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Bacteria-Protozoan Interactions in a Mixed-Species Biofilm Community

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Abstract

Environmental biofilms typically consist of mixed-species communities exhibiting complex inter- and intra-species synergistic/antagonistic interactions. Intense competition in aquatic systems is driven by nutrient and space limitation. Bacterial biofilm members cooperate under such conditions to resist environmental perturbations, processes that subsequently determine community composition. This thesis reports on environmental bacterial screens for amensal/antagonistic metabolites against protozoa and nematodes. Further, defined mixed-species model communities isolated from the surface of the green alga *Ulva australis* were tested for their protozoan predation response under varying nutrient conditions.

Biofilms exhibited higher levels of antiprotozoal and anthelmintic activity than their planktonic counterparts. Biofilm inhibitory activities were highly diverse, commonly targeting either nematodes or protozoa, but rarely targeting both with broad-spectrum activity. Predation resistance was explored using a model marine four-species community. Protozoan grazers of different feeding types were used on different-stage biofilms; *Rhynchomonas nasuta* (early-stage), *Tetrahymena pyriformis* and *Acanthamoeba castellanii* (intermediate- and late-stage). As single-species biofilms, all four species displayed varying levels of grazing. All were susceptible to *R. nasuta* whereas only *Dokdonia donghaensis* and *Acinetobacter lwoffii* were vulnerable to *T. pyriformis*. *A. castellanii* selectively preferred *Shewanella japonica* and *A. lwoffii*.

Mixed-species biofilms facilitated synergistic and antagonistic interactions between consortia members. *R. nasuta* selectively preferred *A. lwoffii*, resulting in a ‘reverse grazer’ effect, allowing other members to benefit. *T. pyriformis* preferred grazing on *D. donghaensis*. The mixed-species community was resistant to *A. castellanii* grazing and thus commensal to *D. donghaensis* and *A. lwoffii*.

Under high nutrient conditions, both three- and four-species biofilms resisted predation. By comparison, low nutrient conditions increased both mixed- and single-species biofilms susceptibility to *A. castellanii* grazing. Under low nutrient conditions, the removal of *Microbacterium phyllosphaerae* from the four-species consortium destabilized the community. Under low carbon conditions, *S. japonica*

demonstrated increased grazing susceptibility, possibly due to resource reallocation from defence to growth/maintenance.

Bacterial grazing resistance depends on survival strategies, e.g. the ability to exploit nutrients and the trade-off between growth and defence. The differential levels of grazing resistance of both mixed- and single-species consortia, resulting from ‘top-down’ and ‘bottom-up’ factors, affected community structure and composition. Successful application of the carbon:nutrient balance hypothesis to *S. japonica’s* defence strategy, highlights the potential for eukaryotic theories as predictive models for microbial systems.

List of Publications

Tan, L. S., Matz, C., McDougald, D. and S. Kjelleberg. 2008. Impact of selective grazing by *Acanthamoebae castellanii* on a marine mixed-species biofilm. *12th International Symposium on Microbial Ecology Abstract Book*.

Tan, L. S., Matz, C., Steinberg, P., McDougald, D. M. and S. Kjelleberg. 2009. Effects of protozoan grazing on a mixed-species marine biofilm. *ASM Conferences Biofilms 2009 Abstract Book*.

Tan, L. S., Steinberg, P., Matz, C., Kjelleberg, S. and D. McDougald. 2010. The effect of marine predators on the community composition in a marine mixed-species biofilm. *13th International Symposium on Microbial Ecology Abstract Book*.

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Abbreviations

°C	Degree Celsius
μ	Growth rate
µm	Micrometers
µg	Micrograms
µl	Microlitres
µmol	Micromoles
d	Day/ Days
mg	Milligrams
ml	Millilitres
mM	Millimolar
M	Molar
ng	Nanograms
nM	Nanomolar
g	Grams
hrs	hours
l	Litres
Hz	Hertz
s	Seconds
vol	Volume
wt	Weight
2.04	*Microbacterium phyllosphaerae*
2.12	*Shewanella japonica*
2.3	*Dokdonia donghaensis*
2.34	*Acinetobacter lwoffii*
2M	Marine minimal media
AHL	Acylated - Homoserine Lactones
AI	Autoinducer

ANOVA	Analysis of Variance
APA	Amoeba Plate Assay
AR	Associational Resistance
ARB	Amoeba Resistant Bacteria
AS	Associational Susceptibility
ATCC	American Type Culture Collection
BLASTn	Basic Local Alignment Search Tool
C	Carbon
CaCl	Calcium Chloride
CCAP	Culture Collection of Algae & Protozoa
CFB	Cytophaga-Flavobacterium-Bacteriodes
CFU	Colony Forming Units
CNB	Carbon Nitrogen Balance Hypothesis
CNP	Carbon Nitrogen Phosphate Ratio
CV	Crystal Violet
DNA	Deoxyribonucleic Acid
DOM	Dissolved Organic Matter
E	Evenness Index
EPS	Extracellular Polymeric Substances
FeSO4	Iron(II) Sulfate
GDB	Growth-Differentiation Balance Hypothesis
g	*g*-force
H_2O	Water
H_3BO_3	Boric Acid
HC	High Carbon
HCN	Hydrogen Cyanide
HEPES	4-2-hydroxyethyl-1-piperazineethanesulfonic acid
HN	High Nitrogen
HP	High Phosphorus
HKB	Heat Killed Bacteria

HNF	Heterotrophic Nanoflagellates
IC_{50}	50% survival
K_2HPO_4	Potassium Hydrogen Phosphate
KBr	Potassium Bromide
KCl	Potassium Chloride
KPO_4	Potassium Phosphate
KtW	Killing the Winner Hypothesis
LB	Luria-Bertani medium
LC	Low Carbon
LN	Low Nitrogen
LP	Low Phosphorus
LPS	Lipopolysaccharides
LT_{50}	Number of days required to kill 50% of the nematodes
$MgCl_2$	Magnesium Chloride
$MgSO_4$	Magnesium Sulfate
Mip	Macrophage infectivity factor
N	Nitrogen
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
$NaHCO_3$	Sodium Hydrogen Carbonate
NGM	Nematode Growth Media
NI	Niche-Specific Index
NSS	Nine Salts Solution
OD	Optical Density
P	Phosphate
PBS	Phosphate Buffered Saline
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PYG	Protease Yeast Glucose
qPCR	Quantitative PCR

