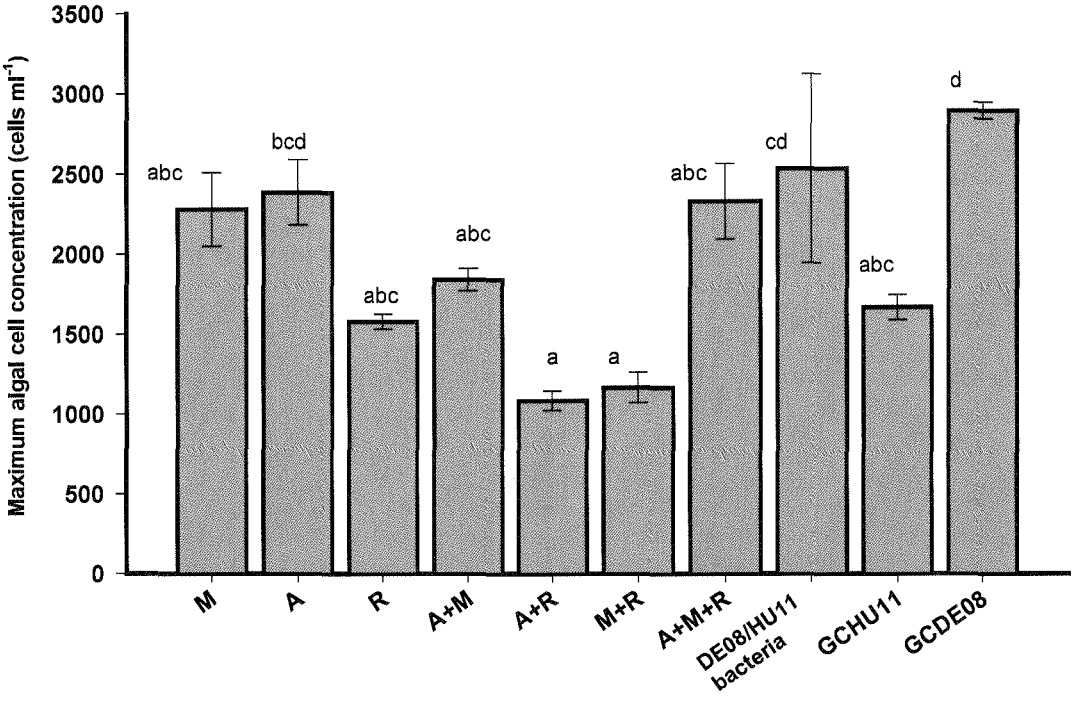

Fig. 4.6: Maximum algal cell concentration (cells mL^{-1}) of replicate *G. catenatum* cultures grown with single bacteria, pair-wise combination of bacteria, cultures with synthetic communities of 3 bacteria, mixed bacterial communities from non-axenic parent cultures, parent GCHU11 and GCDE08 cultures. M= *Marinobacter* sp. DG879; A= *Alcanivorax* sp. DG881; R= *Roseobacter* sp. DG874; A+M+R= all three bacteria mixed. Superscripts indicate significant differences (P= < 0.05)

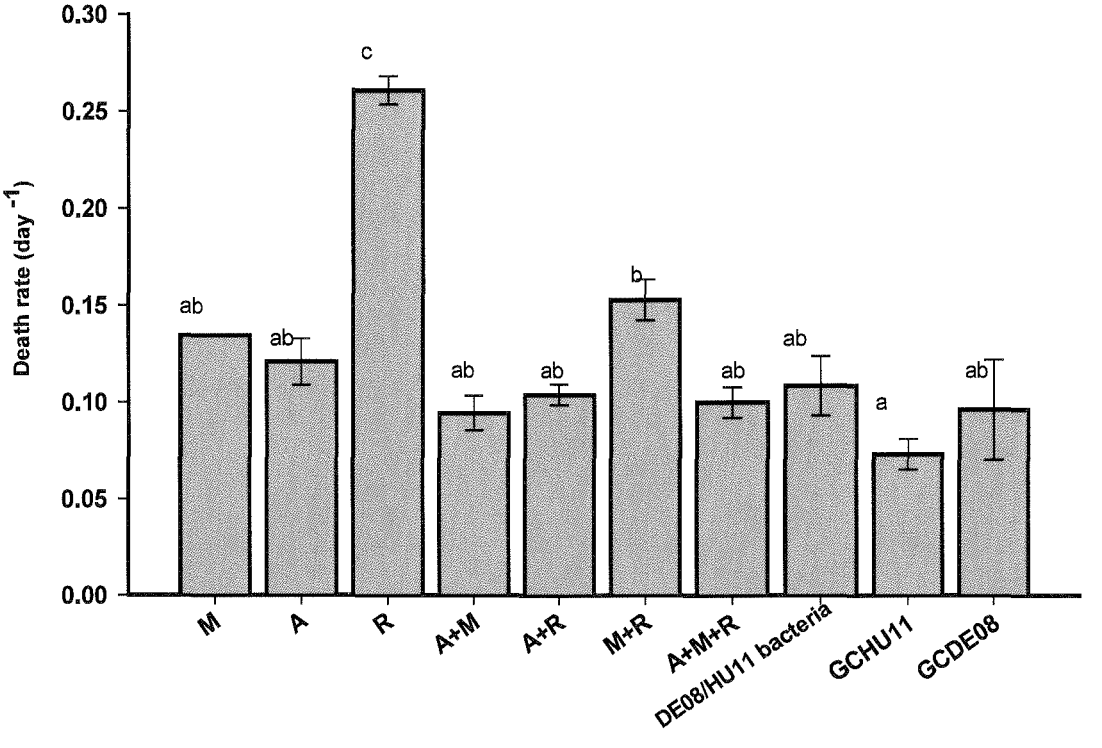

Fig. 4.7: Death rate (day $^{-1}$) of replicate *G. catenatum* cultures grown with single bacteria, pair-wise combination of bacteria, cultures with synthetic communities of 3 bacteria, mixed bacterial communities from non-axenic parent cultures and parent GCHU11 and GCDE08 cultures. M= *Marinobacter* sp. DG879; A= *Alcanivorax* sp. DG881; R= *Roseobacter* sp. DG874; A+M+R= all three bacteria mixed. Superscripts indicate significant differences (P= < 0.05).

Cultures grown in the presence of pair-wise combinations of bacteria exhibited growth curves with combinations of features from that of uni-bacterial cultures (**Fig. 4.8**). Cultures grown with *Marinobacter* sp. and *Alcanivorax* sp. showed a short rapid exponential growth period (days 0-12) similar to that of cultures grown only with *Marinobacter* sp., but a more gradual death phase similar to cultures containing only *Alcanivorax* sp. Similar "hybrid" growth curves are evident in the cultures grown with *Alcanivorax* sp. and *Roseobacter* sp., however, cultures grown with *Marinobacter* sp. and *Roseobacter* sp. showed a growth curve more similar to cultures containing only *Roseobacter* sp. (compare **Fig. 4.4** (c) and **Fig. 4.8** (c)). Mean exponential growth rates of the two-bacterium *G. catenatum* cultures were, in all cases, intermediate between the growth rates of the corresponding uni-bacterial cultures (**Fig. 4.5**). Mean maximum cell concentrations in these cultures were generally lower than either of the single bacterium cultures and the two-bacterium cultures containing *Roseobacter* sp. achieved a significantly lower maximum cell concentration than positive control (**Fig. 4.6**). A sharp decline after day 44 was evident in cultures grown with *Marinobacter* sp. and *Roseobacter* sp., however the overall rate of decline (death rate) to day 68 was not significantly faster than the other two-bacterium combinations (**Fig. 4.7**).

Cultures grown with simple communities composed of three bacterial strains exhibited batch culture dynamics most similar to those of the positive control containing log-phase bacterial communities from HU11 and DE08 (**Fig. 4.9**). Exponential growth rates (**Fig. 4.5**), maximum cell concentrations (**Fig. 4.6**) and death rates (**Fig. 4.7**) were almost identical and only small differences were noted in the onset and length of batch culture phases (**Table 4.2**). Growth curves of the two parent strains GCDE08 and GCHU11 were, however, surprisingly different from both positive control and cultures grown with the three bacteria (compare **Fig. 4.9**; **Fig. 4.10**). Strain GCHU11 exhibited a significantly higher ($f= 23.99$; $df= 9, 10$; $P= 0.041$) growth rate than GCDE08 (**Fig. 4.5**) although the maximum cell concentration reached by both were not significantly different (**Fig. 4.6**).

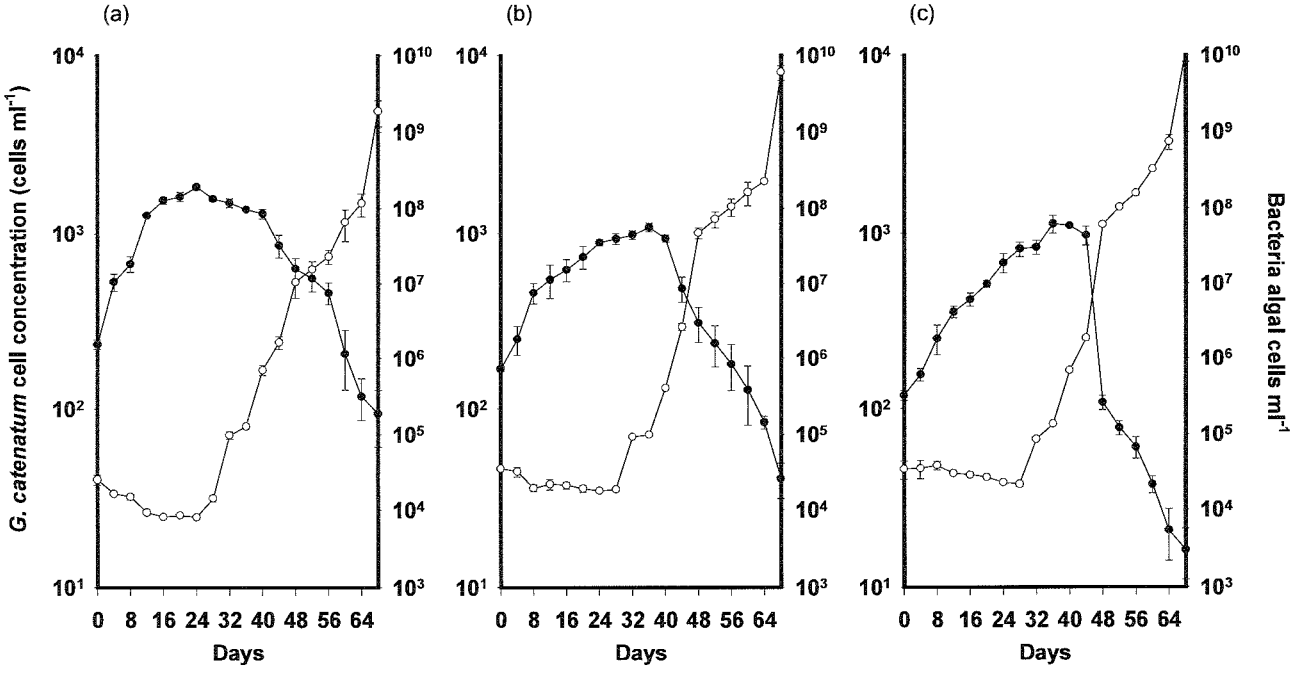

Fig. 4.8: Batch growth curves from replicate cultures of *G. catenatum* grown with pair-wise combination of bacteria (a) *Alcanivorax* sp. DG881 +*Marinobacter* sp. DG879; (b) *Alcanivorax* sp. DG881 + *Roseobacter* sp. DG874; (c) *Marinobacter* sp. DG879 + *Roseobacter* sp. DG874 (± standard error). Curves with closed circles indicate *G. catenatum* cell concentration (cells mL^{-1}) and curves with open circles indicate bacteria/ algal cell concentration (cells mL^{-1}).

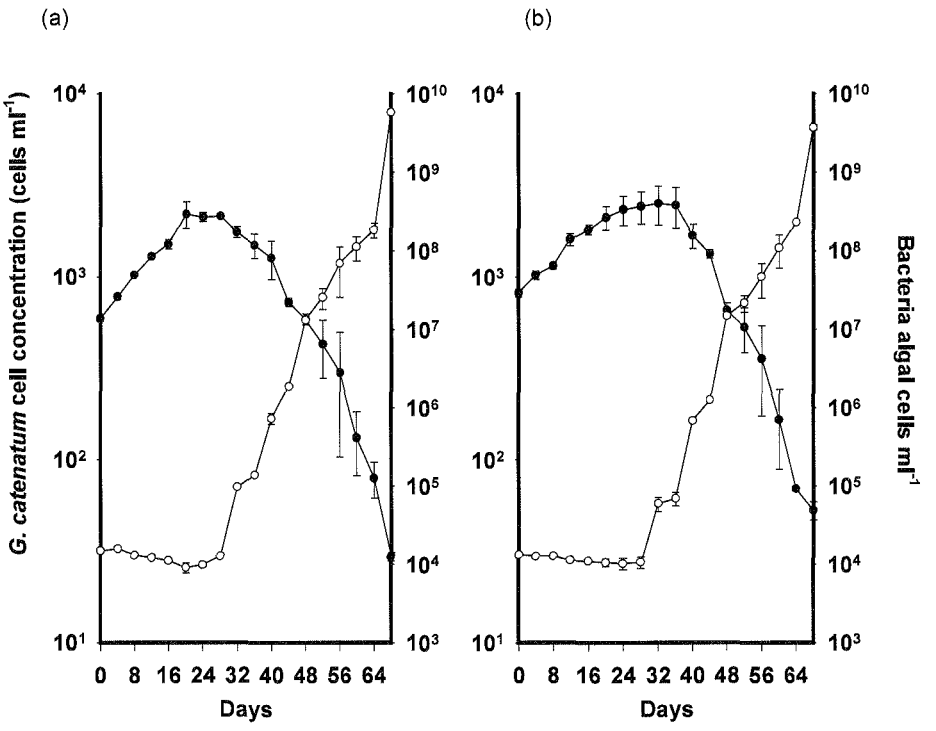

Fig. 4.9: Batch growth curves from replicate cultures of *G. catenatum* grown with (a) synthetic communities of all three bacteria; (b) mixed bacterial communities from non axenic GCHU11/ GCDE08 cultures (± standard error). Curves with closed circles indicate *G. catenatum* cell (cells mL^{-1}) concentration and curves with open circles indicate bacteria/algal cell concentration (cells mL^{-1}).

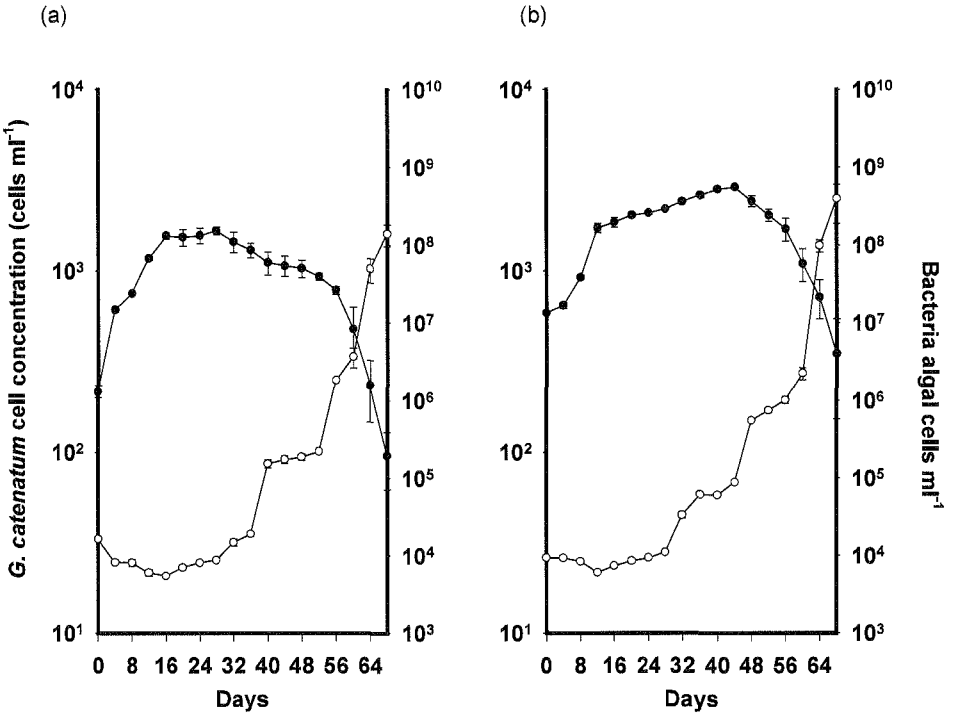

Fig. 4.10: Batch growth curves from replicate parent cultures (a) GCHU11 and (b) GCDE08 (± standard error). Curves with closed circles indicate *G. catenatum* cell concentration (cells mL^{-1}) and curves with open circles indicate bacteria/ algal cell concentration (cells mL^{-1}).

4.3.3 Bacterial growth dynamics in *G. catenatum* cultures

Bacterial growth followed a remarkably consistent pattern in all treatments and positive control established from surface-sterilised resting cysts, therefore only the mean concentration (+/- SE) from all treatments is presented (**Fig. 4.11**). Similar patterns were also evident in the cultured mixed bacterial communities associated with parent cultures GCDE08 and GCHU11, and these are presented for comparison along with the mean of all treatments and positive control. Two distinct phases were evident in the growth of the bacterial communities: From day 0 to 28, bacterial growth was relatively slow (0.02–0.05 $days^{-1}$); from day 28 onward, bacterial growth rate increased dramatically (0.16–0.23 $days^{-1}$). All treatments (including GCHU11 and GCDE08) exhibited regularly spaced periods of limited bacterial growth interspersed with rapid bacterial growth.

Bacterial cells per dinoflagellate cell also followed a consistent pattern in all treatments and controls (see **Fig. 4.4; Fig. 4.8; Fig. 4.9; Fig. 4.10)**, beginning at approximately 5 x 10^4 bacteria $cell^{-1}$ and decreasing steadily to approximately 1 x 10^4 bacteria $cell^{-1}$ around days 24-28. This relatively stable period coincided with logarithmic-phase of the dinoflagellate in most cases. Bacteria per dinoflagellate cell increased sharply at or near the end of the dinoflagellate logarithmic-phase in all cultures coinciding with the increased bacterial growth rate (compare **Fig. 4.4**; **Fig. 4.8**; **Fig. 4.9**; **Fig. 4.10**). At the termination of the experiment on day 68, bacteria per dinoflagellate cell had increased to over 10^8 bacteria $cell^{-1}$ in all treatments.