QS	Quorum Sensing
RER	Rough Endoplasmic Reticulum
rRNA	Ribosomal Ribonucleic Acid
RPM	Revolutions Per Minute
S	Species Richness
SD	Standard Deviation
$SrCl_2$	Strontium chloride
TMA	Tetrahymena Motility Assay
VNSS	Väätänen Nine-Salt Solution
VBNC	Viable but Non-culturable
Amp^R	Ampicillin Resistance
Cm^R	Chloramphenicol Resistance
$Gent^R$	Gentamicin Resistance
Kan^R	Kanamycin Resistance
$PolyB^R$	Polymycin Resistance
$Strep^R$,	Streptomycin Resistance
Tet^R,	Tetracycline Resistance

Chapter 1 : Literature Review

1.1 Introduction

Bacteria have an ancient evolutionary history which has provided them with genotypic and phenotypic plasticity to adapt to any environment (221). In most environmental settings, bacteria preferentially associate as highly cooperative and complex structured sessile multispecies consortia called biofilms, where intra and interspecies interactions occur (61). Such bacterial biofilms are ubiquitous and can be formed on both animate and inanimate surfaces, including alga, endothelia lining of body organs, medical devices, contact lenses, river rocks, and water distribution pipes (reviewed in (62)). Although biofilm communities play a crucial role in the well-being of the planet by mediating biogeochemical cycles and microbial food webs (221), biofilms can also have negative impacts, for example in clinical situations by causing chronic infections (64) and in industrial settings, where they cause significant damage through biofouling and biocorrosion (17).

Predators such as protozoa are one of the major selective pressures presented to bacteria in the environment (155). The coexistence of bacteria and protozoa in the environment has resulted in the evolution of complex interactions that have developed over millions of years, allowing bacteria to evolve a variety of defense strategies for survival and persistence. The defense mechanisms employed by the bacterial cells include the formation of filamentous and ultramicro-cells (204), the ability to survive within food vacuoles (42), the formation of multi-cellular biofilms (198) and the expression of biofilm-related virulence factors (200), all of which are effective against protozoan grazing. There is increasing interest in enhancing the understanding of the complex interactions between protozoa and bacteria, especially those in mixed-species consortia, and to explore how these interactions relate to bacterial persistence. Moreover, there is a great interest in manipulating these interactions to control industrial biofouling problems and to better understand the evolution of pathogens.

1.2 Marine ecosystems

The marine environment, which makes up more than 70 % of the Earth's surface, is a highly structured complex ecosystem where archea, bacteria and eukaryotes participate in biochemical interactions to form fully functional food chains (11). Despite being an oligotrophic environment, the oceans are teeming with a huge diversity of bacteria, whose metabolic activities are responsible for at least half of the global primary production of organic compounds (50, 165).

Primary production in the ocean is dependent on a variety of factors. Light is of fundamental importance to a marine community of phototrophs and mixotrophs, which are responsible for converting solar energy to dissolved organic matter (DOM) through photosynthesis (160, 219). DOM, which is required for growth by heterotrophic bacteria, is made accessible to higher order members in the food cycle when bacteria are grazed by protists, who in turn are grazed by zooplankton, followed by metazoans, fish and humans (154, 255).

The availability of inorganic nutrients such as nitrate and phosphate, required for basic cell assembly, is crucial to controlling primary production. Microbes are capable of responding rapidly to changes in nutrient levels and adapt efficiently to changes in C:N:P ratios, resulting in phenotypic changes (124). The most obvious example is a change in cell size which can indirectly affect bacteriovory and consequently the microbial community composition (86, 98). As nutrient cycles form a feedback loop controlling primary production, the increased grazing resistance arising from such morphological changes can affect nutrient recycling and hence primary productivity in the marine environment (85, 111).

Due to nutrient limitation, bacteria, fungi and protozoa form highly structured communities that often result in complex division of labour on marine biotic and abiotic surfaces (113). Hence, some biotic surfaces such as phytoplankton and algae regulate the production of polysaccharides or inhibitors to deter such communities from attachment and invasion (252). Those incapable of secondary metabolite production, are dependent on their surface epiphytic biofilm community for herbivory defense and biofouling deterrence (125).

1.3 The biofilm mode of life

Biofilms are heterogeneous microbial communities, embedded in their own hydrated matrix of extracellular polymeric substances (EPS), which accumulate at interfaces (78). Biofilm development is characterized by a sequential succession of attachment, proliferation and dispersal, which is likened to the well-studied multicellular life cycle of the differentiating 'higher' bacterium *Myxococcus xanthus* (291, 337). The initial stage involves planktonic cells, which colonize available surfaces through swarming motility, producing adhesive surface-localized proteins, and organelles such as fimbriae and flagella (241). Reversible attachment leads to the upregulation of EPS production for irreversible attachment and biofilm expansion (38). Subsequently, the recruitment of additional cells from the surrounding environment leads to structural and phenotypic differentiation, followed by the development of a mature biofilm (308). The mature biofilm is characterized by microcolonies, which are found principally at the late stage of the biofilm life cycle and are highly stable and resilient to many different types of stresses (62). The release of motile planktonic cells from the sessile consortia, through sloughing and active dispersal in response to particular environmental cues, such as nutrient starvation completes the biofilm life cycle (308). The association of bacteria with abiotic and biotic surfaces mediated by biofilms is proposed as one of the major factors allowing for persistence of bacteria in the environment (146).

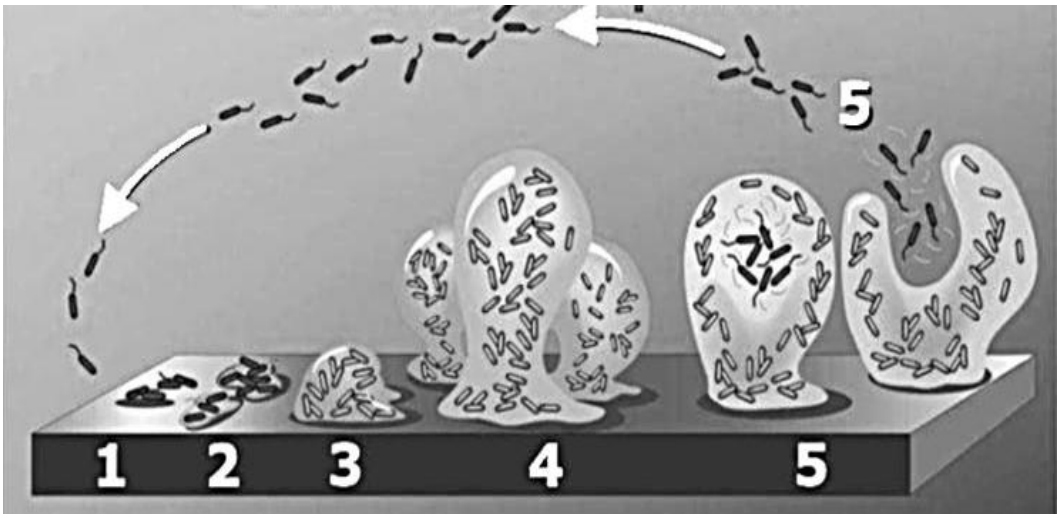


Figure 1.1: Diagrammatic illustration of the developmental life cycle of a biofilm depicting the initial reversible attachment of cells to a substratum (Stage 1), production of EPS under favorable conditions (Stage 2), cell proliferation and differentiation into

juvenile microcolonies (Stage 3), biofilm maturation (Stage 4) and the release of migratory cells, through a passive process sloughing (not shown), or an active dispersal event to colonize new environmental niches (Stage 5) (adapted from (308)).

1.3.1 The biofilm structure

Attachment of microbial cells to a substratum and aggregation is facilitated by the production of EPS, which is crucial for maintaining the three-dimensional structure of the microcolonies. The composition of the biofilm matrix can differ but is generally composed of microbial cells, EPS, water, nutrients and detritus picked up from the surrounding environment (314). The biofilm matrix and in turn, the differentiation process in mature biofilms, are both dependent on physical factors such as hydrodynamic shear (307) and chemical factors (354), and involves alteration in gene regulation for metabolic efficiency (71, 231, 291).

The general architecture of biofilms has been well characterized in a few model organisms, with *Pseudomonas aeruginosa* being the best-studied example. During biofilm maturation, clonal expansion of the biofilm is facilitated by quorum sensing (QS), a form of cell-to-cell communication, which employs the use of autoinducer (AI) signaling molecules, usually acylated-homoserine lactones (AHLs), to assess cell density. Once a certain threshold of AI is achieved, repression or induction of gene expression occurs. For example, biofilms formed by *P. aeruginosa* are highly complex and resemble mushroom-like structures (microcolonies) interspersed with water channels for exchange of nutrients and waste (166). These microcolonies, interspersed with a complex network of water channels, are regulated by the *lasR-lasI* QS signaling system (71). A QS-negative mutant was capable of initial attachment but was deficient in the development of the mature microcolonies and channels. Similarly, a mixed-species biofilm of strains isolated from dental plaque forms a structure similar to that of a *P. aeruginosa* biofilm (314). These findings sparked much interest in exploring QS-regulation of the biofilm life cycle and in particular biofilm development and differentiation in various model organisms (175, 291, 361).

In addition to QS systems, the biofilm development process is dependent on expression of phenotypic traits such as flagella and twitching mediated motility (232). Type IV pili play a significant role in the initial adherence of cells to a

substratum (112), as well as in microcolony formation in *P. aeruginosa* (166). Klausen *et al.* (166) showed that in order for *P. aeruginosa* microcolonies to form, non-motile cells first form the stalks of the microcolonies, followed by active migration of motile cells up the stalks to form the caps. As indicated above, EPS production is an integral part of the biofilm formation process. In addition to providing structural integrity, secretion of EPS at high cell densities provides a competitive advantage by encouraging upward growth of descendents, thereby increasing their exposure to nutrients and at the same time suffocating non-producing competitors (223, 351).

A range of environmental parameters has been shown to influence the structure of a biofilm. Under intense hydrodynamic shear conditions, *P. aeruginosa* forms biofilms that appear as filamentous strands. However, when shear conditions are removed, the filamentous biofilms reverts back to the original microcolony type biofilms (263). Biofilm development by *Serratia marcescens* is dependent on nutritional cues (271). Under conditions of carbon and nitrogen starvation, *S. marcescens* forms microcolony-type biofilms, but with increased nutrient concentrations, *S. marcescens* forms a filamentous biofilm (271). For the invasive plant pathogen *Agrobacterium tumefaciens*, biofilm formation is stimulated when phosphate is limited, but is surprisingly patchy with lower biomass content during phosphate abundance (69). Other environmental processes that contribute to shaping biofilm structure include grazer-mediated sloughing and coordinated dispersal. The process of grazing by protozoa can lead to large portions of the biofilm biomass being detached (222). Active seeding dispersal and passive erosion or sloughing in response to changes in environmental conditions also result in changes in the biofilm structure (309). The process of biofilm formation is therefore highly complex and variable as heterogeneity of the biofilm is dependent on the bacterial consortia forming the biofilm, as well as both intrinsic and extrinsic factors that the cells are exposed to.

1.3.2 Biofilm sociobiology

Sociobiology is defined as the social behavioral interactions of individuals in response to natural selection, i.e. the study of the biological basis of behaviour. The type of interactions that individuals have evolved are those that are beneficial

for their own fitness (222). For example, in biofilms, indirect and direct interactions between members can include QS regulated expression of phenotypes, (206, 240, 257), exchange of genetic material (268), and metabolic complementation, all of which contribute to bacterial social behavior in biofilms (144). In the marine environment, nutrient limitation is a strong selective pressure affecting biofilm formation and community interactions of bacteria within biofilms. Spatial and temporal chemical variations will lead to diffusion gradients of nutrients, oxygen (73) and pH, thus creating microniches within the biofilm, where interactions between members in the consortia become crucial to its fitness and stability.

1.3.2.1 Interactions within mono-species biofilms

The relationship between different individuals in a mono-species biofilm is not transitory as is the case for planktonic cells. Concentrations of nutrients and metabolic by-products govern the type of interactions occurring in these cooperative consortia (63). Altruistic interactions in a mono-species biofilm, where individuals are genetically similar has been reported (191, 338). However, such interactions are usually not observed in multi-species biofilms, where more complex synergistic and antagonistic interactions occur (54, 315). For example, cell death and lysis of subpopulations of cells occurs within the microcolonies in *P. aeruginosa* biofilms (338). This phenomenon is analogous to programmed cell death (PCD) in eukaryotic cells. Such phage mediated processes of cell death in biofilms may be compared to altruism, as the death and lysis of this sub-population of cells provides nutrients to support the remaining viable cells (338). Cell death in biofilm microcolonies has also been observed in a variety of other microorganisms, but the causative factor can differ. In the case of the marine bacterium *Pseudoalteromonas tunicata*, cell death is caused by the expression of an autotoxic protein, AlpP. This autolytic process is believed to maximize biofilm fitness by preventing overcrowding, relieving competition and regenerating nutrients for the residual and dispersed viable cells (191).