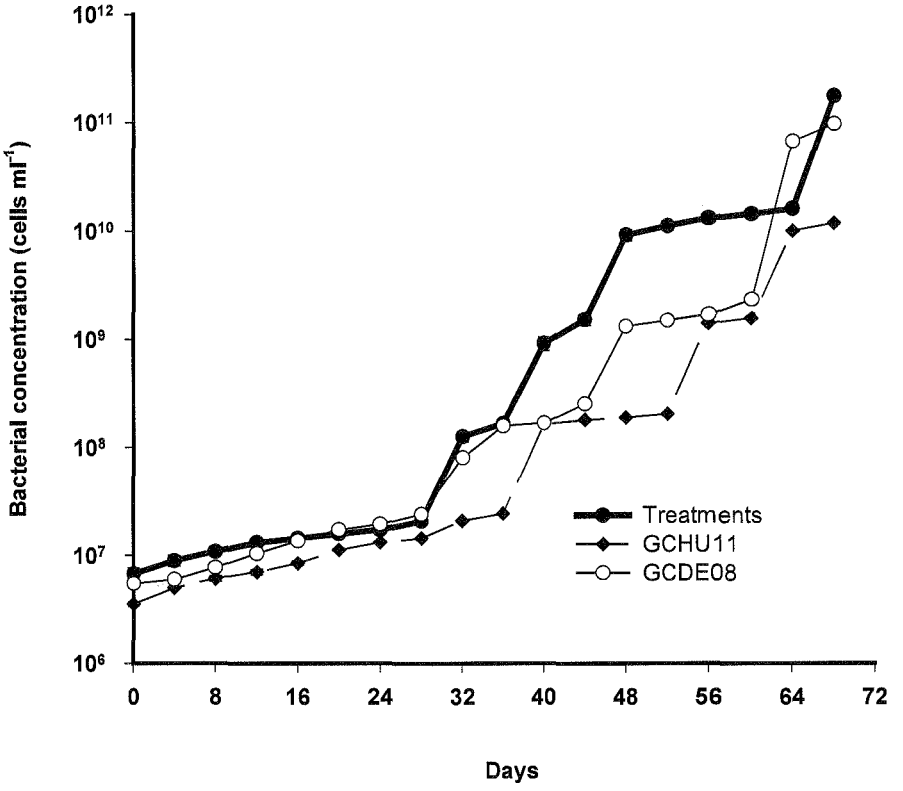

Fig. 4.11: Mean bacterial concentration (cells mL^{-1}) of all treatments and parent GCHU11 and GCDE08 cultures (± standard error).

4.4 DISCUSSION

The lack of growth in bacteria-free control in this study is similar to chapter 2 and previous studies by Bolch *et al.* (2002); Vincent (2003) and Bolch *et al.* (2004). This demonstrates the necessity of a marine bacterial community for growth of laboratory cultures of *G. catenatum*. Cyst germination studies here, and earlier, included a negative control in which *G. catenatum* cysts were germinated in bacteria-free medium. All bacteria-free treatments showed poor germination, poor early growth and long term maintenance of cultures was impossible.

Interactions existing between bacteria and phytoplankton are believed to form an intrinsic component of phytoplankton bloom physiology and ecology (Cole 1982). Evidence suggests that such interactions may play an important role in either algal bloom formation or termination (Doucette *et al.* 1998; Azam 1998). Bacterial communities may selectively promote bloom formation of specific algal species or may exhibit strong algicidal or detrimental activity (Furuki & Kobayashi 1991; Imai *et al.* 2001; Iwata *et al.* 2004). The distinct and significant differences in algal growth dynamics demonstrated by the present work supports these earlier works and the concept that the bacterial community can have a significant effect on algal growth dynamics. Altering the simplified bacterial communities, not only affected logarithmic growth rate, but also the onset and duration of batch culture phases indicating that the composition of the bacterial community has a significant influence on dinoflagellate growth and decline.

Several alcanotrophs or hydrocarbon-utilizing bacteria are known to stimulate algal growth in laboratory cultures (Vincent 2003; current study). However, alcanotrophs are found in relatively low proportion in cultured and natural communities (Green *et al.* 2004) which suggests these bacteria, though in low proportion, may be important for algal growth. They are also reported to restore reproductivity and algal cell tolerance to black oil contamination, stimulating cell growth in both *Chlorella* sp. and *Phormidium* sp. (Safonova *et al.* 1999). *Roseobacter* spp. (α-proteobacteria)

are a typically dominant group in natural dinoflagellate blooms and cultures (Alavi *et al.* 2001; Zubkov *et al.* 2001; Vasquez *et al.* 2001; Allgaier *et al.* 2003; Green *et al.* 2004), yet in this study *Roseobacter* sp. DG874 appears associated with only slow growth of *G. catenatum* cultures while a number of other α-proteobacteria cannot support growth in uni-bacterial culture. Dinoflagellates and prymnesiophytes produce major quantities of dimethylsulfoniopropionate (DMSP) by excretion, grazing or viral lysis that serves as a source of bacterial carbon and sulphur (Keller & Bellows 1996). *Roseobacter* sp. is a predominant degrader of DMSP and is correlated with DMSP producing algal blooms (Gonzalez *et al.* 1999) which may explain its dominance in *G. catenatum* laboratory cultures. *Roseobacter* spp. are also reported to exhibit a close physical relationship (surface attachment) with dinoflagellates (Gallacher *et al.* 1997; Alavi *et al.* 2001; Biegala *et al.* 2002). The closer association could be attributed to chemotactic responses of bacteria to DMSP as the latter has been identified as a chemoattractant (Faust *et al.* 1996; Gonzalez *et al.* 1999; Kiene *et al.* 2000). The increased dinoflagellate death rate associated with cultures containing *Roseobacter* sp. may be due to attachment to *G. catenatum* cells during stationary phase eventually parasitizing or lysing algal cells. Bacteria that lyse blue-green algae by attaching to cell surface have been previously described (see Daft & Stewart 1973). A myxobacterium (strain CP-1) that is attracted towards algae by chemotaxis, attaches and releases lysozyme, breaking the algal cell wall and exposing protoplasmic contents and lysing the algal cell.

In the present study, bacterial growth pattern across all treatments appear to be remarkably consistent. All show an initial phase that appears coupled to (or limited by) dinoflagellate cell concentration. The bacteria may well use algal- exuded organic carbon as a primary substrate for growth and this initial phase is likely due to the limited exudate available for bacterial growth. During exponential growth, phytoplanktons tend to release only a few percent of their photosynthetic products directly (Wiebe & Pomeroy 1999) and thus, limited supply of organic carbon may limit bacterial growth. Once the dinoflagellate cells enter stationary phase the organic

carbon exuded may increase and no longer limit bacterial growth (Paerl 1976; Middelboe *et al.* 1995; Azam *et al.* 1995; Simon *et al.* 2002).

In the controlled mixed bacterial *G. catenatum* cultures, the intermediate patterns of growth, such as reduced log-phase growth rate and rapid death rate suggest that the different bacterial types can influence the growth pattern even in the presence of other bacteria. For example, a reduced log-phase growth rate was a consistent feature of all cultures containing *Roseobacter* sp., as was a steeper decline during death phase. It may be that the bacterial community is dominated by one type at particular phases, or that the effects of bacteria on dinoflagellate growth could be cell concentration dependent at various stages. Similarly, in the natural environment, the presence or dominance of different bacterial types may facilitate bloom formation, maintenance or decomposition.

Mechanisms by which bacteria may influence algal cell growth are not well understood. One possible scenario is that bacteria release dissolved compounds that may stimulate and other compounds that reduce growth. The production and release of stimulators or inhibitors may be regulated by a number of bacterial systems, such as quorum sensing, a mechanism regulating gene expression in response to bacterial population concentration. Quorum sensing is modulated through chemical signals termed "autoinducers" whose concentration increases with increasing cell population (Bassler 1999). Bacteria monitor the threshold concentration of these autoinducers and alter gene expression and a diverse array of physiological activities. One family of signaling molecules are acetylated homoserine lactones (AHL). These are known to regulate secondary metabolite production, antibiotic production, conjugation, toxin synthesis, biofilm formation and exoprotease production (Bassler 1999; Eberl 1999). It may be possible to detect AHL's in the uni-bacterial *G. catenatum* cultures, as evidence of altered gene expression that may be inducing the production of algicidal compounds. Bacterial secondary metabolites and algicidal compounds are

thought to be released during stationary and late log phase (Fukami *et al.* 1992; Mikhailov & Ivanova 1994; Lereclus *et al.* 2000).

Marinobacter sp. and *Alcanivorax* sp. share a common feature of hydrocarbon degrading ability and both support *G. catenatum* cyst germination and growth. Dinoflagellate cells are rich in hydrocarbons and fatty acids (Hallegraeff *et al.* 1991) and hence the ability to utilize these hydrocarbons is of benefit to the bacteria. When the algal cells entered declining or stationary phase, bacterial growth increased substantially. At this point, increasing bacterial concentration may trigger changes to a competitive or parasitic relationship resulting in a decline of algal cell numbers. Bacterial growth leading to phytoplankton cell death could also be associated with bacteria competing with algal cells for use of nutrients (Wheeler & Kirchmann 1986; Jumars *et al.* 1989) or production of algicidal compounds by bacteria (Doucette *et al.* 1999). Compounds excreted by bacteria are believed to either stimulate or inhibit dinoflagellates. For example, a glycoprotein produced by *Pseudomonas* sp. 022 strain stimulated growth of *A. glacialis* (Riquelme 1988) and 50-Kda-serine protease from *Pseudomonas* sp A28 exhibited algicidal properties (Lee *et al.* 2000) particularly during the stationary phase (Mitsutani *et al.* 2001).

Green *et al.* (2004) characterized alphaproteobacteria, gamma proteobacteria and bacteroidetes to be numerically dominant in *G. catenatum* cultures. These bacterial representatives of *G. catenatum* have also been found associated with other dinoflagellates. For example, Alphaproteobacteria (Rhodobacteraceae) has been found to be a dominant phylotype associated with *Pfiesteria* sp. (Alavi *et al.* 2001), *Alexandrium* sp. and *S. trochoidea* cultures (Hold *et al.* 2001). Similar to *G. catenatum*, members of Alteromonadaceae (*Marinobacter* sp. and *Alteromonas* sp.) are reported in various dinoflagellate associations (Alavi *et al.* 2001; Hold *et al.* 2001; Seibold *et al.* 2001; Ferrier *et al.* 2002; Jasti *et al.* 2005).

These earlier studies indicate that the bacterial communities associated with dinoflagellates are broadly similar between species and also between cultures and natural blooms. The model communities used in the current study were selected to provide a representative of the dominant and sub-dominant types associated with *G. catenatum*. Despite the simplicity of the three- bacterium community, the similarity of the growth dynamics to the mixed bacterial (positive) controls, these simple communities appear to approximate the dynamics of cultures grown with more complex "natural" bacterial communities. This indicates that the simplified communities used here are a potentially useful model system for bacterial-dinoflagellate interaction studies.

In conclusion, this chapter demonstrated that after germination, growth and batch culture dynamics of *G. catenatum* growth in laboratory culture is strongly influenced by the make up of the bacterial community, supporting the idea that bacterial communities can be an important factor influencing dinoflagellate and phytoplankton growth dynamics. The simplified experimental models used here approximated phytoplankton responses to more complex bacterial communities in the natural environment and are beneficial in improving the available knowledge on algal bloom dynamics.

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Chapter 5

Spatial distribution of growth promoting bacteria in uni-bacterial *G. catenatum* cultures.

5.1 INTRODUCTION

Algal-bacterial interactions have recently received substantial attention as a potential factor influencing phytoplankton population growth and decline (Doucette *et al.* 1998). Phytoplankton excretes organic compounds, a major input to the pelagic energy flow or food web (Lancelot 1983) and stimulate bacterial growth (Schafer *et al.* 2002). In the phycosphere, bacteria can be free living, attached to the algal cell surface (Kogure *et al.* 1982; Vaque *et al.* 1990; Worm & Sondegaard 1998; Simon *et al.* 2002) or exist as intracellular algal symbionts (Silva & Franca 1985, Lewis *et al.* 2001). The presence of bacteria within dinoflagellates has been reported for decades (Silva 1962; Gold & Pollingher 1971; Silva 1978; Lucas 1982). However, only recently has the taxon specificity of bacteria and/or spatial association with the dinoflagellate cell been hypothesised to influence bacterioplankton interactions, population dynamics and toxicity of these algae (Doucette *et al.* 1999).

Bacteria associated with natural phytoplankton populations are not homogeneously or randomly distributed. Alphaproteobacteria dominate the free-living bacterial population while members of Cytophaga-Flavobacteria, Gammaproteobacteria and Planctomycetes dominate the attached bacterial population(DeLong *et al.* 1993, Gonzalez & Moran 1997). Several studies

have considered the spatial proximity of bacteria and phytoplankton cells as a regulating factor of toxin production (Silva 1982; Kodama *et al.* 1990; Franca *et al.* 1995; Gallacher *et al.* 1997). Higher intensity of interaction is believed to exist among phytoplankton and attached bacteria than with free-living bacteria (Rooney-Varga *et al.* 2005). Intracellular or attached bacteria have also been considered to influence PST production in *Alexandrium tamarense* (Silva 1982; Kodama *et al.* 1990). Hence an understanding of the spatial relationship between bacteria and algal cells may be useful in determining or predicting the nature and mechanism of interaction between bacteria and algal cells.

The aim of this chapter is to examine the spatial relationship of bacteria in *G. catenatum*-bacteria experimental model cultures without disrupting the cells, therefore a non-destructive technique was employed. Staining with fluorescent dyes, the most accepted technique (Fry 1990; Ward & Johnson 1996), was applied to estimate the number of bacteria attached to *G. catenatum* cells compared to those unattached. A wide range of stains are available currently such as TOTO-1, TO-PRO-1, YOYO-1, YO-PRO-1, acriflavine, bisbenzimide (and other Hoechst dyes), erythrosine, rhodamine, euchrysine, ethidium bromide, phenolic alanine blue, methylene blue, SYBR Green I+II, SYBR gold, PICO Green and Ribo Green (Zimmermann & Reil 1974; Porter & Feig 1980; Kepner & Pratt 1994; Yu *et al.* 1995; Noble & Fuhrman 1998). The most commonly used fluorochromes are 4', 6–diamidino-2-phenylindole (DAPI) and acridine orange (AO) (Kepner & Pratt 1994). DAPI and AO are both nucleic acid stains used to identify bacteria based on colour, size and shape. DAPI is a DNA-specific dye that produces a blue fluorescence when bound to DNA and excited with light at wavelength 365 nm. Unbound DAPI and DAPI bound to non-DNA material fluoresce yellow. AO binds to both DNA and RNA and, on staining actively growing bacteria, fluoresces red-orange due to RNA dominance, while inactive bacteria fluoresce green due to DNA dominance (Hobbie *et al.* 1977). Direct counting with AO is not recommended as (a) growth media, cell taxonomy and staining procedure may affect AO colour reaction and (b) particles like clay and detritus could also be stained or autofluoresce, causing difficulties in distinguishing bacteria and other non-living substances. While DAPI also stains some non-living

material, the fluorescent intensity is much higher than AO and background fluorescence is typically much lower (Porter & Feig 1980; Kepner & Pratt 1994).

This chapter employs DAPI-staining coupled with epifluorescence microscopy to examine changes in the spatial distribution of bacteria in uni-bacterial *G. catenatum* model cultures at three points in the batch culture cycle. Intracellular or endonuclear bacteria are rare or absent in *G. catenatum* cells (Rees & Hallegraeff 1991), therefore we compare only the ratio of unattached to attached bacteria (surficial) observed in the uni-bacterial cultures generated in chapter 4.

5.2 MATERIALS AND METHODS

Uni-bacterial cultures of *G. catenatum* from chapter 4 were examined for bacterial distribution studies using DAPI staining. A concentrated stock solution of 1 $mgmL^{-1}$ DAPI was prepared with filtered sterile seawater. The stock solution was diluted to 0.1 μgmL^{-1} before use (Porter & Feig 1980). Replicate cultures grown with *Marinobacter* sp., *Alcanivorax* sp., or *Roseobacter* sp., were sub-cultured regularly in 100 mL flasks to maintain cells in early log-phase. Sub-samples (1 mL) of cultures were removed at mid-exponential phase (day 9 for *Marinobacter* sp. and *Alcanivorax* sp. cultures and day 28 for *Roseobacter* sp. cultures), stationary phase (day 28 for *Marinobacter* sp., *Alcanivorax* sp. cultures and day 42 for *Roseobacter* sp. cultures) and death phase (day 40 for *Marinobacter* sp., *Alcanivorax* sp. cultures and day 52 for *Roseobacter* sp. cultures). For staining, 1 mL of *G. catenatum* culture was aliquoted to 1.5 mL centrifuge tubes and 0.1 mL of the diluted DAPI solution was added. The cells were incubated for 15 mins, mounted on a glass slide and viewed under epifluorescent microscope at 100 x magnification (Croft *et al.* 2005). Ten randomly selected fields of view that included a *G. catenatum* cell were examined and the number of cells closely associated with the cell surface and unattached bacteria were recorded.

5.2.1 Statistical analysis

Significant changes in proportion of unattached bacteria compared to bacteria attached to *G. catenatum* cell over a batch culture cycle, was determined using a χ^2 test for independence ($P< 0.05$). Data is depicted as bar graphs of uni-bacterial treatments studied during different batch culture phases. Normalized residuals were used to determine significant differences between observed and expected values at >±2. All analyses were carried out using the statistical analysis software SPSS ver. 11.5 (LEAD technologies, Chicago, USA).

5.3 RESULTS

All bacterial cells (100%) of all the three bacterial strains, *Alcanivorax* sp., *Marinobacter* sp. and *Roseobacter* sp. were observed to be unattached during exponential phase of *G. catenatum* culture (**Fig. 5.1-Fig. 5.4**) During this phase (days 0-12 for *Marinobacter* sp. and *Alcanivorax* sp. and days 0-40 *Roseobacter* sp. cultures) the bacterial cell density near algal cells was very low in all treatments (<5 bacterial cells algal cell^{-1}). As the cultures reached stationary phase bacterial cell numbers increased, but 100% of the cells remained unattached to the dinoflagellate cell. Cultures of *G. catenatum* grown with *Alcanivorax* sp. averaged <15 cells algal cell^{-1} while cultures with *Marinobacter* sp. averaged <20 cells algal cell^{-1} (**Fig. 5.1**; **Fig. 5.2**); *Roseobacter* sp. averaged < 30 cells algal cell^{-1} (**Fig. 5.3**).

During death phase more bacteria were associated closely with dinoflagellate cell surface. *Marinobacter* sp., *Alcanivorax* sp. and *Roseobacter* sp. cultures all showed bacterial cells closely associated with dinoflagellate cell wall. *Roseobacter* sp. showed a higher proportion (18%) of attached bacteria compared to *Alcanivorax* sp. (7.9%) and *Marinobacter* sp. (11.6%) during death phase of the dinoflagellate culture (**Fig. 5.1**; **Fig. 5.2**; **Fig. 5.3**). A χ^2 independence test performed

for different growth phases in all three bacteria showed a significant increase in bacteria associated with the cell surface during death phase in all treatments ($P < .006$).

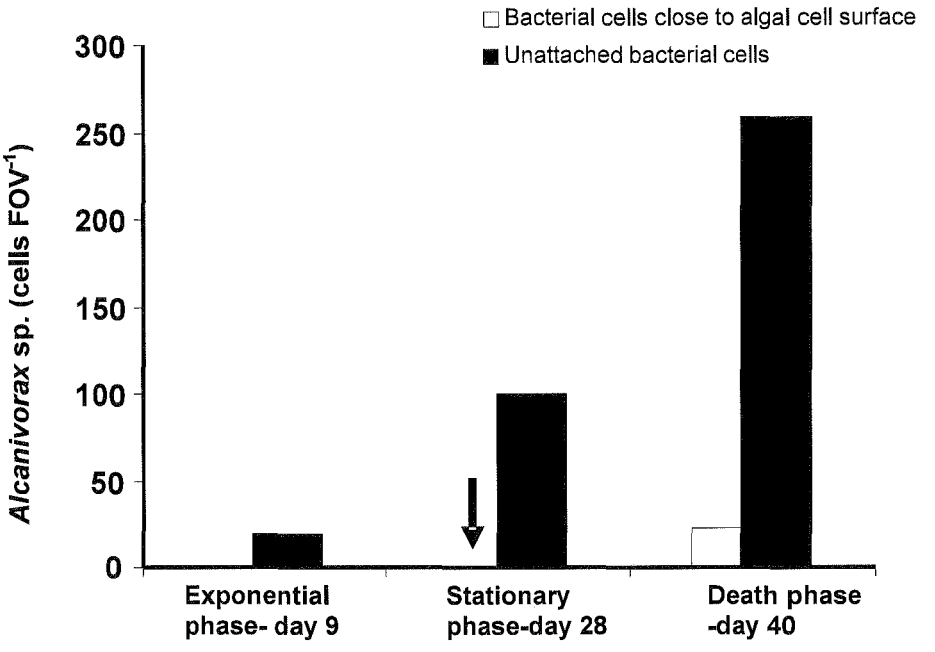

Fig. 5.1: Mean number of *Alcanivorax* sp. DG881 cells attached or unattached (free-floating) in 10 different fields of view (FOV). Arrow indicates where frequency of bacterial cells close to algal cell surface (tested by χ^2 independence) was significantly less (↓) than expected.