In addition to altruism, antagonistic interactions can occur within a mono-species biofilm, where cheaters are able to exploit producers of various metabolites and benefit without providing any metabolic returns (89, 344). The degree of

population bottleneck (an evolutionary event whereby a significant proportion of the population is purged) determines if cooperation or competition will occur between variants in a pure culture biofilm (40). For example, a study done on *P. aeruginosa* biofilms by Brockhurst (40) demonstrated that ecological factors such as colonization or periodic disturbances can encourage cooperation under circumstances of small population bottlenecks, as high levels of genetic relatedness with low frequency of cheats are observed. However, under high population bottlenecks, a higher frequency of evolutionary cheats was observed, leading to competition and constant removal (40-41, 250).

Cooperation in the form of synergism has also been observed within mono-species biofilms. Intra-species interactions can lead to synergistic biofilm development in biofilms of genetically diverse strains of *Escherichia coli*, whereby biofilm expansion is induced by lateral gene transfer of conjugative plasmids (268). These conjugative plasmids, which belong to different incompatibility groups and thus express different conjugative pili are capable of stimulating biofilm formation (268). This observation highlights the possibility of synergistic biofilm development in single-species consortia by lateral gene transfer.

1.3.2.2 Interactive behavior of mixed-species biofilms

Environmental biofilms are usually mixed-species consortia consisting of highly complex communities of microorganisms (144). For example, oral biofilm communities are composed of more than 700 coexisting phylotypes. The ability of oral bacteria to persist in such a dynamic environment, where there is constant exposure to salivary flow and nutrient limitation has been attributed to the formation of multi-species consortia (245). The high cell densities and close proximity of members in mixed-species biofilms facilitate a variety of interspecies interactions such as mutualism, commensalism, competition, ammensalism and cheating (144, 197). These interspecies microbial interactions can be neutral, where the presence of a particular focal member has no influence on other members (144). However, truly neutral interactions may not occur in nature. Beneficial interactions may occur where both members are better off together than on their own, in either obligate (mutualism) or facultative (synergism)

relationships (67, 246). Antagonistic interactions occur when members compete for limited resources (359) or regulate the production of inhibitory products (ammensalism) to increase individual fitness (189). Therefore, in mixed-species biofilm communities, members may exhibit more than one type of multi-cellular behaviour in parallel, such as cooperation and conflict, which can have significant impacts on the physiology and structure of the biofilm (44, 144).

Most work aimed at comprehending biofilm sociobiology has been performed on well-characterized mono-species systems such as *P. aeruginosa* (as described above). However, in recent years, increasing emphasis has been placed on understanding mixed-species biofilms and unraveling inter-species interactions. The construction of defined mixed-species systems has increased in an effort to understand the complex interactions occurring (143, 162, 246). These defined systems provide the benefit of creating biofilms that are more analogous to natural biofilms and thus may reflect more accurately how the latter function. In addition, these systems can also be easily manipulated in the laboratory to elucidate the roles of each member in the community (162).

The most common symbiotic interactions observed are commensal interactions, which usually occur when the metabolic by-products of one species are utilized as carbon sources for other species (116) or where the inhibitory effect of the growth media on one member is mitigated by the other (67, 144). For example, Kolenbrander and his colleagues (244-246) have made significant contributions to our understanding of mixed-species biofilm interactions, by work done on two, three and four member biofilms, where they showed that the formation of oral biofilms follows a succession pattern of colonization. First, early colonizers adhere to the oral substratum and coaggregate with other early colonizers by surface receptor recognition and binding (244). In cases where members are deficient in receptor recognition and binding, cooperative interactions are crucial for adhesion and aggregation to facilitate the initial stages of dental plaque biofilm formation (174, 237). Subsequently, metabolic commensalism between early and middle colonizers occurs where the metabolic by-products and altered growth conditions encourage the formation of a coaggregation bridge by middle colonizers with the early colonizers. This coaggregation bridge serves as a substratum for attachment and proliferation of late colonizers, resulting in the expansion of the biofilm (244-246). Coaggregation of different stage colonizers

facilitates intergeneric interactions and the success of these interactions is crucial for the success of each species in the biofilm community (93, 143). Commensalism is also seen in other dual-species systems. For example, *Staphylococcus sciuri* is able to gain protection from chlorine treatment by spatially distributing itself around chlorine resistant *Kocuria* sp. microcolonies (182). In a five-member cellulose degrading mixed community structural and functional stability requires a balance of positive and negative interactions for stable coexistence (162). Mutualism may also occur, such as in the case of mixtures of aerobes and anaerobes, where the aerobes consume oxygen, making the environment suitable for growth of anaerobes, in exchange for metabolic by-products (162).

However, the tight association between members in a mixed consortium can lead to conflicts of interest when limitations in space or nutrients arise, leading to competition or production of antagonistic compounds (ammensalism) that inhibit the growth of competitors (144). In a dual-species biofilm of *Acinetobacter* sp. and *Pseudomonas putida*, the absence of a substratum for attachment and biofilm formation led to competition between members (54). When a surface was provided for biofilms formation in flow chambers however, a metabolic commensal relationship developed, highlighting the importance of spatial orientation of one member relative to the other (54).

In nature, most surfaces (e.g algae) are a source of nutrients and thus intense competition between colonizers. The metabolic by-products of the primary colonizers can either be synergistic or antagonistic, thereby determining the composition of secondary and tertiary colonizers and thus the eventual shape of the biofilm community (62). Marine biofilm-forming bacteria produced more inhibitors then free-living bacteria, as a form of deterrence against potential competitors (189). The inhibitor, AlpP, upregulated by the marine bacterium, *P. tunicata* in the presence of other colonizers, provides it with a competitive advantage by inhibiting the settlement of potential competitors (266). The ecological success of a biofilm is therefore dependent on how members interact and respond to the surrounding environment to generate a stable sessile biofilm with metabolic flexibility. Understanding these interactions would be essential to develop measures to manipulate biofilms in a way beneficial to medicine and industry.

1.3.3 Biofilms as a reservoir of bioactives

Biologically active compounds such as antibiotics have been conventionally isolated from a variety of environmental sources through laborious culturing of microorganisms, followed by isolation and screening libraries of compounds (259). Currently, there are two parallel streams of novel bioactives discovery: screening of natural products produced in the environment and the synthesis of target-specific drugs using synthetic chemistry and metagenomics (53, 332). The screening of natural bioactives by traditional methods is effective only for the culturable fraction of environmental isolates. Despite improvements in assays for natural product screening and purification and characterization methodologies over the last 60 years, the necessity for culturing and the lack of accessibility to the greater not-yet culturable diversity are limiting factors (58, 331). The use of synthetic chemistry to generate analogues of antibiotics with greater specificity provides an alternative approach for bioactive discovery (332). The availability of completed genomes of many microbes and parasites have revealed novel drug targets, for which synthetic drugs can be engineered, providing a more directed approach (53). Even with the discovery of new synthetic antibiotics, natural selection will inevitably lead to a rise in antibiotic resistance or, worse, multi-drug resistance (187). This constant demand for novel drugs serves as a driving force for the use of novel organisms and habitats, such as biofilm communities, as potential providers of novel bioactives (332).

Marine pelagic systems are examples of the most diverse and underexplored habitats, ranging from continental shelves to deep ocean trenches, from tropical to sub-zero temperatures, from eutrophotic to oligotrophic and from aerobic to anoxic conditions (287). These habitats host a vast array of marine plants and invertebrates, that have in turn evolved a diverse range of positive and negative interactions with surrounding microbes (147-148). In terrestrial ecology, the main mode of protection against predators is by flight (273). However, for plants that are sessile, the production of defense metabolites has evolved as a form of deterrence against herbivory (273). Metabolites, such as pyrethrin (pesticide), can make the host less palatable by inhibiting digestion, interrupting metabolic pathways or causing acute toxicity to grazers (273). Marine plants behave in a

similar way (208, 252). For example, the marine red alga, *Delisea pulchra* prevents biofouling on its surface by interfering with bacterial regulatory systems through the production of secondary metabolites, which are analogues of bacterial signaling molecules (99). Subsequent research demonstrated that certain species of algae, such as *Ulva australis*, are also capable of remaining free from fouling despite the absence of secondary metabolite production. The anti-fouling properties of such algae are attributed to the production of inhibitory compounds by their epiphytic bacteria. These bioactive compounds serve as a form of chemical defense for the algal host and at the same time limit competition for space and nutrients from higher fouling organisms (127, 148, 184). As such, marine epiphytic biofilm communities are portrayed as undiscovered reservoirs, potentially rich in inhibitor production.

Biofilm formation has been adopted by bacteria as a strategy to maximize survival against environmental stresses. Due to their sessile mode of life, this adaptive strategy has been proposed as a rich source of inhibitory metabolite production (208). As inter-species interactions within biofilms are common, members may produce bioactive compounds to prevent resource competition (allelopathy) (273). For example, attached marine bacteria display more antibacterial activity than free-living isolates (224). This chemical competition was suggested to increase the fitness of the producers within the biofilm (189). The genus *Pseudoalteromonas*, which is exclusive to marine environments, contains many species that produce bioactives. The extracellular inhibitory agents display a broad range of activities such as antibacterial, antialgal and antifungal activities, providing benefits to the producer during intense competition. These traits have been suggested as crucial for survival in marine oligotrophic environments (127). For example, *Pseudoalteromonas aurantia*, *P. luteoviolacea* and *P. rubra* produce two classes of antibacterial compounds that function through inhibition of bacterial respiration (127). In addition, *Pseudoalteromonas* strains also produce pigments that display inhibitory activities (81). In another example, pigment production by a range of organisms (e.g. phenazines and quinones(355)) was correlated with antifouling activities, where 90% of pigmented isolates tested exhibited antagonistic activities against colonizers (81). The production of toxins, enzymes or pigments as chemical defenses by surface-attached biofilms members is likely given that these organisms are constantly evolving and adapting to

environmental pressures such as competition for resources and space (200). Thus, such organisms present significant opportunities for discovery of novel natural product chemistries.

1.4 Protozoa

In addition to competition for resources and space, marine bacteria face another challenge from grazing by protozoa. Eukaryotic protozoa are the primary consumers of phytoplankton and bacterioplankton. They are responsible for 25-100% reduction of the daily bacterial primary production in marine environments, thereby controlling and keeping these planktonic populations in check (286-287). The predatory activities of phagotrophic protists also affect the nutrient flux in such systems by stimulating microbial communities through nutrient remineralization (88, 288), as well as releasing the carbon, nitrogen and phosphorus 'locked up' in bacterial biomass, making these nutrients accessible to other members of the marine community (90). As protozoans are the major cause of bacterial mortality, their interactions with biofilms are crucial in structuring microbial community composition on marine surfaces (109). Despite certain aspects of protozoa-bacteria biology being well studied, many interactions between these microbes remain poorly understood. The diversity of protozoa, the formation of biofilms and the interactions between both is discussed in further detail in the following sections.

1.4.1 General classification of protozoa

Protozoa are ubiquitous unicellular phagotrophic eukaryotes ranging in size from 2 – 200 μm (239). The majority of protozoa in the environment are free-living and survive through consumption of detritus or microorganisms (phagocytosis) or through absorption of soluble dissolved organic matter (pinocytosis) (242). Some protozoa have also been found to be opportunistic pathogens capable of causing parasitic diseases in higher organisms, while others are involved in complex symbiotic relationships with a multi-cellular host (90). The generation time for protozoans is similar to other microbes and as such they are the most abundant eukaryotes in the oceans (286, 292). The diversity and abundance of bacterial prey

of various shapes and sizes has resulted in the evolution of a variety of feeding mechanisms, and morphological diversification of protozoan predators (90, 288). Protozoa are generally classified into three main groups, flagellates, ciliates and amoebae, based on their locomotion organelles (flagella, cilia and pseudopodia, respectively) (242). These organelles usually determine the ecological niche that the protist occupies.