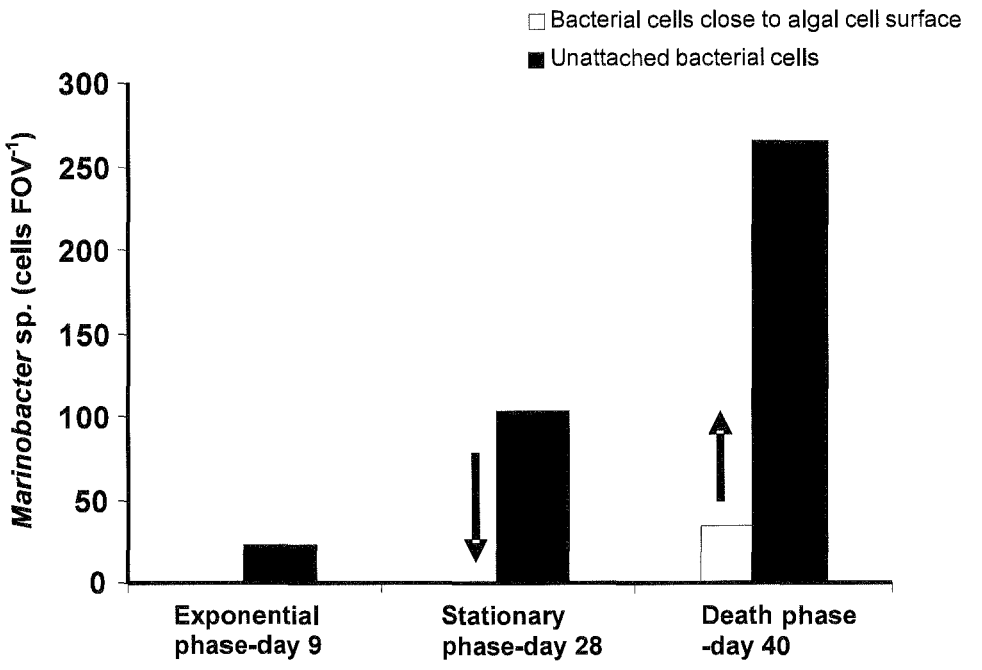

Fig. 5.2: Mean number of *Marinobacter* sp. DG879 cells attached or unattached (free-floating) in 10 different fields of view (FOV). Arrows indicate where frequency of bacterial cells close to algal cell surface (tested by χ^2 independence) showed significantly more (↑) or less (↓) than expected.

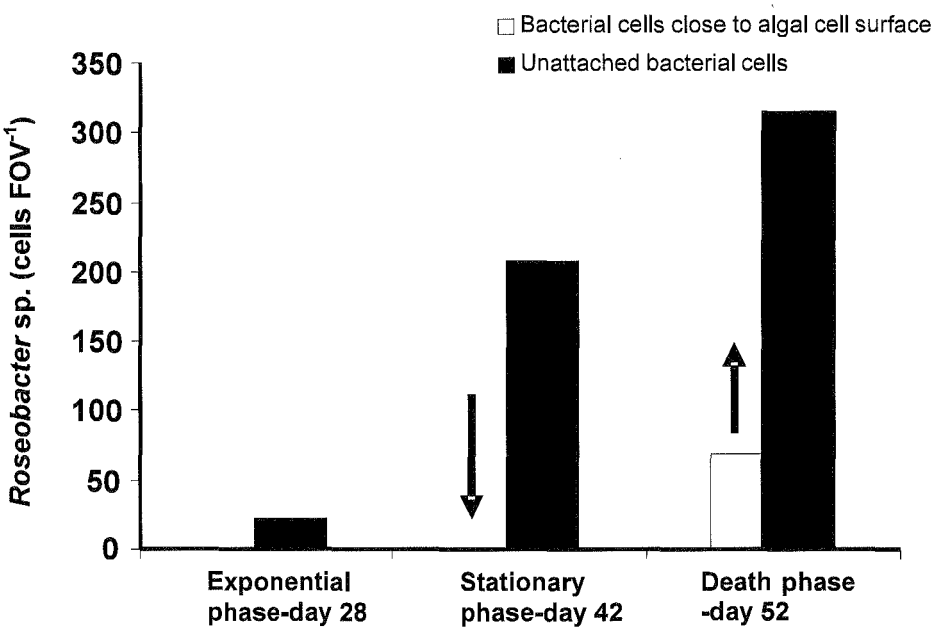

Fig. 5.3: Mean number of *Roseobacter* sp. DG874 cells attached or unattached (free-floating) in 10 different fields of view (FOV). Arrow indicates where frequency of bacterial cells close to algal cell surface (tested by χ^2 independence) was significantly more (↑) or less (↓) than expected.

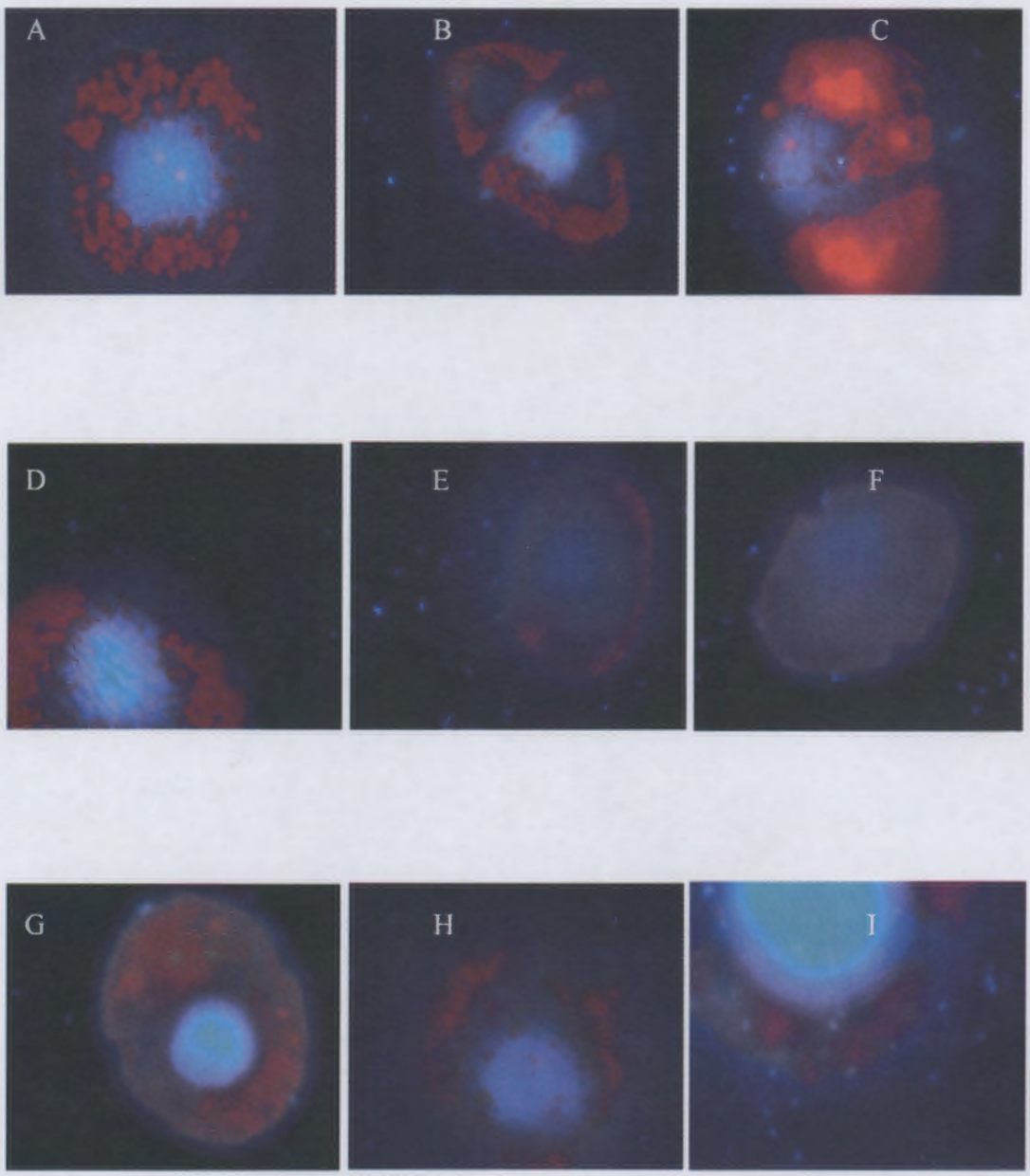

Fig. 5.4: Epifluorescense microscopy images of DAPI stained *G. catentaum* cells with *Alcanivorax* sp. (A-C) or *Marinobacter* sp. (D-F) or *Roseobacter* sp. (G-I). Culture samples were examined during mid exponential phase (day 9 A, D; day 28 G), stationary phase (day 28 B, E; day 42 H) and death phase (day 40 C, F; day 52 I).

5.4 DISCUSSION

Previous studies have identified the physiological state of phytoplankton cells to have a major influence upon the distribution of bacterial cells associated with algal cells (Bell & Mitchell 1972; Fogg 1983; Albright *et al.* 1986; Simon *et al.* 2002). The results of this study also suggest a strong correlation between physical state of phytoplankton cells and the distribution of associated bacteria. It is well known that algal cells are the major organic substrate for heterotrophic bacteria (Paerl 1976; Azam *et al.* 1995). Bacteria are known to transform phytoplankton derived particulate organic carbon (POC) to dissolved organic carbon (DOC) and maintain pelagic carbon turn-over (Jensen 1983; Bratbak & Thingstad 1985; Cole *et al.* 1988; Doucette 1995).

Bacteria begin to colonize algal cells only when POC and other nutrients are depleted in the environment. In this work, the exponential phase of all three algal cultures was dominated by free–living bacteria, suggesting that algal exudates of polymeric compounds were readily available in the culture medium as a major carbon source for bacterial growth (Middelboe *et al.* 1995). As the phytoplankton cells reached stationary phase, the bacterial distribution changed, with cells beginning to closely associate with the dinoflagellate cell surface. Several reports have shown that bacteria colonize phytoplankton cells only when the latter becomes more senescent (Vaque *et al.* 1989, 1990; Simon *et al.* 2002). In this study firm bacterial attachment to the algal cell wall was evident only during the death phase of algal culture.

Studies measuring changes in hydrolytic activity of bacteria have reported concentration of monosaccharides to decrease as bacterial concentration increases, implying that monomers are used for bacterial production during initial phase of blooms. Middleboe *et al.* (1995) observed a significant correlation between free-living bacterial population and chlorophyll-a concentration suggesting algal exudates as an important carbon source for free living bacteria. In the above study a significant proportion of bacterial attachment was observed only during death phase and late senescence. The reduced concentrations of monomers and other depleting nutrients due to algal

senescence may have drawn the bacterial cells more closer to algal cells in search of nutrition. Bacterial attachment has also been reported during initiation phase and death phase of *Skletonema costatum* and *Dunaliella tertiolecta* in both laboratory cultures and natural assemblages (Albright *et al.* 1986), possibly due to release of newly synthesised organic nutrients from the phytoplankton inducing bacterial attachment during bloom initiation.

Various types of interactions can be expected in complex associations such as algae and bacteria. Earlier studies have reported antibiosis effects that alter both bacterial and algal physiology. Antibiotic-like substances synthesised by algae can also strongly affect bacterial behaviour (Berland *et al.* 1972; Cooper *et al.* 1983). The data here suggests, when the algae are growing exponentially, growth inhibitors such as antibiotics may keep the bacteria away from algal cells. In *S. costatum,* fatty acids are believed to have antibiotic effects and are synthesised during the middle to late exponential growth phase of algae (Albright *et al.* 1986).

The attachment of bacteria to algal cells is clearly influenced by the physical state of the algal culture, but little is known of differences among bacterial types. For example, *Roseobacter* sp. which showed slower growth promoting ability in *G. catenatum* uni-algal cultures (Chapter 4) had 18% of bacterial cells associated with the algal cell surface. *Alcanivorax* sp. and *Marinobacter* sp., which supported *G. catentaum* growth to a greater extent in the current and earlier studies (Vincent 2003) showed only 7.9 and 11.8% attached cells during death phase. The rapid decline in *G. catenatum* cultures grown with *Roseobacter* sp. could be well related to algicidal activity by *Roseobacter* sp. Members of the genus *Roseobacter* are known to have algal-lytic activity when in close physical contact with toxic dinoflagellate *Alexandrium catenella* (Amaro *et al.* 2005). However, the exact mechanisms by which these bacteria can express algal-lytic properties are still unknown (Amaro *et al.* 2005). Algicidal bacteria may show direct cell to cell contact (Imai *et al.* 1993) or release extracellular algicidal compounds affecting growth of phytoplankton (Jeong *et al.* 2003). Hence the increased activity and closer association of *Roseobacter* sp. to *G. catenatum* cells may have resulted in the more rapid decline in algal cell numbers in these cultures.

This study suggests that in our simplified models, different bacteria exhibit varied level of attachment in death phase and the closer association of bacterial cells to the outer membrane of *G. catenatum* cells may have lead to the death of uni-algal cultures. Further studies on enzymatic activities and soluble compounds mediating algal cell lysis in cultures is essential to confirm the possible use of bacterial control of algal blooms.

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Chapter 6

G. catenatum growth - Is genetic variation or bacterial composition a dominant factor?

6.1 INTRODUCTION

Biotic and abiotic factors influencing algal growth dynamics have been widely studied and discussed (Morel & Hudson 1985; Hallegraeff 1993; Doucette *et al.* 1999; Thompson 1999; Ferrier *et al.* 2002). Another factor which may possibly influence phytoplankton growth is genetic variation, or the individual genotype of the algal cell. The effect of genotype on algal growth has received little attention from ecologists and physiologists studying phytoplankton growth dynamics. Most studies imply that there is either no genetic variability in their population, or that it has little influence on phytoplankton growth. Only a few studies have investigated variation in dinoflagellate reproductive rates due to mixed genotypes (Nelson & Brand 1979; Brand 1981).

In chapter 4, *G. catenatum* non-clonal progeny cultures generated with mixed bacterial community from parent GCHU11 and GCDE08 (clonal) showed different growth patterns compared to their parent cultures (compare **Fig. 4.9** (b); **Fig. 4.10**). The exponential growth rates and maximum algal cell concentrations also differed markedly among

the parent and progeny cultures regardless of the microbial composition. This suggested that growth dynamics was at least partly influenced by differences among strains (i.e. algal genotype) rather than the dominant bacterial community.

This chapter uses the *G. catenatum*-bacteria experimental model to investigate whether algal genotype or bacterial community has a dominant influence on algal growth dynamics. The chapter compares the batch culture growth dynamics, exponential growth rate and bacterial community composition of two clonal parent cultures (GCHU11 & GCDE08) with non-clonal progeny established with bacterial communities from each of the parent cultures. Bacterial community composition in the experimental cultures is analysed using the Terminal Restriction Fragment Length Polymorphism (t-RFLP) fingerprinting technique. T-RFLP is a powerful technique for characterizing complex bacterial communities (Moeseneder *et al.* 1999). The method detects differences in the position of restriction sites among DNA sequences and determines the length of fluorescently labeled terminal restriction fragments (TRFs) by high resolution gel electrophoresis on an automated DNA sequencer (Avaniss-Aghajani *et al.* 1994; Clement *et al.* 1998). The t-RFLP method has been reported to be more effective and consistent in determining microbial communities compared to denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene cloning (Moeseneder *et al.* 1999; Tiedje *et al.* 1999; Dunbar *et al.* 2000).

6.2 MATERIALS AND METHODS

6.2.1 Cyst production

Gymnodinium catenatum GCHU11 and GCDE08 were grown at 19°C (+/- 2.5°C) in 150 mL Erlenmeyer flasks in GSe medium (Blackburn *et al.* 1989) under white fluorescent light of 90±10 μmoles m^{-2} s^{-1} with 12L:12D photoperiod. Cyst production and surface sterilisation of cysts was based on the techniques described in chapter 2 (refer sections 2.2.1; 2.2.2).

6.2.2 Establishing *G. catenatum* cultures

Surface sterile resting cysts were aliquoted into 36 mm petri dishes and 8 μm bacterial filtrates (Millipore, USA) from GCHU11 and/or GCDE08 were aseptically added to the cysts as described in **Table 6.1**. All controls and treatments were replicated 4 times. Each dish contained 25-30 resting cysts in 1.9 mL of sterilized GSe medium. Dishes were sealed with Parafilm™ and incubated at 19°C +/- 2.5°C at a light intensity of 90±10 μmoles m^{-2} s^{-1} with a 12L:12D photoperiod. All treatments and controls containing resting cysts were monitored by stereomicroscope. Once significant healthy swimming cells were seen, replicates from each treatment and the positive control were transferred to sterile 150 mL Erlenmeyer flasks containing 100 mL of sterile GSe medium. The established 100 mL cultures were transferred to fresh sterile 150 mL Erlenmeyer flasks with 100 mL of GSe medium. At the same time clonal cultures of *G. catenatum* strains GCHU11 and GCDE08 were transferred for comparison studies. Triplicate subsamples were withdrawn from cultures every 4 days to estimate *G. catenatum* cell concentration by cell count using Sedgwick-Rafter counting chamber (Guillard 1973). Bacterial cell concentration (CFU mL^{-1}) in treatments and controls was estimated by serial dilution-plating (Buck & Cleverdon 1960) onto ZM1 agar.

Table 6.1: Description of treatments and controls used in cyst germination studies. All treatments and controls (except the media sterility control consisted of 36 mm petri dishes, containing 25-30 surface–sterilized *G. catenatum* resting cysts in 1.9 mL of Gse medium with the addition of the treatment described below. All treatments and the positive control were replicated 4 times.