Flagellates are the most significant consumers of bacterioplankton due to their ability to alter their growth rate in response to rapid changes in bacterial numbers. They are responsible for limiting both daily primary production and the standing stock of pelagic systems (242, 286). These heterotrophic eukaryotes range in size from 3 – 100 μm and are usually characterized by one, two or four flagella (87, 239, 242). Based on their feeding preferences, they can be sub-divided into four broad groups: stramenopiles that feed on planktonic bacteria, bodonids that prefer attached bacteria, euglenids that feed on detritus and kathabelpharids that graze on other protozoa and algae (242). The bicosoecid nanoflagellate, *Cafeteria roenbergensis*, is a stramenophile, which uses its flagella for motility, attachment to substratum and for generating circular water currents to bring potential planktonic prey within a manageable distances for consumption (Figure 1.2A) (27). An example of a widely studied, morphologically distinct bodonid with a cosmopolitan distribution in aquatic ecosystems is the kinetoplastid, *Rhynchomonas nasuta* (Figure 1.2B) (90). Kinetoplastids are characterized as having a mouth, and in the case of *R. nasuta*, it is found inside its distinct proboscis. In contrast to the free-living bicosoecid flagellates, *R. nasuta* usually attaches loosely to the substratum by its posterior flagellum. Grazing on detritus or bacterial prey occurs, when the prey are encountered during gliding movements over surfaces (242). As flagellates are usually small in size, the resultant food vacuoles formed can only process one prey at any one time, with a size limit of 2 - 20 μm (25). Bacterial prey that are larger than this size are usually regurgitated without food vacuole formation (27), so that flagellates effectively display size selective feeding habits (239).

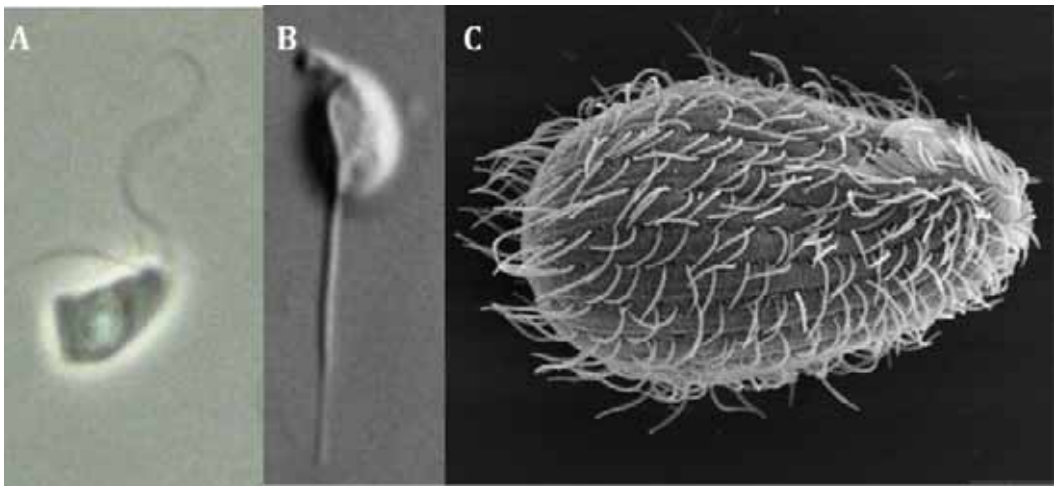


Figure 1.2: Images of protozoa commonly found in marine and terrestrial environments (A) *Cafeteria roenbergensis*, a planktonic marine bicosoecid nanoflagellate; (B) *Rhynchomonas nasuta*, a kinetoplastid surface browser of marine, fresh water and soil and (C) *Tetrahymena* sp., a freshwater ciliate.

Ciliates are the most active multi-nucleated protists, easily distinguished by rows of cilia found on the cell surface, which are used for locomotion and feeding purposes (242). They come in various shapes (e.g. spheres, ovals or cones) and have the most diverse feeding mechanisms amongst protozoans due to their vast array of predatory and reinforcement organelles (221). Compared to flagellates, they are larger in size, ranging from 20 μm to 1 mm. Their feeding preferences are therefore not limited to bacterial prey but can take advantage of other prey types such as bacterial clumps, detritus, algae, flagellates, rotifers and other microzooplankton which can be found on surfaces, suspended in the plankton or in active motion (239, 242). These predatory ciliates are either suctorian or haptorids. Suctorian ciliates are sessile diffusion feeders that have tufts of cilia, or cirri, that capture colliding prey or generate a water current to filter prey (90, 242). In the dispersal stage of their life cycle, ciliated larvae are released and search for new niches for colonization. Peritrich ciliates are conical shaped suctorian ciliates commonly found in sewage and are known for their rapid efficacy in bacterial removal (242). Haptorids are filter feeders (242) and the quality of the filters they possess determines the type of prey consumed. Haptorids with fine-mesh filters such as *Tetrahymena* are bacterivorous predators that actively search for prey in suspension and flourish in bacterial enriched environments (Figure 1.2C) (90, 239). Ciliates with coarser filters like *Pleuronema* use the cilia to collect algae (90). Ciliates can either undergo sexual

or asexual reproduction depending on the environmental conditions (242). Ciliated protozoa are also capable of forming desiccation-resistant cysts during environmental stress (90).

The last group of protozoa, the amoebae, can be found in diverse habitats on both animate and inanimate surfaces, in aquatic and soil environments, dust particles and even on the human host (342). Amoeboid protozoa are the largest of the free-living protozoa, ranging in size from 20 μm to 2 mm and can be sub-divided into the rhizopod and the testate amoebae (242). There is a huge diversity of amoebae, including phagocytic slime molds and symbiotic benthic foraminifera (90). In general, amoebae are slow moving and are found associated with solid-liquid or liquid-air interfaces (239). Those amoebae that rely on temporary extensions from their cell surface (pseudopodia) for locomotion and phagocytosis are termed rhizopod amoebae, and can be further sub-divided into filose (thin-thread like pseudopodia) and lobose (broad pseudopodia) amoebae (90, 242). Much work has been done on the rhizopod group of amoebae due to opportunistic pathogenic members such as *Acanthamoeba castellanii*. *A. castellanii* is a heterotroph found in all types of environments, with two distinct stages in its life cycle (Figure 1.3) (35-36). The first stage, the trophozoite, occurs when the amoeba is actively feeding. The amoebae will contain a variety of visible vacuoles for prey capturing (phagosomes), prey digestion (lysosomes) and maintenance of homeostasis (contractile vacuoles) (163). In this stage, *A. castellanii* is well adapted for feeding on surface-attached microorganisms though phagocytosis (pseudopodia surrounds the prey, forming a phagosome which later fuses with a lysosome) or by taking up nutrients via pinocytosis (163). *A. castellanii* trophozoites is characterized by spiny protrusions (acanthapodia) on the cell surface used for attachment, locomotion and that have even been suggested as a prey capturing mechanism (36, 163). Like all other protozoans, the phagocytic vesicles limits the prey size *A. castellanii* can handle (163).

The second stage of the amoeba life cycle is encystment, which involves trophozoites undergoing complex cellular differentiation to form cysts. This process is a survival response triggered by environmental stresses such as space and nutrient limitations (342). Once encystment is initiated, trophozoites become committed and undergo dehydration, reduction in cytoplasmic mass and gradual changes in cell wall structure and composition, resulting in a mature cysts,

characterized by a distinct double-layered cell wall, which is resistant to desiccation and capable of surviving prolonged starvation (35, 342). Excystment is initiated when nutrients become available in the surrounding environment. This process is characterized by the emergence of a trophozoite from ostioles, a distinct area in the cyst wall, resulting in an empty ‘shell’ (342). Both encystment and excystment are distinctive cellular differentiation processes, which contribute to the opportunistic lifestyle of *A. castellanii*, allowing it to persist in the environment.

There are constant interactions between the huge diversity of prokaryotes and eukaryotes (206). Flagellated and ciliated protozoa that are commonly found in plankton are involved in transient interactions, grazing on bacterioplankton and bacterial biofilms. Sessile protozoa like amoebae or suctorian ciliates, which only feed on attached biofilms are involved in more permanent interactions with their prey (239). An improved understanding of the interactions of different classes of protozoa with the type of prey that they are adapted to graze on, would facilitate our understanding on how microbial communities are shaped by predation.

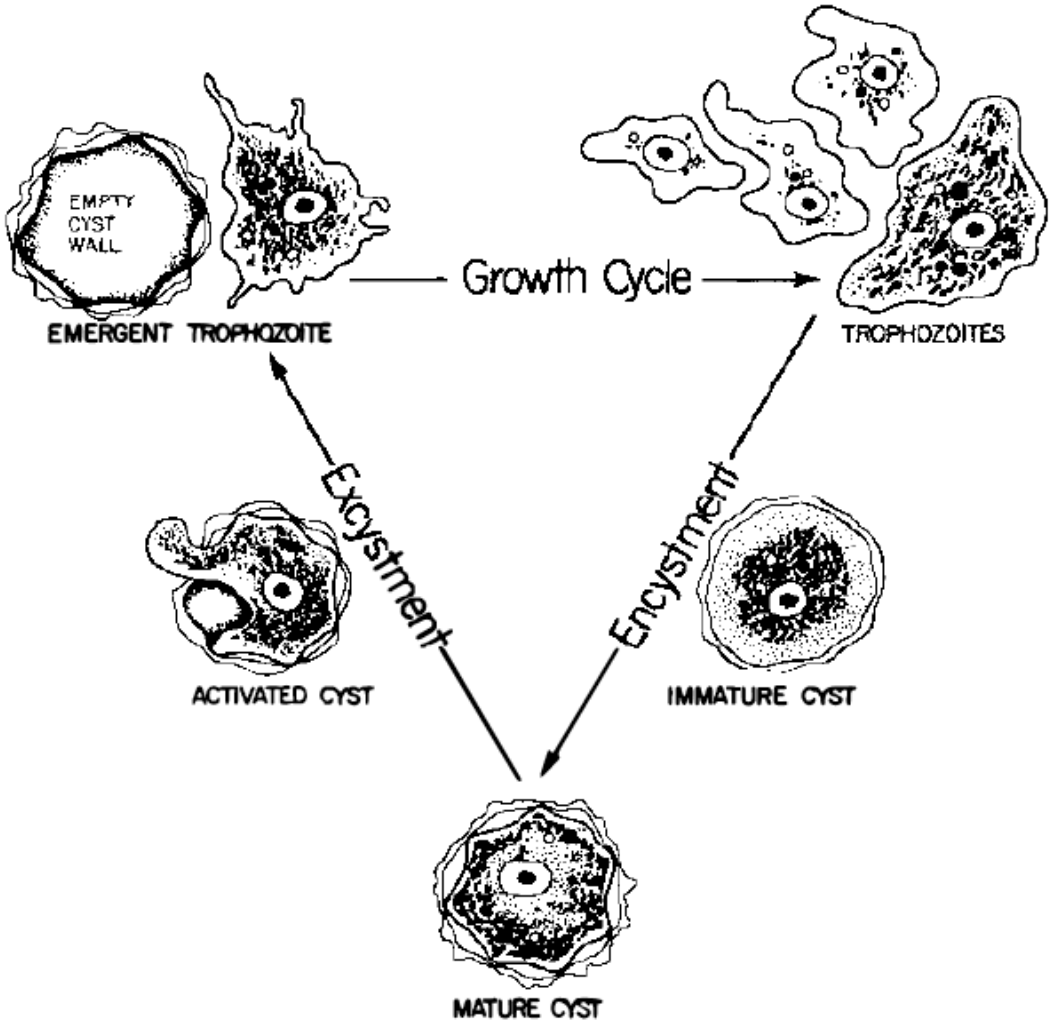


Figure 1.3: A simplified diagram portraying growth and differentiation in the opportunistic pathogen, *Acanthamoeba castellanii*. The process of encystment can be initiated either spontaneously or when nutrients become depleted. Excystment occurs when nutrients become available resulting in the exit of a trophozoite, which proliferates until environment stresses again induce encystment (342).

1.4.2 Protozoan metabolism

Free-living protozoa are generally heterotrophs which rely on phagotrophy, such as bacteriovory or herbivory and adsorption of nutrients for survival. In aquatic environments where there is distinct stratification in the oceans from pelagic euphotic/aphotic to benthic systems, protozoa have adopted different trophic habits (autotrophy and mixotrophy) in order to survive (288). Autotrophic eukaryotes are the largest primary producers of organic compounds in the oceans. However, there has been ongoing debate as to whether these photosynthetic

eukaryotes should be classified as protozoa or algae. Dinoflagellates, which consist of phagotrophic and autotrophic members, are understood to have evolved from obligate heterotrophs (137). These autotrophic dinoflagellates are involved in a long-standing endosymbiotic relationship with their algal prey, through which they have acquired their remnant chloroplasts, now used to supplement their nutrient intake (thus making them mixotrophs) (137). Mixotrophs are expected to have lower fitness due to the energetic cost of having to produce and maintain the apparatus for both photosynthesis and phagotrophy as compared to specialists, which only need to maintain one system (323). However, the benefits of maintaining a mixotrophic lifestyle outweigh the cost for *Orchomonas minima* by allowing it to outcompete autotrophic microalgae under oligotrophic and limited light conditions (92). Thus, under low light and nutrient conditions, mixotrophy might in fact be a more efficient alternative survival strategy than specialization (161, 305).