Treatments	**Sterile *G. catenatum* resting cysts plus**
Positive control	200 µl of 8 µm filtrate from mid-log phase cultures of GCDE08 and GCHU11 (non- clonal)
Negative control	Sterile Gse medium
Cysts + HU11	200 µl of 8 µm filtrate from mid-log phase cultures of GCHU11 (non- clonal)
Cysts + DE08	200 µl of 8 µm filtrate from mid-log phase cultures of GCDE08 (non- clonal)
GCHU11	75 mL of log phase, GCHU11 (clonal) grown in Gse
GCDE08	75 mL of log phase, GCDE08 (clonal) grown in Gse

6.2.3 DNA extraction and PCR amplification

Bacterial genomic DNA from mid-log phase *G. catenatum* cultures were extracted using a cetylmethylammonium bromide purification (CTAB) method (Ausubel *et al.* 1999) (Appendix 3). For t-RFLP analysis, primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 518R (5'-ATTACCGCGGGCT -GCTGG-3') (Weisburg *et al.* 1991) were used to amplify an approximately 500 bp region of the SSU-rDNA from the bacterial community of experimental cultures. The primers were synthesized and labeled with WellRED^{TM} cyanine-based fluorescent dyes D3 (primer 27F) and D4 (primer 518R) by Proligo, Australia Pty Ltd. The PCR was performed using a MJ Research PTC-200 Thermal Cycler (MJ Research, USA). Reactions were carried out in a 50 µl reaction volumes containing 0.2 mM of each primer, 1.25 U of Taq polymerase (BioTaq, Bioline, UK), 3 mM $MgCl_2$, 200 mM of each dNTP and Bioline ammonium PCR buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris HCl (pH 8.8), 0.1% Tween -20). PCR amplification included: an initial denaturation at 96°C for 5 mins, followed by 30 cycles of: denaturation at 95 °C for 15 s, annealing at 49°C for 30 s, extension at 72°C for 1 min; and a final extension at 72°C for 5 mins. Amplified products were purified using Montage-PCR ultrafilters (Millipore, USA) using the manufacturer protocols and the DNA resuspended in MilliQ water. The DNA concentration was determined using a Turner TBS 380 DNA fluorometer (Turner Designs, USA) according to standard protocols.

6.2.4 Restriction digestion and t-RFLP analysis

Restriction enzymes for t-RFLP analysis were selected to discriminate the dominant components of the microbial community of GCDE08 and GCHU11 (described by Green *et al.* 2004). Both communities are dominated by *Roseobacter/Rhodobacter* genotypes but with different SSU-rDNA genotypes dominating each culture. From comparison of the dominant SSU-rDNA sequences in each culture, the enzymes *Hha*I and *Bfa*I were selected due to the differing restriction sites between the representative *Roseobacter/Rhodobacter* species. Approximately 100 ng of the purified PCR amplicons were digested in a total volume of 20 µl for 4 hrs with restriction enzymes *Hha*I (20 U), *Bfa*I (5 U), (New England Biolabs Inc, Massachusetts, USA) according to manufacturer instructions. Following digestion, the enzymes were heat-inactivated according to manufacturer recommendations. For t-RFLP analysis, fluorescently labeled fragments were separated on a CEQ™ 8000 genetic analysis system (Beckman Coulter™, Fullerton, CA, USA), by adding 25 µl of Beckman Sample Loading Solution (SLS) and 0.25 µl Beckman 'WellRED' size standard (600 bp) (Beckman Coulter, Fullerton, CA, USA) to 1 µl of digested product. The fragment patterns were visualized and sized by comparison with size-standards using Beckman-Coulter CEQ™ 8000 genetic analysis software (version 8.0) (Beckman Coulter, Fullerton, CA, USA).

6.2.5 Phylogenetic analysis

The TRF size matrices generated by the genetic analysis software were examined and scored manually to produce presence/absence matrix of homologous fragments from both forward- and reverse-labeled t-RFLP traces for each replicate of all culture treatments. Fragments differing by < 1bp in size were binned by manual inspection, and by reference to the electropherograms, and recorded as present (1) or absent (0) for each fragment in each sample. The matrix was then exported to the phylogenetics software program PAUP 4.0* (version 4.0b10, Swofford 1998) for further analysis. The presence/absence matrix (Appendix 7) was used to calculate a pairwise distance matrix among all treatments and replicates using the mean distance metric of PAUP 4.0*. The mean distances were subjected to unweighted pair-group mean-average (UPGMA) cluster analysis and support for clusters assessed by performing 1000 bootstrap randomizations of the dataset.

6.2.6 Statistical analysis

Dinoflagellate growth rates were calculated according to the methods of Guillard (1973) and expressed as instantaneous growth rate ($days^{-1}$). Growth rates and maximum algal cell numbers were analysed using one-way ANOVA with planned contrasts to test for significant differences (**Table 6.2**). All analyses were carried out using the statistical analysis software SPSS ver. 11.5 (LEAD technologies, Chicago, USA).

Table 6.2: Description of planned contrasts of non-clonal progeny cultures grown with GCHU11 or GCDE08 bacterial community, compared with parent GCHU11 & GCDE08 cultures. Possible outcomes and interpretation are described.

Planned Contrast	**Possible Outcomes**	**Interpretation**
Cysts+ HU11 bacteria Versus Clonal HU11 culture	Significant difference in growth rates or maximum algal cell numbers (P<=0.05)	Supports hypothesis that genotype is the dominant influence on growth rate
	No difference in growth rate or maximum algal cell numbers (P>0.05)	Supports hypothesis that bacterial community is the dominant influence on growth rate
Cysts+ DE08 bacteria Versus Clonal DE08 culture	Significant difference in growth rates or maximum algal cell numbers (P<=0.05)	Supports hypothesis that genotype is the dominant influence on growth rate
	No difference in growth rate or maximum algal cell numbers (P>0.05)	Supports hypothesis that bacterial community is the dominant influence on growth rate
Cysts+ HU11 bacteria Versus Cysts+ DE08 bacteria	Significant difference in growth rates or maximum algal cell numbers (P<=0.05)	Supports hypothesis that bacterial community is the dominant influence on growth rate
	No difference in growth rate or maximum algal cell numbers (P>0.05)	Supports hypothesis that genotype is the dominant influence on growth rate
Cysts+ HU11 bacteria Versus Cysts+ HU11/DE08 bacteria	Significant difference in growth rates or maximum algal cell numbers (P<=0.05)	Supports hypothesis that bacterial community is the dominant influence on growth rate
	No difference in growth rate or maximum algal cell numbers (P>0.05)	Supports hypothesis that genotype is the dominant influence on growth rate
Cysts+ DE08 bacteria Versus Cysts+ HU11/DE08 bacteria	Significant difference in growth rates or maximum algal cell numbers (P<=0.05)	Supports hypothesis that bacterial community is the dominant influence on growth rate
	No difference in growth rate or maximum algal cell numbers (P>0.05)	Supports hypothesis that genotype is the dominant influence on growth rate

6.3 RESULTS

Cyst germination and cells moving/germinant data is not included in this study as there was no significant difference seen among non clonal cultures, similar to results obtained in chapter 2 and chapter 4 and are not presented here. Negative control (cyst germinated in the absence of bacteria) showed poor germination and survival similar to earlier chapters (2 & 4).

6.3.1 Algal growth dynamics in clonal and non clonal *G. catenatum* cultures

Clonal and non-clonal cultures of *G. catenatum* showed growth curves similar to patterns obtained in chapter 4. Growth curves obtained from all non-clonal progeny cultures were more similar to each other and less similar to either clonal parent culture. Both GCHU11 and GCDE08 clonal cultures showed a short exponential phase, entering stationary phase on day 12, while non-clonal cultures showed an extended growth phase and more gradual declining growth phase, with some cultures not reaching stationary phase until day 20 (**Fig. 6.1**). The onset of death phase (day 44) was similar for all cultures.

Exponential growth rates and maximum algal cell concentrations varied among clonal and non-clonal cultures. Growth rate of non-clonal cultures ranged from 0.07- 0.09 day^{-1} while clonal GCHU11 and GCDE08 cultures showed 0.11 day^{-1} and 0.06 day^{-1} respectively (**Fig. 6.2**). Maximum dinoflagellate cell concentrations in non-clonal cultures of *G. catenatum* ranged from 4.8 - 8.4 x 10^3 cells mL^{-1} while clonal GCHU11 and GCDE08 cultures showed 9.4 x 10^3 cells mL^{-1} and 3.5 x 10^3 cells mL^{-1} respectively (**Fig. 6.3**). Statistical support for significant differences among clonal and non-clonal cultures are discussed in **Table 6.3** (growth rate) and **Table 6.4** (maximum algal cell concentrations).

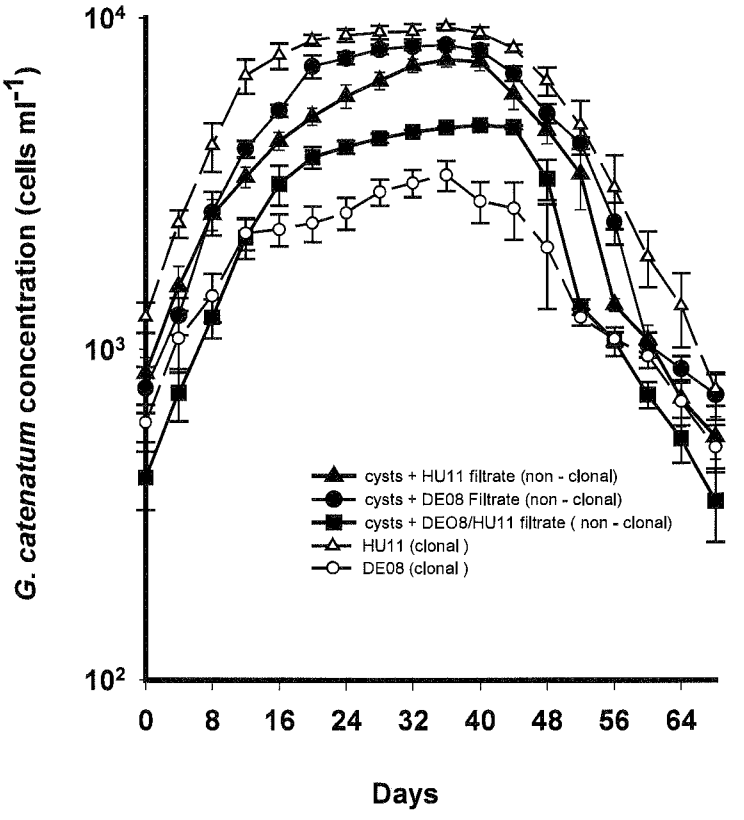

Fig. 6.1: Algal growth dynamics from replicate (4) *G. catenatum* cultures (non-clonal cultures) grown with bacterial communities from non-axenic parent cultures, parent GCHU11 and GCDE08 (clonal cultures).

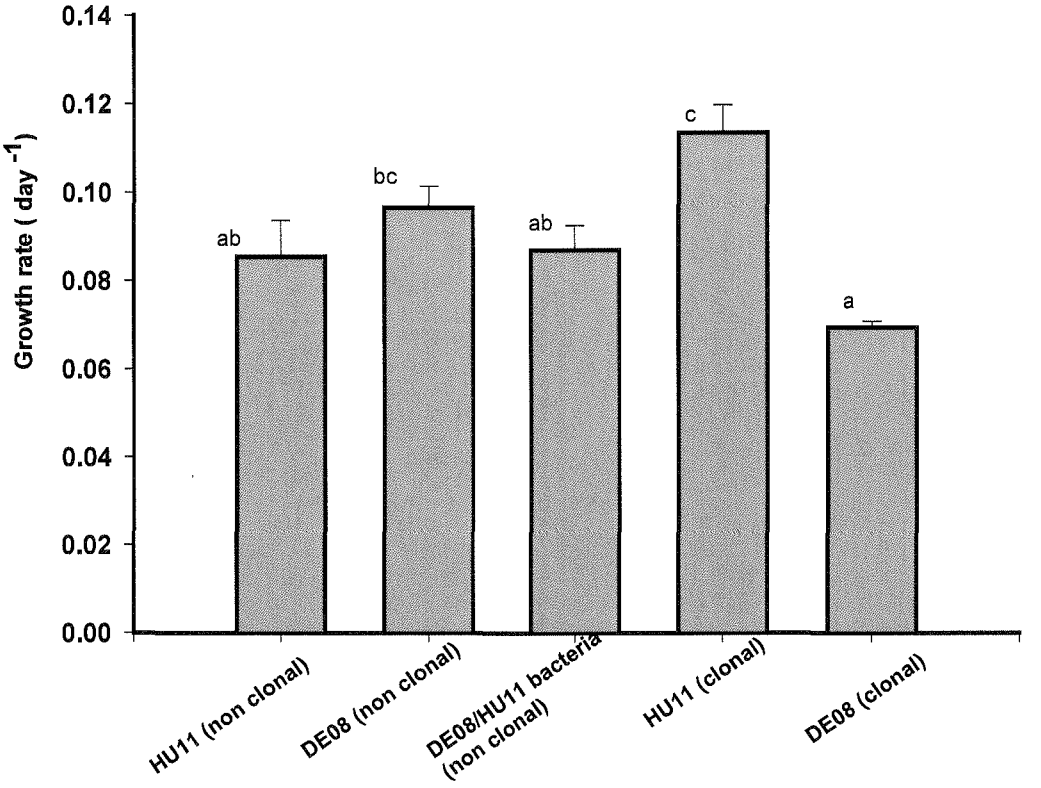

Fig. 6.2: Exponential growth rates (day^{-1}) from replicate (4) *G. catenatum* cultures (non-clonal cultures) grown with bacterial communities from non-axenic parent cultures and parent GCHU11 and GCDE08 (clonal cultures). Superscripts indicate significant differences (P= < 0.05)

Table 6.3: Interpretation of ANOVA with planned contrasts comparing exponential growth rates of non-clonal progeny cultures with clonal parent GCHU11 and GCDE08 cultures.

Planned Contrast	**Outcome**	**Interpretation**
Cysts+ HU11 bacteria Versus Clonal HU11 culture	Significantly different growth rates (t= -3.537; df= 4; *P*= 0.003)	Supports hypothesis that genotype has a dominant influence on growth rate
Cysts+ DE08 bacteria Versus Clonal DE08 culture	Significantly different growth rates (t= 3.405; df= 4, 15; *P*= 0.004)	Supports hypothesis that genotype has a dominant influence on growth rate
Cysts+ HU11 bacteria Versus Cysts+ DE08 bacteria	No difference in growth rate (t= -1.393; df= 4; *P*= 0.184)	Supports hypothesis that genotype has a dominant influence on growth rate
Cysts+ HU11 bacteria Versus Cysts+ HU11/DE08 bacteria	No difference in growth rate (t= 2.032; df= 4; *P*= 0.060)	Supports hypothesis that genotype has a dominant influence on growth rate
Cysts+ DE08 bacteria Versus Cysts+ HU11/DE08 bacteria	No difference in growth rates (t= 3.425; df= 4; *P*= 0.054)	Supports hypothesis that genotype has a dominant influence on growth rate

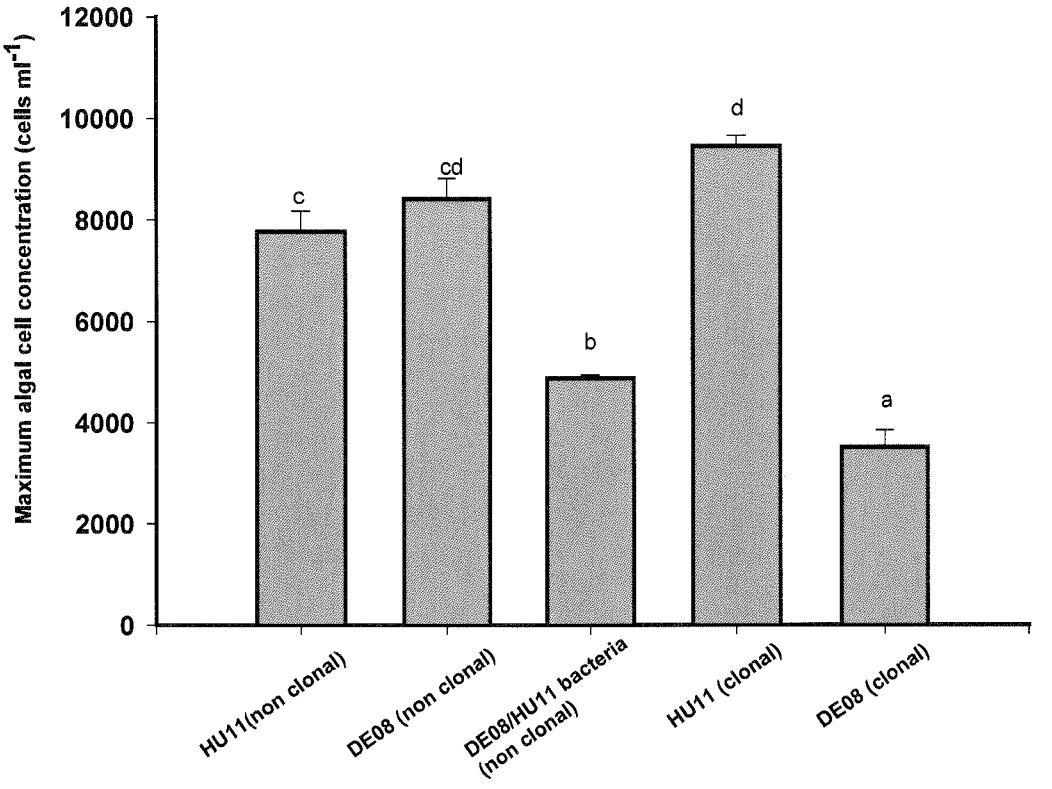

Fig. 6.3: Maximum algal cell concentration (cells mL^{-1}) in replicate *G. catenatum* cultures (non-clonal cultures) grown with bacterial communities from non-clonal progeny cultures and parent GCHU11 and GCDE08 (clonal cultures). Superscripts indicate significant differences ($P = < 0.05$).

Table 6.4: Interpretation of ANOVA with planned contrast comparing maximum algal cell concentration of non-clonal progeny with that of clonal parent GCHU11 and GCDE08 cultures.

PlannedContrast	**Outcome**	**Interpretation**
Cysts+ HU11 bacteria Versus Clonal HU11 culture	Significant difference in maximum algal cell numbers (t= -3.829; df= 4; *P*= 0.002)	Supports hypothesis that genotype has a dominant influence on algal cell numbers
Cysts+ DE08 bacteria Versus Clonal DE08 culture	Significant difference in maximum algal cell numbers (t= 11.142; df= 4; *P*= 0.000)	Supports hypothesis that genotype has a dominant influence on algal cell numbers
Cysts+ HU11 bacteria Versus Cysts+ DE08 bacteria	No significant difference in maximum algal cell numbers (t= -1.457; df= 4; *P*= 0.166)	Supports hypothesis that genotype has a dominant influence on algal cell numbers
Cysts+ HU11 bacteria Versus Cysts+ HU11/DE08 bacteria	Significant difference in maximum algal cell numbers (t= 6.583; df= 4; *P*= 0.000)	Supports hypothesis that bacterial community has a dominant influence on algal cell numbers
Cysts+ DE08 bacteria Versus Cysts+ HU11/DE08 bacteria	Significant difference in maximum algal cell numbers (t= 8.040; df= 4; *P*= 0.000)	Supports hypothesis that bacterial community has a dominant influence on algal cell numbers

6.3.2 Bacterial growth dynamics in clonal and non-clonal *G. catenatum* cultures

Bacterial growth dynamics followed a similar pattern across clonal and non-clonal treatments. Progeny, non-clonal HU11 and DE08 cultures and their parent, clonal GCHU11 and GCDE08 cultures had $>10^{7}$ cells mL^{-1} on day 0 and reached $>10^{11}$ cells mL^{-1} on day 68. In contrast, progeny HU11/ DE08 mixed bacterial culture alone started with a higher bacterial cell concentration ($>10^{8}$ cells mL^{-1}) on day 0 and reached $>10^{11}$ cells mL^{-1} on day 68 (**Fig. 6.4**)

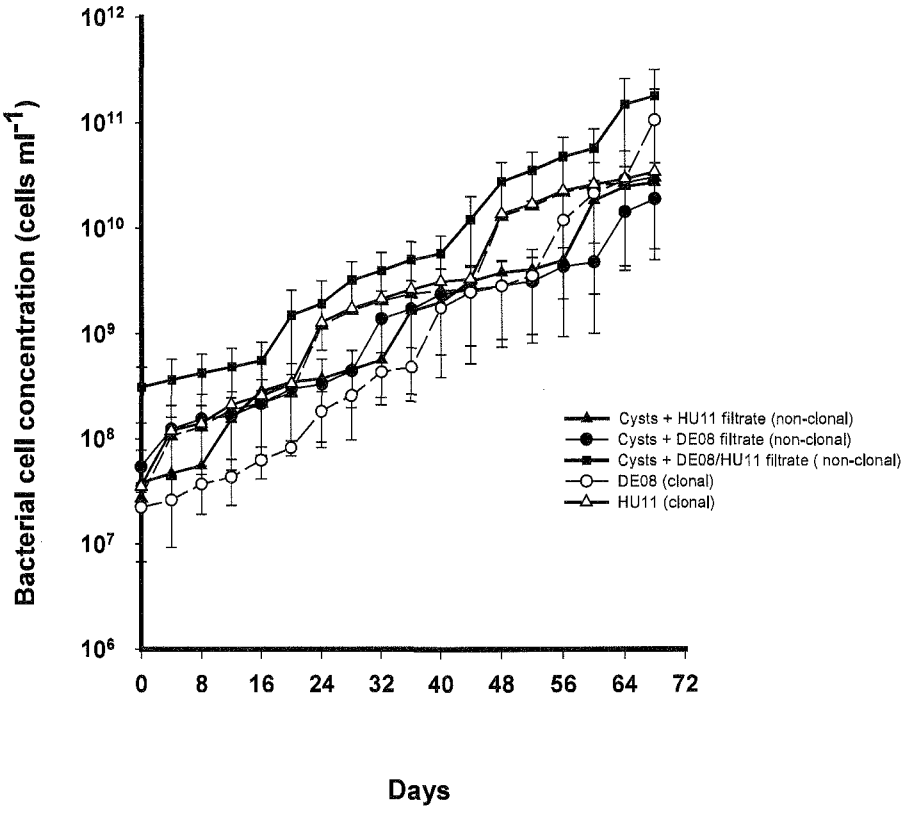

Fig. 6.4: Bacterial growth dynamics from replicate *G. catenatum* cultures (non-clonal progeny cultures) grown with bacterial communities from non-axenic parent cultures, parent GCHU11 and GCDE08 (clonal cultures) (± standard error).

6.3.3 t-RFLP analysis of bacterial communities

The t-RFLP fragment electropherograms from replicate culture treatments were highly reproducible. Representative examples of TRF patterns obtained from parent GCHU11, GCDE08 and progeny DE08 cultures are shown in **Fig. 6.5**; **Fig. 6.6** and **Fig. 6.7**. One replicate of the cysts + HU11/DE08 bacteria treatment produced a weak and noisy TRF trace for both the forward and reverse labeled TRFs. Scoring the fragments was difficult and unreliable therefore this replicate was not included in the cluster analysis.

T-RFLP profiles from clonal, GCHU11 and GCDE08 cultures showed that 16S rDNA patterns varied markedly between the parent cultures. *HhaI* digestion of GCHU11 (**Fig. 6.5**) cultures showed TRF's at 58, 60, 67, 69, 71, 79, 80, 84, 113, 115, 146, 192, 230 (bp) whereas GCDE08 cultures showed TRF's at 77, 118, 121, 137, 141, 151, 182, 210 (bp) (**Fig. 6.6**). Similarly *BfaI* digestion of GCHU11 cultures showed TRF's at 72, 73, 74, 81, 82, 112, 131, 143 (bp) while GCDE08 cultures showed TRF's at 70, 77, 126, 128, 133, 134, 155, 162 (bp)

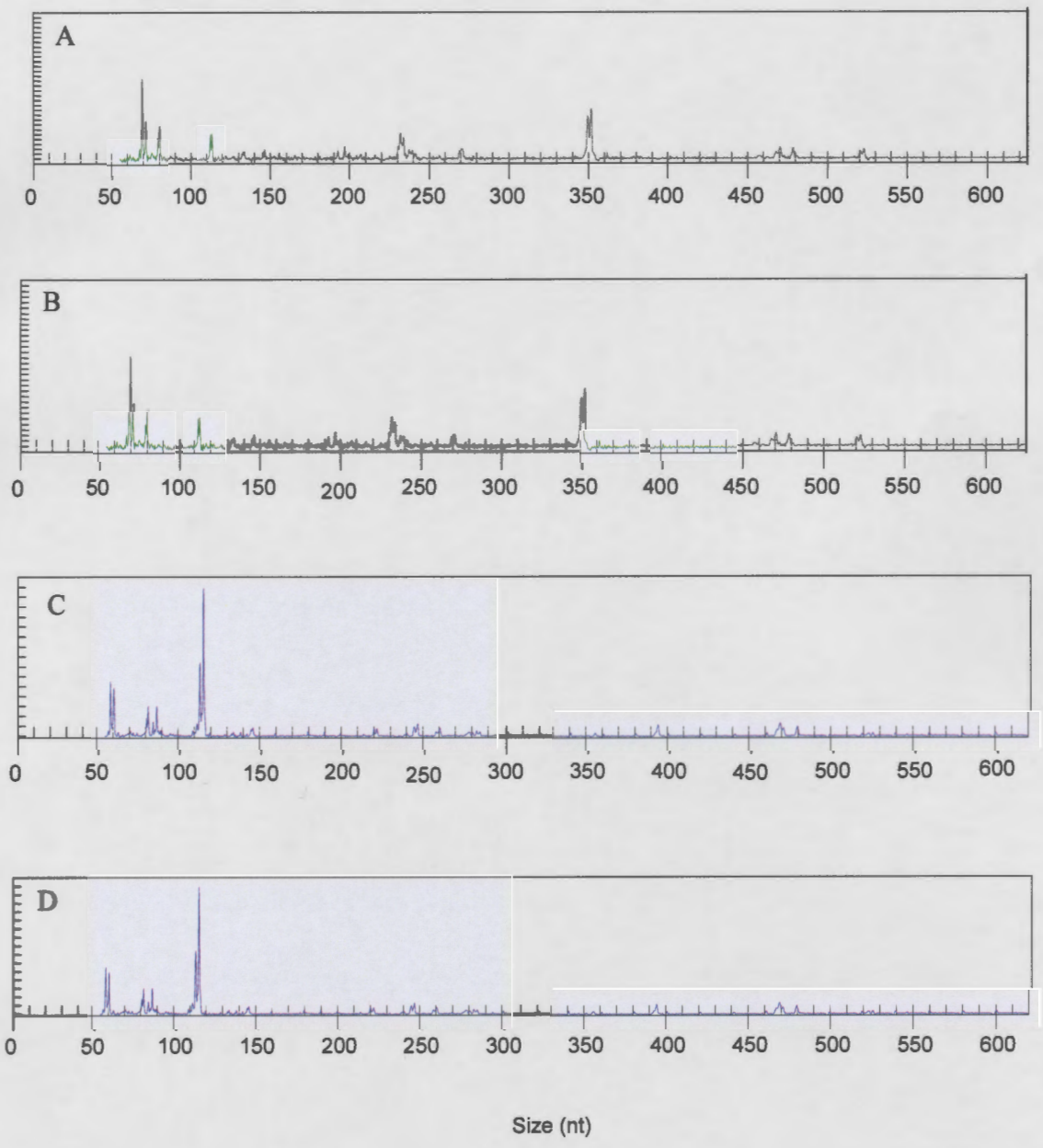

Fig. 6.5: t-RFLP traces from replicate cultures of GCHU11. A and B = Forward primer labeled (27F, dye D3) fragment traces from two independent replicate cultures. C and D = Reverse primer labeled (518R, dye D4) fragment traces from two independent replicate cultures. The PCR amplified 16S rRNA genes were digested with restriction enzyme *HhaI* and fluorescent labeled fragments were separated on CEQ ™ 8000 genetic analysis system. Fragment size in base pairs is shown on the x-axis.

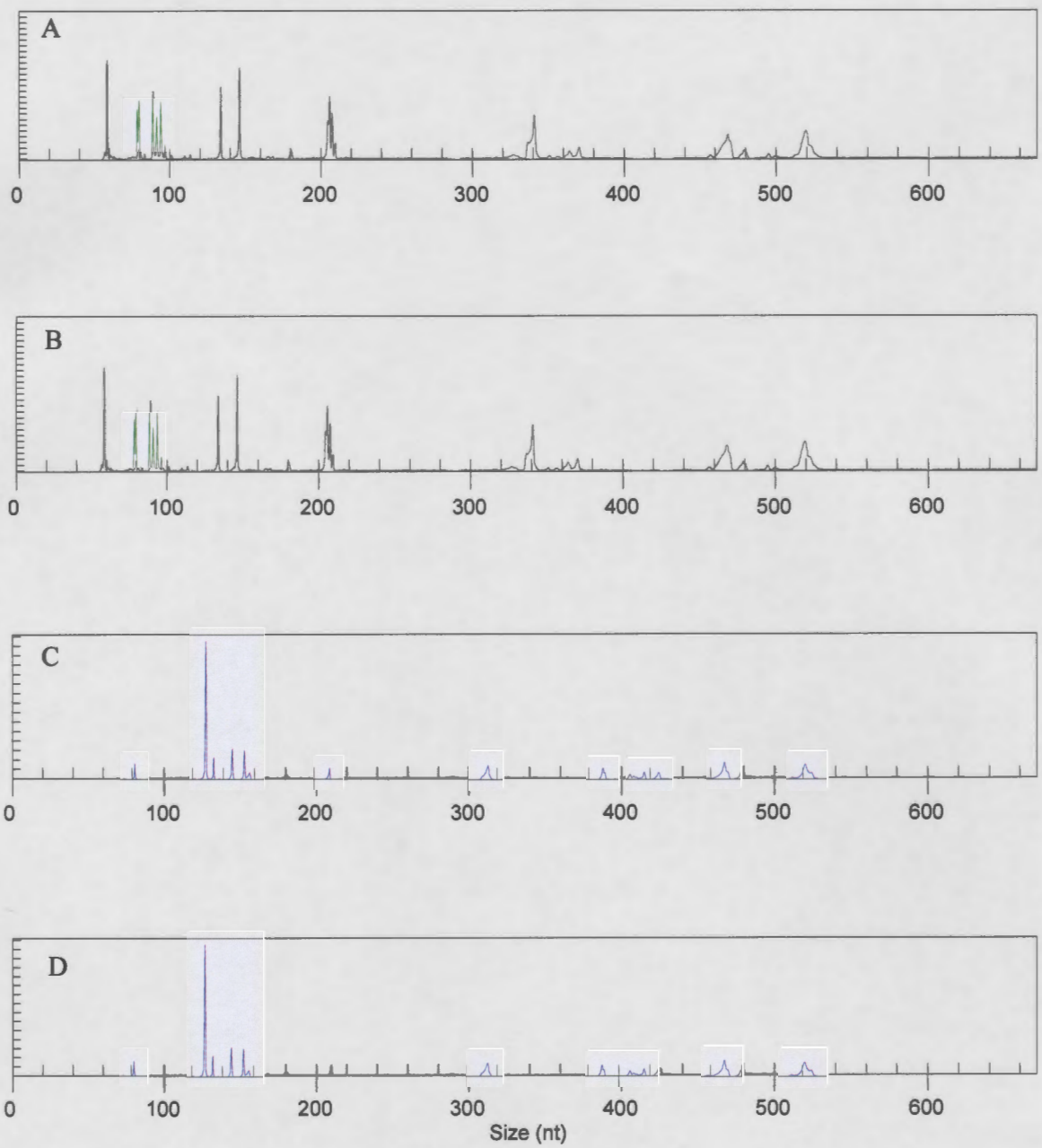

Fig. 6.6: t-RFLP traces from replicate cultures of GCDE08. A and B = Forward primer labeled (27F, dye D3) fragment traces from two independent replicate cultures. C and D = Reverse primer labeled (518R, dye D4) fragment traces from two independent replicate cultures. The PCR amplified 16S rRNA genes were digested with restriction enzyme *HhaI* and fluorescent labeled fragments were separated on CEQ ™ 8000 genetic analysis system. Fragment size in base pairs is shown on the x-axis.

Comparison of the t-RFLP patterns from non-clonal progeny cultures and parent cultures indicated that the non-clonal progeny cultures were more similar to each other than to either clonal parent culture. Non-clonal progeny treatments that received a GCHU11 bacterial community shared TRF's with treatments receiving the GCDE08 bacterial community and treatments receiving a mix of GCHU11 and GCDE08 bacteria, but TRF's from these treatments differed markedly from those of the parent cultures GCHU11 and GCDE08. For example, after cleavage with *Hha*I, all progeny cultures showed TRF's of 63, 79, 80, 83, 89, 91, 94, 97, 133, 146 (bp) that were absent from the parent cultures from which the bacterial community was substituted. Parent cultures and progeny cultures shared very few common TRF's (79, 80, 145 bp) with the majority of the TRF's in parent cultures being unique (67, 69, 71, 77, 112, 113, 118, 120 bp) and not present in any of the progeny cultures. Similarly, digestion with *Bfa*I also showed that progeny cultures shared more TRF's in common with each other than with either of the clonal parent cultures. For example, fragments 61, 67, 71, 77, 79, 117, 118, 119, 133, 134, 144, 174, 180 (bp) were common among progeny treatments but absent in both clonal parent cultures. Parent cultures possessed unique fragments of 73, 121, 126, 128, 132, 140, 143, 155, 156, 162, 201 (bp) and only 4 TRF's in common with progeny cultures (113, 133, 134, 145, 146 bp).

The t-RFLP approach using restriction enzymes *Hha*I and *Bfa*I both showed 1.8 to 2.1 times the number of TRF's from the non-clonal progeny cultures compared to the non-clonal parent cultures. *Hha*I cleaved 16S rDNA from non-clonal progeny cultures on an average showed 44.6 TRF's, while *Bfa*I digested 16S rDNA from progeny cultures averaged 43.7 TRF's. Clonal parent cultures GCHU11 and GCDE08 showed an average of 21.7 TRF's on digestion with *Hha*I and 24.5 TRF's on digestion with *Bfa*I.

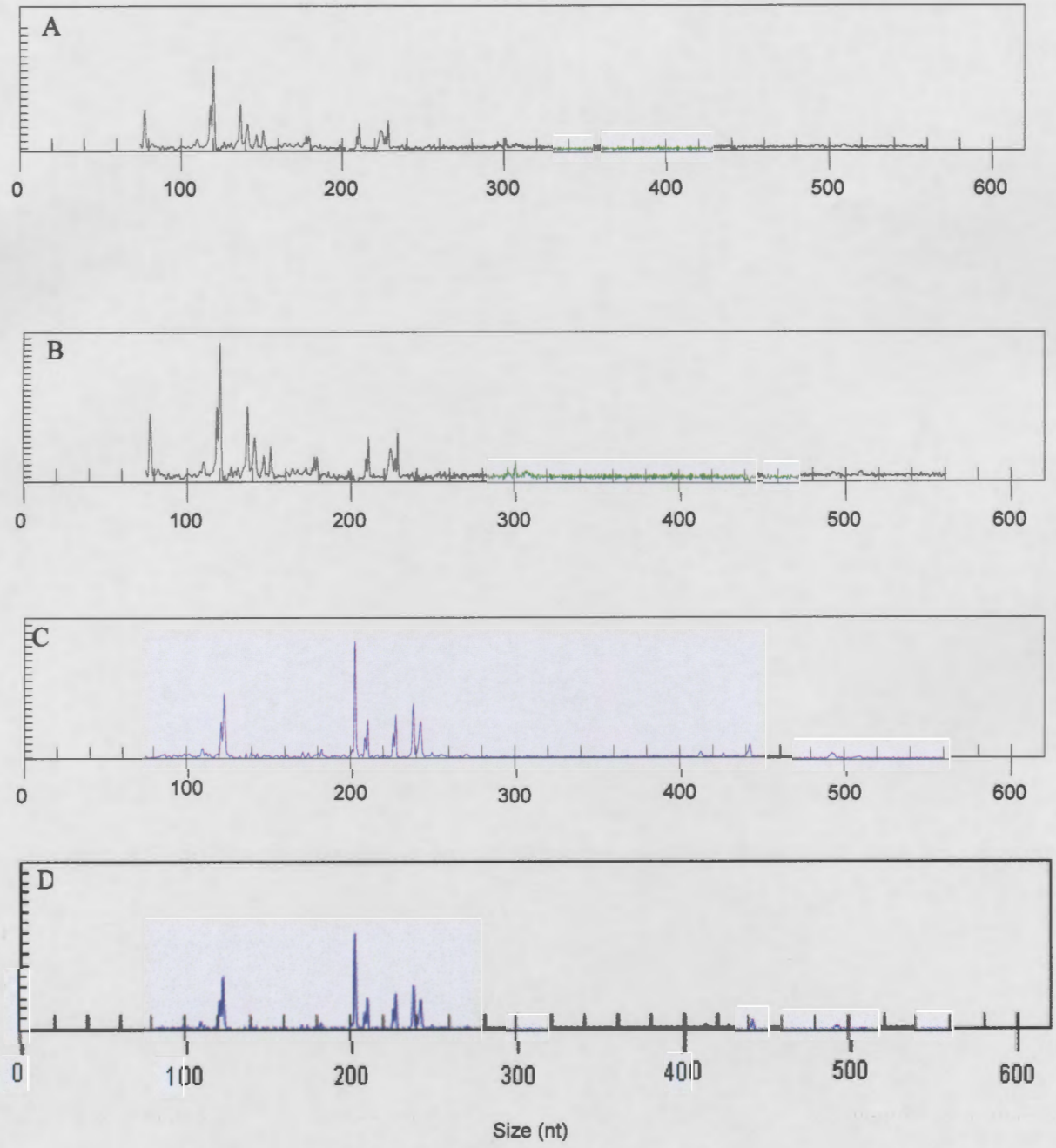

Fig. 6.7: t-RFLP traces from replicate progeny cultures of cysts + DE08 bacteria. A and B = Forward primer labeled (27F, dye D3) fragment traces from two independent replicate cultures. C and D = Reverse primer labeled (518R, dye D4) fragment traces from two independent replicate cultures. The PCR amplified 16S rRNA genes were digested with restriction enzyme *HhaI* and fluorescent labeled fragments were separated on CEQ ™ 8000 genetic analysis system. Fragment size in base pairs is shown on the x-axis.

The UPGMA cluster analysis **(Fig. 6.8)** of t-RFLP data clearly grouped the bacterial community of all progeny cultures together in one cluster. The replicate parent cultures are present as outliers to this main cluster. The consistency of t-RFLP patterns among replicate cultures is evident by the clustering of replicates together in the analysis.

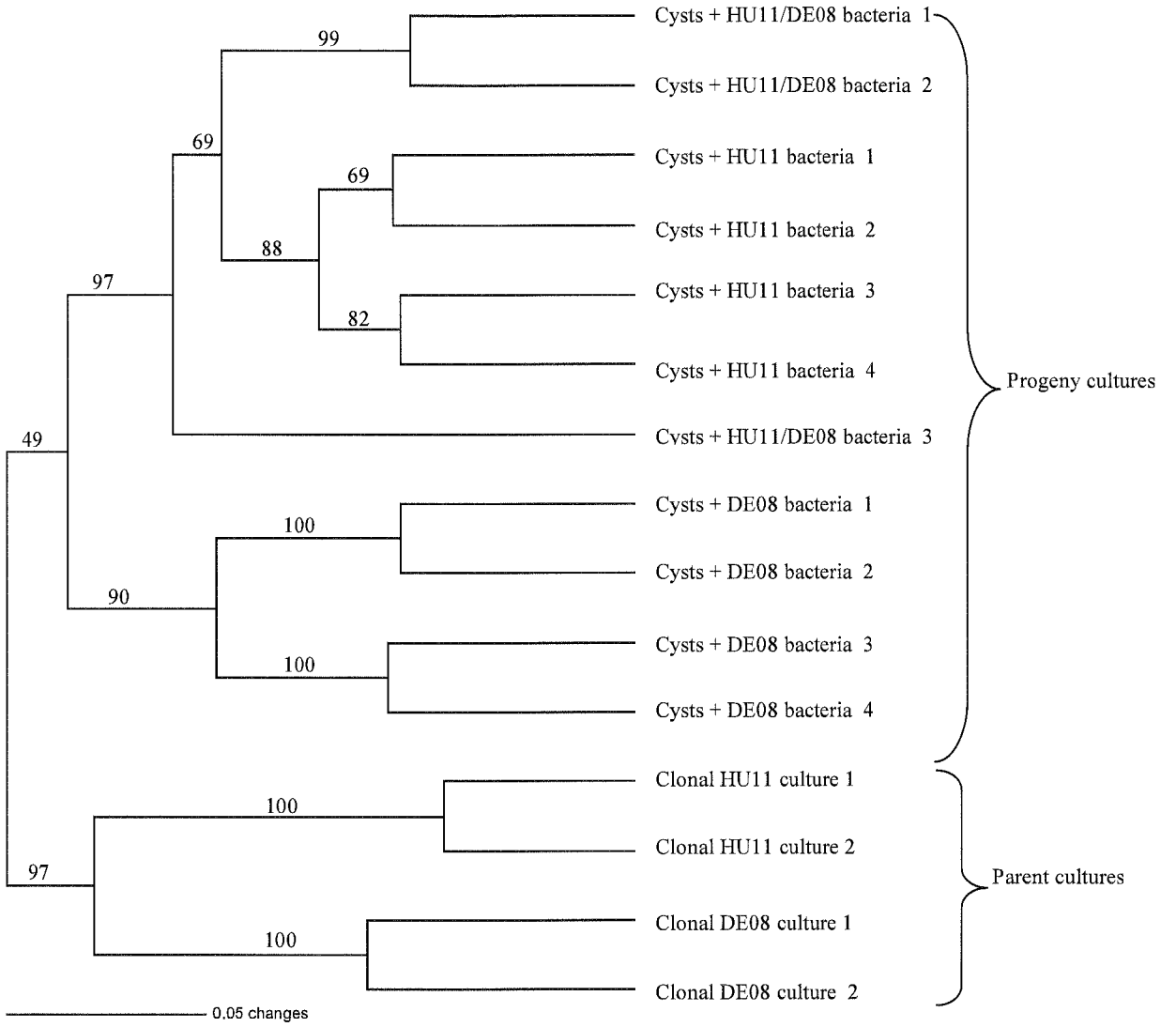

Fig. 6.8: UPGMA cluster analysis constructed from TRF presence/absence matrix of *BfaI* and *HhaI* cleaved 16SrDNA amplicons from non-clonal progeny cultures grown with either GCDE08 or GCHU11 bacterial communities, and clonal parent GCHU11 and GCDE08 cultures. The tree was constructed from PAUP mean distances and support for clusters assessed by bootstrap re-sampling with 1000 replicates.

6.4 DISCUSSION

This study is the first to systematically compare the influence of algal genotype with the influence of the bacterial community on algal cell growth dynamics. The working hypothesis for the chapter was that bacteria were the dominant source contributing markedly different batch culture dynamics between the non-clonal progeny cultures and parent clonal cultures GCHU11 and GCDE08. Using the *G. catenatum*- bacteria model, the experimental aim was to compare and generate a consistent genetic background composed of a mix of non-clonal progeny against which it would be possible to assess the influence of bacterial communities substituted from either or both the clonal parent cultures. If the bacterial community has a dominant influence, then the growth dynamics of the non-clonal progeny cultures receiving a GCDE08 bacterial community (cysts+DE08 bacteria) should closely match that of the clonal GCDE08 culture. Similarly growth dynamics of progeny cultures receiving GCHU11 bacteria should match that of the clonal GCHU11 culture. Assuming that the effect of the bacterial community on growth is approximately additive (as suggested in Chapter 4), then treatments receiving a mix of GCDE08 and GCHU11 bacteria might reasonably be expected to show an intermediate growth pattern.

In this experiment, the mixed-progeny cultures showed exponential growth rates and maximum cell densities intermediate between those of the clonal parents. While this effect is consistent with either genetic or bacterial influences on growth dynamics, there were notable exceptions among the maximum cell concentrations. All non-clonal progeny cultures exceeded the low maximum cell concentrations attained by clonal HU11 cultures despite the bacterial community added to the germinating cysts at the beginning of the experiment.

One possible explanation for the similarity of growth dynamics among the non-clonal progeny is that these cultures are effectively populations of multiple related genotypes; equivalent to a collection of "brother/sisters" for a single set of parents. Under the culture conditions used here, this "population" may be subject to selection of individuals, making the non-clonal cultures

more able to adapt to culture conditions than the single clonal parent cultures GCDE08 and GCHU11 (Bulmer 1980). In effect, the overall growth response approximates the average response of the population whereas the parent cultures are constrained and potentially extreme response of single individuals under the conditions used in the experiment.

The planned contrast analysis of the batch culture dynamics presented here showed that non-clonal progeny cultures exhibited growth patterns more similar to each other than either clonal parent cultures regardless of the bacterial community added to the resting cysts prior to germination. Given the working hypothesis, this supports the hypothesis that dinoflagellate cell genotype is the dominant factor responsible for the different growth dynamics of the *G. catenatum* strains GCDE08 and GCHU11 used in this experiment.

The t-RFLP analysis of non-clonal progeny cultures provides a means to compare the bacterial community composition established in each of the treatments. This approach proved to be a highly discriminatory and powerful tool that could clearly discriminate the bacterial communities of both clonal parent cultures and establish whether the same or similar communities were established in the non-clonal progeny. The t-RFLP analysis clearly showed that, despite the addition of either GCHU11 or GCDE08 or a mixture at the time of germination, the community composition of the established non-clonal progeny cultures were consistently more similar to each other than either parent culture (see **Fig. 6.8**), and also more complex (more t-RFLP fragments) than either parent culture. The similarity of growth patterns among the non-clonal progeny can now be seen largely as a function of the bacterial community associated with each culture. This finding supports earlier work presented in this thesis (Chapters 2-4), that the bacterial community has a major influence on the growth dynamics of *G. catenatum*. However, this finding forces a reinterpretation of the algal cell growth dynamics data.

The more complex TRF patterns obtained from the bacterial community associated with all the non-clonal progeny cultures indicates a more diverse bacterial composition in non-clonal

cultures than in clonal parent cultures. This suggests that a low level bacterial community may have survived the initial cyst-sterilisation when progeny cultures were originally established. In this experiment, sub-samples of the sterile cyst suspensions were routinely plated onto marine agar at establishment and no bacteria were detected at this time, indicating that the external bacterial community had been removed by surface-sterilisation. However, it is possible that bacteria have survived treatment within the resting cyst and contributed to the community of bacteria established by addition of GCDE08 and/or GCHU11 bacteria. As additionally as much as 95% of marine bacterial strains are considered to be uncultivable (Schut *et al.* 1993), it is possible that internal or external bacteria that survived the sterilization process were not detected by growth on marine agar.

The establishment of a more diverse bacterial community in progeny cultures may make a wider range of bacterial metabolites readily available for algal growth. Grossart & Simon (2007) reported algal growth to greatly depend on the presence of bacterial communities. Bacterial exudates such as re-mineralised nutrients (Golterman 1972), vitamins (Croft *et al.* 2005), phospholipids (Kimura & Ishida 1989), glycopeptides (Riquelme *et al.* 1988) and other growth factors are well-known to stimulate algal growth. Bacterial community associated with phytoplankton and diatoms species have been documented by various authors using phylogenetic studies (Schafer *et al.* 2002; Green *et al.* 2004; Jasti *et al.* 2005). All these studies have revealed different phytoplankton species to have highly specific associations with different bacterial species. For example, Jasti *et al.* (2005) reported bacterial community to differ among non-toxic phytoplankton and *Alexandrium* sp. cultures. Accordingly, *S. costatum*, *Thalassiosira gravida*, *N. granulate*, *Prorocentrum minimum* shared no bacterial isolates with *Alexandrium* sp. cultures.

The establishment of remarkably similar bacterial communities, in the progeny cultures (as evidenced by the t-RFLP data), regardless of which parent community was added at germination, is surprising and difficult to explain. One explanation is that the dinoflagellate genotype may play a role in selecting the bacterial community. Microalgae exude a wide range of complex organic carbon molecules during growth (lipids, sugars, polysacharrides, fatty acids, sterols etc) that are

utilised by the marine bacterial community (Bell & Mitchell 1972). For example, *G. catenatum* is rich in fatty acids, sterols, lipids and oils (Hallegraeff *et al.* 1991) and may select or favour growth of bacteria capable of utilizing complex carbohydrates and hydrocarbons (e.g. *Marinobacter* sp. and *Alcanivorax* sp.; Green *et al.* 2004). On the other hand algae may also require specific bacterial products and hence may only support the growth of bacteria releasing such products. For e.g vitamins (Haines & Guillard 1974), chelated iron from siderophores (Soria-Dengg *et al.* 2001) or cytokinins (Maruyama *et al.* 1986).

The differing bacterial communities associated with different strains of microalgae have traditionally been considered a combination of artifacts of isolation (e.g. the random subset of co-contaminants present after isolation) and selection by the growth medium and culture environment (Jasti *et al.* 2005). However, it is also possible that different dinoflagellate genotypes may express some metabolic activities and pathways at different levels, leading to subtle differences in the mixture of metabolic by products exuded into the growth medium. This may provide substrate-selective conditions for the bacterial growth, leading to establishment of different bacterial communities with different dinoflagellate genotypes. In this experiment, the mixed genotype progeny represent an "average" of the two parent genotypes. A similar "average" organic carbon profile exuded into the growth medium by these cultures, compared to the more divergent single genotype cultures of the parents, provides a substrate selection mechanism by which different dinoflagellate genotypes could select different bacterial communities.

In conclusion, this chapter reinforces the importance of the bacterial community composition as a major factor influencing dinoflagellate growth dynamics in laboratory cultures. The data presented, indicate that variation in growth dynamics among different *G. catenatum* strains is likely a result of interaction with or modulation by the bacterial community. The potential interaction of genotypic selection of bacterial communities provides further evidence of the dynamic complexity of interactions among phytoplankton cells and the bacterial community.

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Chapter 7

Summary and conclusion

Global expansion, frequent episodes and economic impacts of harmful algal blooms demand deeper knowledge and understanding of factors influencing algal growth (Landsberg 2002). Both biotic and abiotic factors are believed to control HAB's (Barlaan *et al.* 2007). Among biotic factors bacterial interactions with algae have been widely cited as a potential factor controlling phytoplankton community.

Studies of interactions among algae and bacteria are currently hampered by the complexity and diversity of the bacterial community associated with algal cells and populations. The simplified *Gymnodinium catenatum* experimental model of bacteria–phytoplankton interactions removes this complexity and simplifies studies on influence of specific bacteria on growth of the dinoflagellate. The range of experiments presented in Chapters 2 to 6 demonstrate the flexibility of the experimental model as a tool to address specific hypotheses relating to the interaction of *Gymnodinium catenatum* with marine bacteria. Using the model, this thesis has shown that marine bacteria are essential to the growth of the dinoflagellate and that the compositon of the bacterial community has a direct and significant effect on the growth rate and dynamics of *Gymnodinium catenatum* in laboratory cultures. Furthermore, the bacterial community appears a more important factor than any variation associated with the individuals' genotype of the

dinoflagellate. There is ample evidence that similar interactions can influence natural dinoflagellate (and other algal) populations (Keshtacher-Liebson *et al.* 1995; Doucette *et al.* 1998; Kim *et al.* 1998; Hold *et al.* 2001), thus providing a mechanism driving the observed coupling of algal and bacterial populations in the world's oceans. The specific outcomes and implications of each experimental chapter are summarised in the following sections.

7.1 *G. catenatum* requires bacteria for growth

Although bacterial interactions with algae are believed to affect phytoplankton growth, studies have reported removal of bacteria with antibiotics to have less or no effect on growth of dinoflagellate cultures (Ho *et al.* 2006; Uribe & Espejo 2003). To investigate whether the presence of bacterial community is essential for growth of *G. catenatum*, surface-sterile *G. catenatum* cysts were germinated in the presence of antibiotic sensitive or resistant *Brachybacterium* sp. or *Marinobacter* sp. treated with antibiotics and examined both bacterial and algal growth dynamics. The removal of antibiotic-sensitive bacteria with antibiotics significantly reduced *G. catenatum* cell numbers while cultures grown with antibiotic resistant bacteria were unaffected by antibiotics, demonstrating that the reduction in *G. catenatum* was not due to antibiotic toxicity. The results confirmed that *G. catenatum* has an obligatory requirement for marine bacteria to support growth in the laboratory.

7.2 Breakdown of bacteria- dinoflagellate interaction in *G. catenatum* cultures

Bacterial influence controlling phytoplankton dynamics is well-known (Mouget *et al.* 1995; Sakami *et al.* 1999; Ho *et al.* 2006; Grossart & Simon 2007), while cell death or mortality in algal cultures still needs more attention (Sheldrake 1974). Rapid decline in algal cell numbers does occur in a healthy environment where growth promoting bacteria and other nutrients are available, however not many studies have drawn attention towards exploring such incidents (Fogg & Thake 1987; Usup & Azanza 1988; Heiskanen 1993). *G. catenatum* cultures grown with *Brachybacterium*

sp. or *Marinobacter* sp. showed reduced dinoflagellate cell concentrations after several months of sub-culturing suggesting that the bacterial-dinoflagellate relationship may have degraded over time. To test this hypothesis, "failing bacteria" were replaced with "fresh bacteria" and subsequent growth was studied. The addition of "fresh bacteria" appeared to stimulate algal growth initially, however, the "rescued" cultures scaled up to 100ml volumes gradually declined even in the presence of fresh bacteria, suggesting a gradual loss in algal growth stimulating activity by the bacteria over several months.

7.3 Bacterial community composition influences *G. catenatum* growth dynamics.

Interactions between algae and bacteria are highly specific and stimulation or inhibition of algal growth may vary depending on the associated bacterial taxa (Doucette *et al.* 1998). This study also showed bacteria isolated from *G. catenatum* cultures to have varied responses on the growth of the phytoplankton. *Marinobacter* sp. DG879, *Alcanivorax* sp. DG881 and *Roseobacter* sp. DG874 showed distinct differences in algal growth patterns with varying onset and duration of growth phases. *Marinobacter* sp. and *Alcanivorax* sp. supported growth to a greater extent comparing *Roseobacter* sp. *Roseobacter* sp. also caused a rapid decline in algal cell numbers. These results, indicate that bacterial community composition has a strong influence on *G. catenatum* growth. The growth dynamics seen in cultures grown with "synthetic communities" composed of three bacterial types were similar to that of cultlures grown with a typical mixed bacterial community, indicating that the simplified experimental model provides a good approximation of bacteria and phytoplankton interactions of more complex bacterial communities associated with natural populations.

7.4 The physical association of bacteria to *G. catenatum* cells.

Chemotactic responses of bacteria to algal exudates may draw bacteria closer to algal cells resulting in strong physical attachment to external membranes of algal cells (Bell & Mitchell 1972; Kogure *et al.* 1982; Albright *et al.* 1986). Ageing algal cultures generally release higher amount of

exudates (Fogg 1983) which suggests bacteria may become closely attached during stationary or death phase. This study supports the above suggestions as *G. catenatum* cultures showed bacterial attachment only during the death phase of algal cultures. *Roseobacter* sp. showed higher percentage of attachment to *G. catenatum* cells compared to *Alcanivorax* sp. or *Marinobacter* sp.. The rapid decline of *G. catenatum* cell concentration during death phase when grown with *Roseobacter* sp. (Chapter 4) may be related to direct algicidal attack by *Roseobacter* sp., perhaps through changes in bacterial metabolism induced by quorum-sensing.

7.5 *G. catenatum* growth: Is genotype or bacterial composition a dominant factor?

This thesis and earlier studies have established that algal growth is significantly influenced by the associated bacterial community. However, whether this influence is more or less important than genotypic variation is not clear. The *G. catenatum* experimental model was used in this chapter to examine the growth response of equivalent non-clonal cultures established from resting cysts. Cysts germinated in the presence of bacterial community from either of the parent cultures showed growth patterns more similar to each other than to either parent clonal culture, regardless of the bacterial community added during culture establishment. While the culture dynamics suggested genotype as the dominant influence, a molecular analysis of the bacterial communities of the cultures (using tRFLP) showed that the bacterial communities established with the non-clonal progeny cultures were more similar to each other than to either parent culture. The combined data supports the hypothesis that the bacterial community is the primary influence on culture dynamics and growth. The establishment of similar communities in progeny cultures suggests that the algal genotype (or mix of genotypes) may select for particular bacterial community structure possibly through subtle differences in metabolism that affect the type and amount of of complex carbon released by the dinoflagellate cells.

7.6 Key aspects for future research

To improve the existing knowledge regarding bacterial effects on harmful algal species the following key aspects of the complex interaction needs to be addressed.

1. Using simplified experimental models, investigate whether the requirement for marine bacteria is widespread among dinoflagellates.

   Currently, only a few dinoflagellates have been reported to have an obligate requirement for marine bacteria. The current model could be applied for other dinoflagellate species to identify similar requirement for marine bacteria. This will widen existing knowledge on mechanism of bacteria-algae interactions. Further, it would be interesting to determine whether dinoflagellate requirement for bacteria is linked to the bio-availability of micro-nutrients such as iron, other trace metals, or vitamins. With such models it is easier to control and manipulate both physical and biological factors influencing dinoflagellate growth in laboratory cultures than in natural environment reducing the complexity associated with similar studies.

2. Other bacterial types associated with *G. catenatum* cultures need to be tested for growth stimulating activity.

   This study has established experimental models based on few bacterial types that have already been identified to influence *G. catenatum* growth. However, other bacteria associated with *G. catenatum* should be studied for growth promoting activity to determine specific characteristic features of bacteria enhancing growth. This study also identified *Brachybacterium* sp. to support growth which has not been reported in association with algal cells till date. Further studies could include *Brachybacterium* sp. as a possible growth supporting bacteria and identify biochemical factors enhancing interaction. Further this

model can also be developed to include toxicity studies.

3. Does bacterial dominance change during different stages of growth?

This study has examined changes in the spatial distribution of bacteria with respect to algal cell. Future work could develop primers or probes for use in real-time PCR and *in situ* hybridization techniques to precisely locate and determine relative abundance of bacterial cells in the dinoflagellate environment. This would give clues to the mechanisms underlying growth stimulation. Probes could also be developed to identify bacterial types dominating various growth phases in relation to proximity to the dinoflagellate cell. This would deepen knowledge on whether contact mediated factors are important for the interaction between bacteria and algae.

4. Is it possible to interrupt the interaction?

G. catenatum-bacteria model is a flexible and tractable model for examining algal bacterial interactions. Future studies could focus on physiological responses of these interactions and the influence on growth dynamics at biochemical and molecular level to determine if these interactions could be interrupted and provide a means to control growth of specific dinoflagellate species. Finally samples from natural environment could be modified similar to our models to identify if the developed models approximate interactions in the natural environment. Using our models, it is possible to identify key bacterial types that may be algicidal. With this effort quantitative methods for using algicidal bacteria in controlling algal populations could be developed.

7.7 Conclusions

This thesis confirms the need for growth stimulating bacteria in *G. catenatum* cultures and the bacterial composition to have a strong influence on algal growth dynamics. Further the simplified experimental models approximate phytoplankton responses to more complex bacterial communities.

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Medium GSe Preparation method

1. Sea water

Autoclave filtered seawater in 1000 mL Teflon bottles.

2. Distilled water

Autoclave distilled water to sterilise.

3. Stock Solution

1. KNO_3	100.0 gL^{-1} H_2O
2. K_2HPO_4	34.8 gL^{-1} H_2O
3. Vitamins	
Biotin	0.2 mg 100 mgL^{-1} H_2O
Vitamin B_{12}	0.1 mg 100 mgL^{-1} H_2O
Thiamine HCl	100.0 mg 100 mL^{-1} H_2O
4. PII Metal Mix	
Na_2 EDTA	6.0 gL^{-1} H_2O
$FeCl_3$. 6 H_2O	0.29 gL^{-1} H_2O
H_3BO_3	6.85 gL^{-1} H_2O
$MnCl_2$.4 H_2O	0.86 gL^{-1} H_2O
$ZnCl_2$	0.06 gL^{-1} H_2O
$CoCl_2$. 6 H_2O	0.026 gL^{-1} H_2O
5. Selenium H_2SeO_3	1.29 mg L^{-1} H_2O

Make up each stock solution separately and add (adjust pH to 7.8-8.0 with NaOH)

4. Nutrient solution

To prepare Gse medium, solution of nutrients is made up for 100 mL mix.

Nitrate stock	20 mL
Phosphate stock	10 mL
Vitamin stock	10 mL
PII Metal Mix	50 mL
Selenium stock	10 mL

Make up to 200mL with distilled water.

Nutrient solution was autoclaved in Schott, then filter sterilised using 0.22 μm filter.

5. To prepare final GSe medium

In sterile 1 litre Teflon bottle the following were added.

900 mL sterile filtered sea water (1)
100 mL sterile distilled water
20 mL nutrient solution (4)

Modification of GSe medium

GSe medium without extracted soil was used in culture flasks. GSe –N-P was also used in this study that contains same amount of nutrients but without nitrate and phosphate.

Bacterial Agar

Zobell marine agar (ZM1)

5g Bacto Peptone
1g Yeast extract (Difco)
15g Bacto-Agar (omit for broth)
750 mL 0.2 μm filtered sea water
250 mL MilliQ water

* For *Alcanivorax* sp. and *Brachybacterium* sp. add 1% Na- acetate

5 mL 100 x Marine Supplement (see below)

Zobell Marine Agar (ZM10)

0.5 g Bacto Peptone
0.1 g Yeast extract (Difco)
15 g Bacto-Agar (omit for broth)
750 mL 0.2 μm filtered sea water
250 mL MilliQ water

* For *Alcanivorax* sp., and *Brachybacterium* sp. add 1% Na- acetate

5 mL 100 x Marine Supplement (see below)

100 x Marine Supplement

1 mL 10 x Trace elements
10 mL 2 mgL^{-1} Na_2SeO_3
84 mL Milli Q water

Prepare and autoclove the above stock. Once cool add 5 mL 0.2 μm filter sterile 2 x vitamin stock. Store in the dark at 4°C

10 x Trace elements (100 mL)

4.36 g Na_2EDTA

3.15 g $FeCl_3$. 6 H_2O

0.022 g $ZnSO_4$.7 H_2O

0.01 g $CoCl_2$. 6 H_2O

0.18 g $MnCl_2$. 4 H_2O

0.0063 g Na_2MoO_4. 2 H_2O

Combine and filter sterilise. Store in the dark at 4°C

2 x Vitamin mixture (100 mL)

0.0005 g Cyanocobalamin (B_{12})

0.1 g Thiamine HCl (B_1)

0.0005 g Biotin

Combine and filter sterilise. Store in the dark at 4°C

Bacterial Genomic DNA isolation from *G. catenatum* cultures.

Lysis Buffer

100 mM Tris –HCl pH 8.0
150 mM NaCl
10 mM EDTA

Lysozyme

50 mg mL^{-1} in Lysis Buffer

Add lysozyme to a sterile 15mL tube and dissolve in the appropriate amount of sterile lysis buffer. 0.2 μm filter sterilise and dispense 500-1000 μl volumes and store at -20°C. Stock can be freeze/thawed a couple of before it needs to be chucked.

CTAB/NaCl

0.7 M NaCl
10% CTAB

Prepare stock by adding 4.1 g NaCl to a 200 mL bottle and dissolve in 80 mL MQ-dH_2O. Add a magnetic stir-bar and autoclave. While the solution is still hot or pre-heat to *ca.* 60-70°C, progressively add small quantities of the 10 g CTAB (*ca.* 1 g) to the solution with stirring and heating to dissolve. Autoclave. Warm to *ca.* 60°C before use, as it is very viscous.

1. Pellet cells from 1-5 mL *G. catenatum* culture by centrifugation at 13,000 x rpm for 15-20 s in a sterile 1.5 mL centrifuge tube. Immediately remove as much of the supernatant as possible. Repeat to pellet all cells.
2. Resuspend the cell pellet in 470 μl of Lysis buffer. Add *ca.* 52 μl 50 mg mL^{-1} lysozyme in lysis buffer. Mix and incubate for 30 min at 37°C.
3. Add 28 μl 10% SDS (0.5% final concentration) and 10 μl 20 mg mL^{-1} Proteinase K (in 10mM Tris-HCl, pH 8.0; 374 μg mL^{-1}). Mix and incubate at 56°C for 30 min.
4. Add 70 μl 5M NaCl and mix thoroughly (to make solution 0.7 M NaCl final concentration). Add 70 μl CTAB/NaCl solution (to 1% CTAB). Mix and incubate at 65°C for 10 min. Final volume at this point should be 700 μl.

5. Add an equal volume of Chloroform:Isoamyl alcohol (24:1), mix by repeated inversion for 30 s and centrifuge for 5 min at 13,000 x rpm.
6. Remove the aqueous phase to a new tube (the interface can be removed with a toothpick if it interferes with removal of the aqueous phase).
7. Add an equal volume of Phenol:Chloroform:Isoamyl alcohol (25:24:1), mix by 30 s inversion and centrifuge for 5 min.
8. Remove the aqueous phase to a new tube and add 0..6 vol. 100% isopropanol alcohol and mix by inversion and incubate at room temperature for 15-30 mins. Collect precipitate by centrifugation at 13,000 x rpm for 15 mins. Wash once with 70% ethanol.
9. Resuspend DNA in dH_20 at approximate 50-150 µl. Store at -20°C.
   Quantitative DNA by fluorometry
10. For the PCR, use 1 µl per 50 µl reaction with 2.0mM Mg^{2+}.

CTAB DNA extraction protocol for bacterial genomic DNA (1.5 mL)

Reagents

1. 1 x TE buffer
   10 mM Tris- HCl pH 8.0
   1 mM EDTA
2. 10 % SDS
3. 20 mg mL^{-1} Proteinase K
   Store at -20 °C
4. 5 M NaCl
5. CTAB/NaCl
   0.7 M NaCl
   10% CTAB

Prepare stock by adding 4.1 g NaCl to a 200 mL bottle and dissolve in 80 mL MQ-dH_2O. Add a magnetic stir-bar and autoclave. While the solution is still hot or pre-heat to *ca.* 60-70°C, progressively add small quantities of the 10 g CTAB (*ca.* 1 g) to the solution with stirring and heating to dissolve. Autoclave. Warm to *ca.* 60°C before use, as it is very viscous.

6. Chloroform: Isoamyl alcohol (24:1)
7. Tris buffered phenol. Store in the dark at 4°C
   * Buffered phenol more than 2months old can damage DNA
8. 100% isopropanol alcohol. Store at -20°C
9. 70% Ethanol. Store at -20°C

Method

1. Label tubes and add 567 µL 1x TE Buffer. Using an inoculating loop under sterile conditions (laminar flow chamber, flame, etc.) remove a good streak of cell material from agar and suspend in TE buffer. Vortex suspensions thoroughly until no 'clumps' can be seen in the solution.
2. Add 30 µL 10% SDS and mix thoroughly by shaking. Add 3 µl 20mg mL^{-1} Proteinase K. Mix thoroughly by shaking, incubate at 56 °C for 60 min.
3. Add 100 µL 5M NaCl and mix thoroughly. Add 80 µl CTAB/NaCl solution, mix by shaking and incubate at 65 °C for 30 min.

----------Perform steps 5-9 in FUMEHOOD----------

4. Add an equal volume of Chloroform:Isoamyl alcohol (24:1) (should be about 780 µL), mix thoroughly, holding cap on with finger, and centrifuge for 5 min at 13,000 rpm.
5. Remove the aqueous phase (should be the top layer) to a new tube making sure to get none of the interface or organic phase.
6. Add an equal volume of tris buffered phenol to the aqueous phase, mix thoroughly, holding cap on with finger, and centrifuge for 5 min at 13,000 rpm.
7. Remove the aqueous phase (should be the top layer) to a new tube making sure to get none of the interface or organic phase.
   Repeat step 4.

.....

8. Remove the aqueous phase to a new tube and add 1:1 volume cold isopropanol alcohol and mix thoroughly by repeated inversion. A visible precipitate should for. Centrifuge to 15 min at 13,000 rpm.
9. Remove supernatant by tipping out and wash pellet by adding 400 µL 70% ethanol, making sure not to dislodge pellet. Centrifuge for 5 min at 13,000 rpm.
10. Remove supernatant. Resuspend DNA in 50-200 µL TE buffer.

Store at - 20°C. Extract can then be quantified for concentration or dilute approximately 1:10 with MQ H_2O; 2.5-5 µL of dilute should be used in a 25 µL.

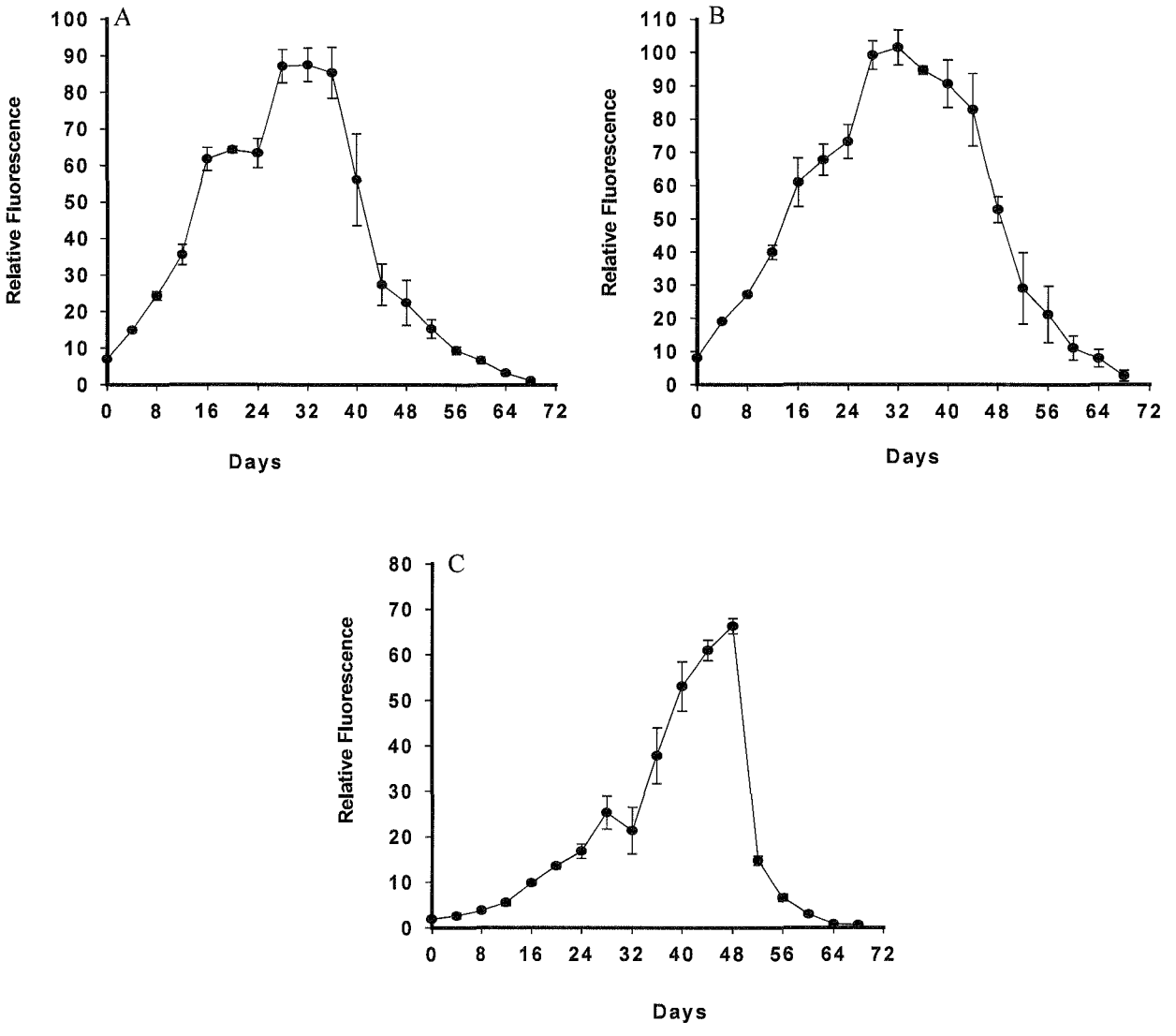

Fig. 1: *In-vivo* chlorophyll fluorescence of *G. catenatum* cultures grown with single bacterial types (A) *Marinobacter* sp. DG879 (B) *Alcanivorax* sp. DG881 (C) *Roseobacter sp.* DG 874. (± standard error). Growth curves obtained from *in-vivo* fluorescence and dinoflagllate cell count data appeared similar (**Fig. 4.4**)

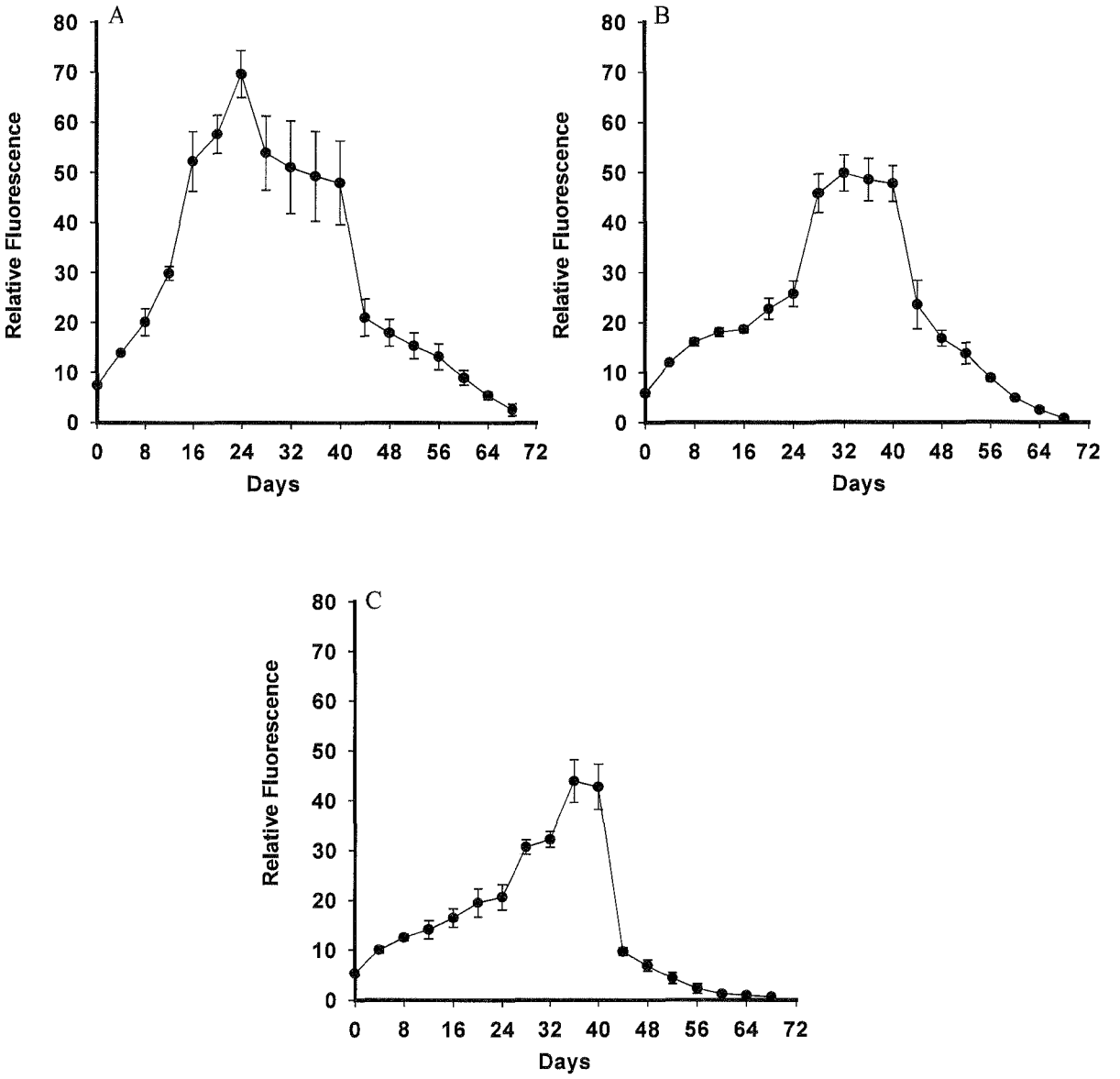

Fig. 2: *In-vivo* chlorophyll fluorescence of *G. catenatum* cultures grown with pair-wise combination of bacteria. (A) *Alcanivorax* sp. DG881 and *Marinobacter* sp. DG879 (B) *Alcanivorax* sp. DG881 and *Roseobacter* sp. DG874 (C) *Marinobacter* sp. DG879 and *Roseobacter* sp. DG874. (± standard error). Growth curves obtained from *in-vivo* fluorescence and dinoflagllate cell count data appeared similar (**Fig. 4.8**)

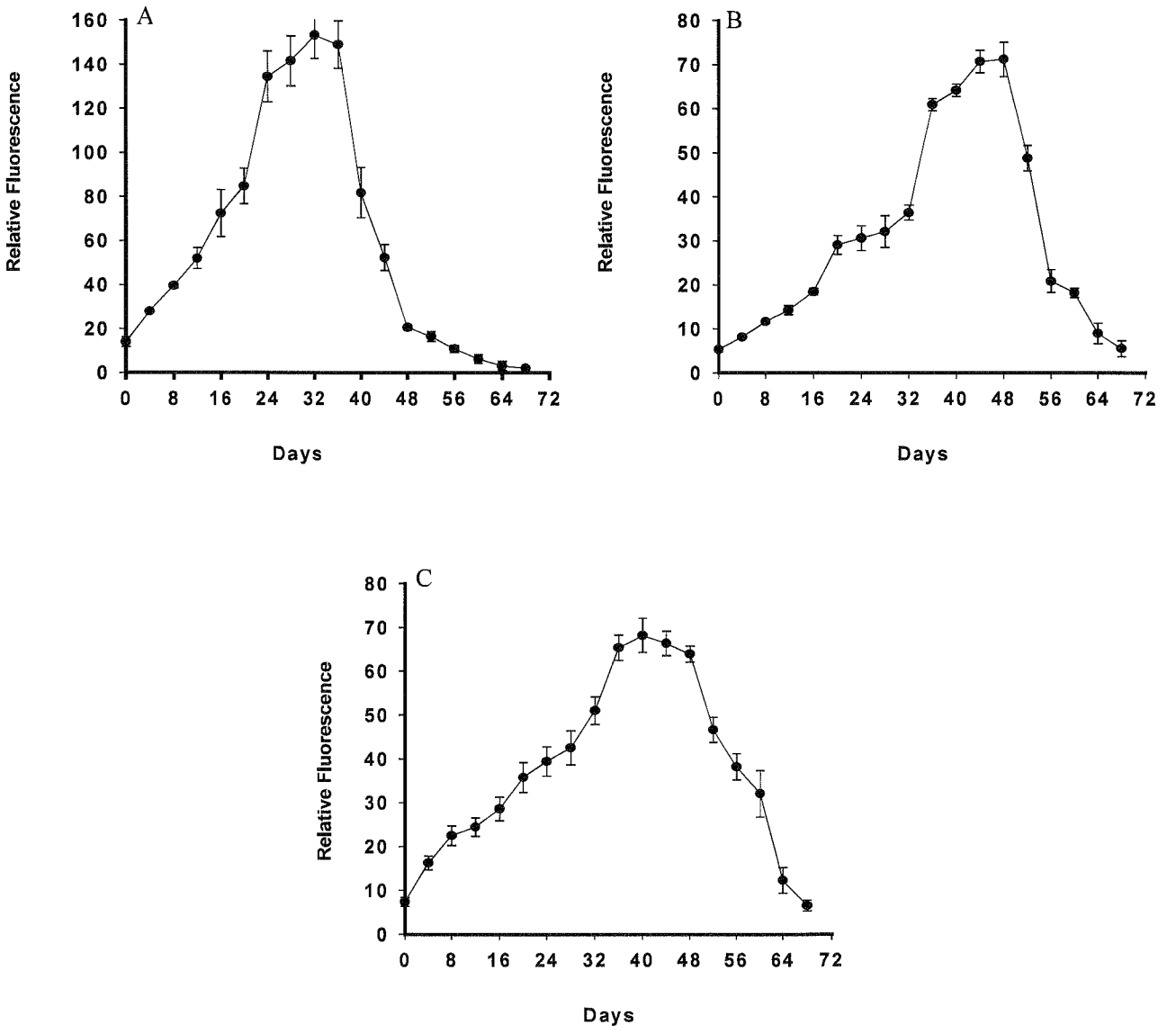

Fig. 3: *In-vivo* chlorophyll fluorescence of mixed *G. catenatum* grown with (a) synthetic communities of all three bacteria; (b) mixed bacterial communities from non axenic GCHU11/GCDE08 cultures (c) parent GCHU11 and GCDE08. (± standard error). Growth curves obtained from *in-vivo* fluorescence and dinoflagllate cell count data appeared similar (**Fig. 4.9; Fig. 4.10**)

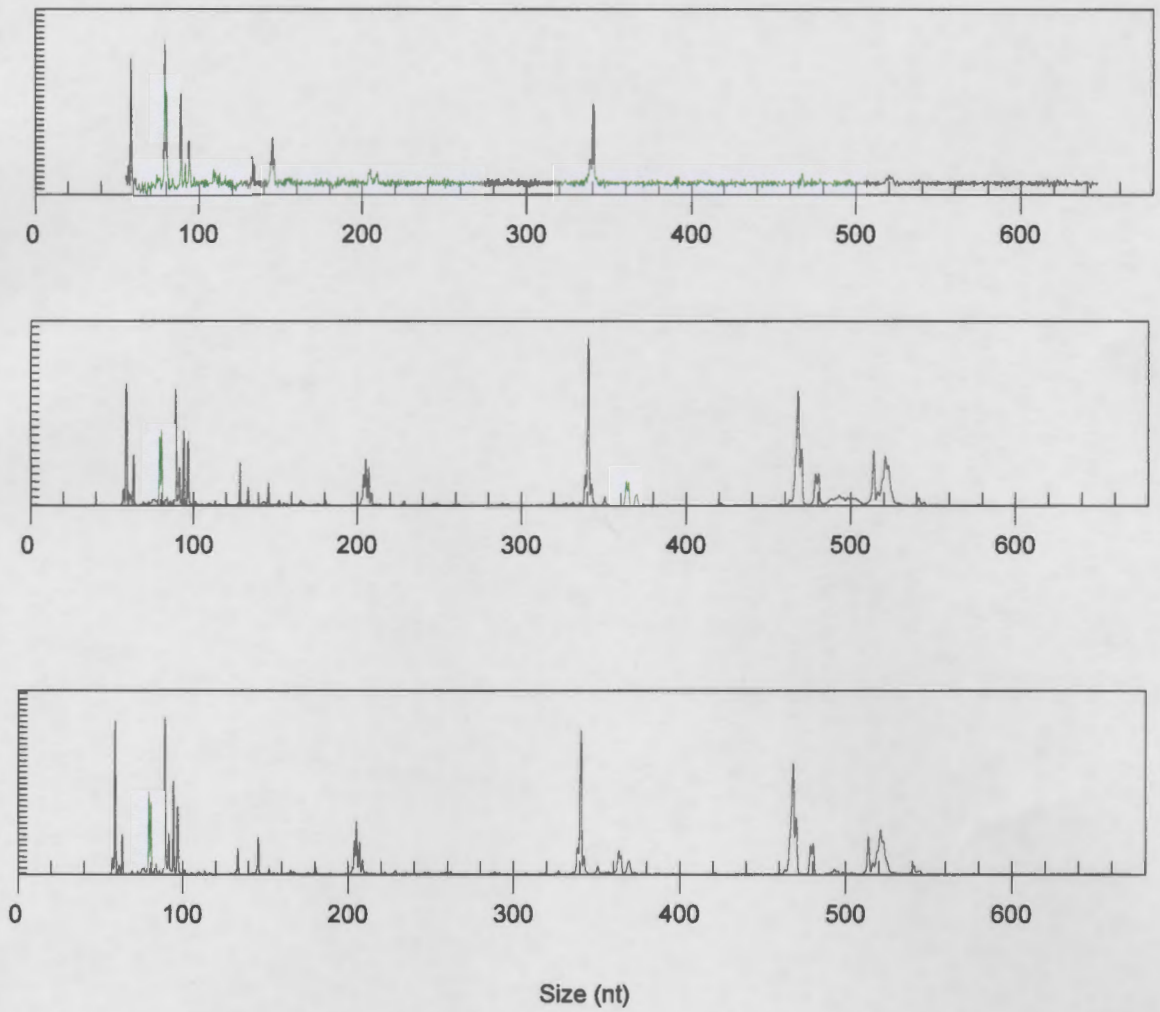

Fig. 1: t-RFLP traces from replicate progeny cultures of cysts + HU11/DE08 bacteria. Forward primer labeled (27F, dye D3) fragment traces from three independent replicate cultures. The PCR amplified 16S rRNA genes were digested with restriction enzyme *HhaI* and fluorescent labeled fragments were separated on CEQ ™ 8000 genetic analysis system. Fragment size in base pairs is shown on the x-axis.

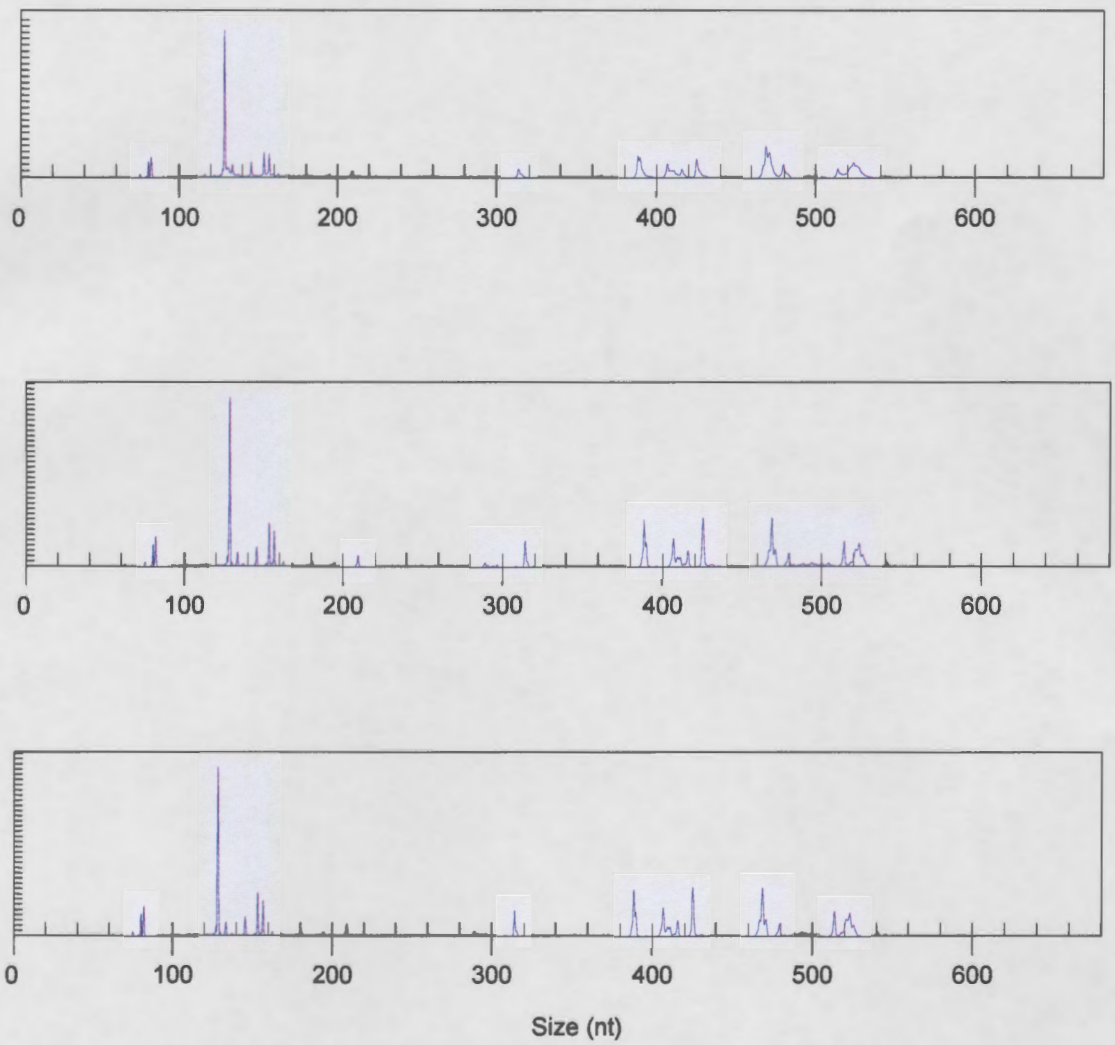

Fig. 2: t-RFLP traces from replicate progeny cysts + HU11/DE08 bacteria. Reverse primer labeled (518R, dye D4) fragment traces from three independent replicate cultures. The PCR amplified 16S rRNA genes were digested with restriction enzyme *HhaI* and fluorescent labeled fragments were separated on CEQ ™ 8000 genetic analysis system. Fragment size in base pairs is shown on the x-axis.

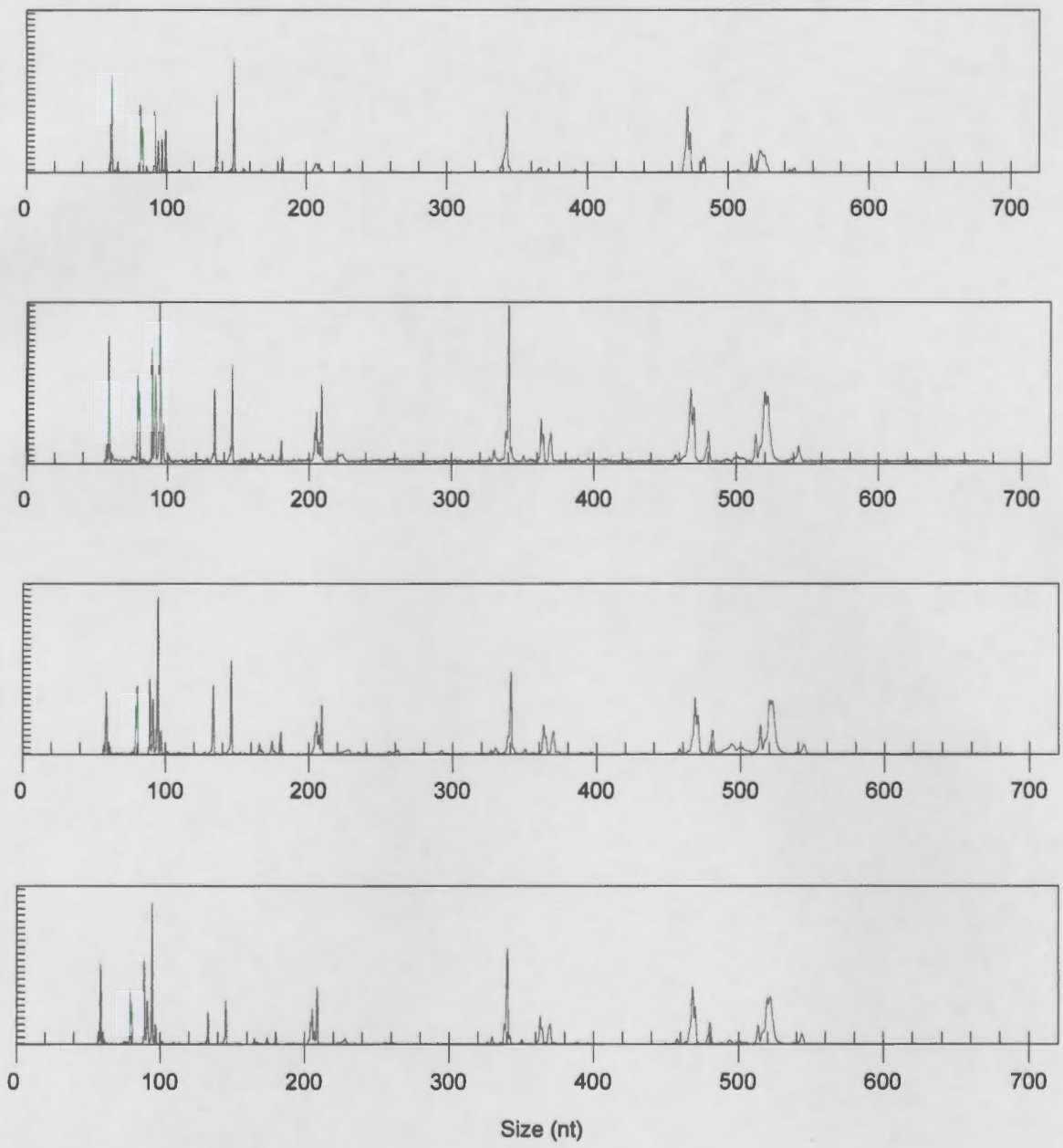

Fig. 3: t-RFLP traces from replicate progeny cultures of cysts + HU11 bacteria. Forward primer labeled (27F, dye D3) fragment traces from four independent replicate cultures. The PCR amplified 16S rRNA genes were digested with restriction enzyme *HhaI* and fluorescent labeled fragments were separated on CEQ ™ 8000 genetic analysis system. Fragment size in base pairs is shown on the x-axis.

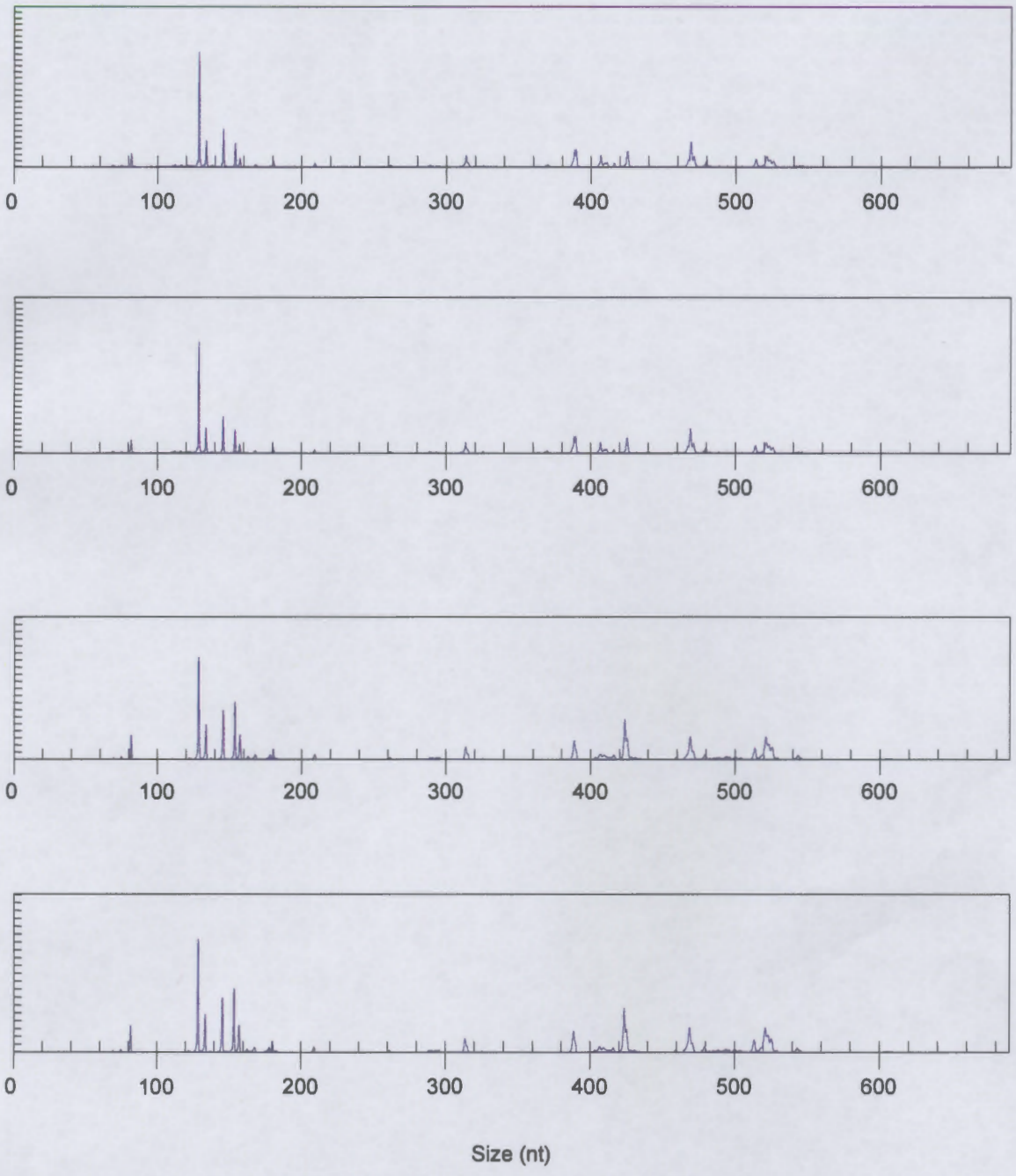

Fig. 4: t-RFLP traces from replicate progeny cultures of cysts + HU11 bacteria. Forward primer labeled (27F, dye D3) fragment traces from four independent replicate cultures. The PCR amplified 16S rRNA genes were digested with restriction enzyme *HhaI* and fluorescent labeled fragments were separated on CEQ ™ 8000 genetic analysis system. Fragment size in base pairs is shown on the x-axis.