Heterotrophic protozoa, are as abundant and diverse as autotrophic protists but they lack chloroplasts and therefore depend on phagotrophy for nutrient acquisition (90). These heterotrophic protists are capable of ingesting a wide variety of prey ranging from bacteria to small bacterivorous flagellates and algae, making them important links in the microbial food web (286-287). Predator ingestion rates of bacteria, vary depending on the type and size of predator, the concentration of the bacterial prey and the bacterial phenotypic form (planktonic, attached immature biofilm or mature biofilm coated with EPS) (27, 103, 239). Despite these varying bacterial clearance rates, the ubiquity of heterotrophic protozoa makes them one of the major predators in the microbial food web. As such, they play a significant role in shaping bacterial communities by controlling bacterial diversity and biomass (110).

1.5 Predator-prey interactions

1.5.1 Protozoan feeding preferences

Phagotrophic protozoa are major consumers of environmental bacteria and, as such, are a major factor in bacterial mortality. The intense predation pressure exerted has the potential to control bacterial biomass and causes phenotypic and

genotypic changes in prey, resulting in community and/or species level responses (110, 156). An example of a community level response is the morphological and taxonomical shifts induced by size-selective grazing in bacterial communities (110, 149).

Major characteristics affecting grazing are bacterial prey cell size, morphology and motility. Different categories of protozoa graze efficiently on different size ranges of bacteria (83). Filter feeders, like ciliates, display a preference for larger prey, whereas flagellates are interception feeders that prefer to prey on medium sized bacteria, leaving the smallest (< 0.020 μm^3) and largest (> 0.1 μm^3) bacteria untouched (56, 83). This size–dependent grazing, was further confirmed by Simek (293), who exposed heterotrophic nanoflagellates (HNFs) to a mixed community of different sized bacteria. The grazing pressure exerted on the medium-sized bacteria (0.4 – 0.6 μm^3) were three orders of magnitude higher than that experienced by the small bacteria (293).

Size-selective grazing by ciliates and HNFs generally occurs due to the correlation between contact probability and prey cell size with small cells evading predation as a result of lower contact probabilities. Nevertheless, not all larger prey are edible and the formation of filaments, flocs, and aggregates is an example of a negative correlation between contact probability and size (198). Although increased rates of encounter are observed, the increase in bacterial size due to filamentation or aggregation induced by grazing pressure results in grazing resistance as bacterivorous predators experience ingestion inefficiency (60, 109, 186). These reports suggest that size-dependent preferential grazing can cause dramatic morphological and taxonomical shifts in the bacterioplankton community. Hence, in aquatic systems, protozoan bacteriovory is one of the contributing factors for the maintenance of a stable bacterioplankton distribution by controlling bacterial abundance and metabolic diversity within the assemblages (104, 285).

The swimming speed of prey is another factor governing predator feeding preference. Matz and Jürgens (203) showed that even though contact rates between predator and prey increase at high motility rate, protozoa experience difficulties in capturing and ingesting prey moving above the threshold of 25 μm/s (203). In addition to size and motility of prey, preferential grazing by protistan

predators is also affected by a variety of other characteristics displayed by prey. HNFs and ciliates in aquatic systems have graze selectively, based on the quality of their prey, discriminating and avoiding surrogate prey such as latex beads and heat killed bacteria (105, 218, 293). Planktonic bacterial prey that are actively metabolizing and have undergone active division in such systems are usually larger in size compared to the others and support better growth of their protozoan grazers (72). Based on the optimal foraging theory, the phenomena of predators selectively cropping larger cells and preferring live over surrogate prey, can be attributed to their preference for improved nutritional quality (285, 293).

The production of pigments by bacterial prey also affects protozoan grazers (284). A positive correlation between pigment production and bacterial toxicity, results in amoebae displaying a preference for non-pigmented over pigmented prey (339). The cell surface properties expressed by a bacterial prey are significant contributing factors to successful phagocytosis and grazing discrimination, as predation by protozoan grazers such as amoebae is mediated by interactions between predator receptors and prey epitopes (3, 284). Predator receptors bind to prey epitopes with different affinities such that slight changes in prey epitopes are sufficient to trigger discrimination in amoeboid predators (34, 346). Amoebae such as *A. castellanii* respond to the chemical stimuli produced by different bacteria via membrane receptors (284). These receptor-mediated recognitions of prey are essential for the binding and internalization of prey during the initial stages of phagocytosis. In addition, the chemosensory response benefits the predator by reducing prey search time (3, 218).

Prey discrimination strategies are also affected by prey concentration and can vary between different species of predators. For example, in HNFs, the expression of such selective feeding strategies is upregulated in an ecosystem with high prey concentration as there are no detrimental effects on the predator. Conversely, in a system where scarcity of prey exists, prey selection due to nutritional quality could prove fatal to HNFs and is therefore terminated (29). On the other hand, low prey concentration induces an increase in food selectivity in ciliates which increases nutritional benefits of ingested prey (218).

The satiation state of the protozoan grazer is another factor influencing grazing habits of protozoa. Starved flagellates feed indiscriminately on bacterial prey and

their surrogates at a faster initial rate than their exponential phase non-starved equivalents (28). The retention time of prey surrogates in their food vacuoles is also significantly longer (28). Similarly, in a separate study done with an amoeboid grazer, starved amoebae were shown to have higher grazing rates compared to their satiated counterparts (186). This grazing disparity could be attributed to the digestive potential of the grazers as satiated amoebae contained a higher number of food vacuoles and therefore were unable to accommodate additional prey. Alternatively, starved amoebae, with their accumulation of digestive enzymes could readily respond to the presence of prey (186). Constant exposure of a grazer to a certain prey results in increased grazing rates of that particular prey as the predator becomes adapted to prey capturing and processing, further contributing to prey discrimination (30).

In general, selective feeding in protozoa is not a simple process only dependent on prey characteristics. Instead, the grazing process is affected by both intrinsic (characteristics expressed by predator) and extrinsic factors (environment and prey characteristics). Because of the complexity of predator-prey interactions and the factors influencing a predator's choice of food, the effects of predation on an ecosystem is multifaceted and multifactorial as the underlying mechanisms driving predation.

1.5.2 Ecological impacts of grazing on the marine ecosystem and its microbial communities

As major consumers of bacteria, protozoa account for major mortalities in bacterial communities and have a significant role in controlling bacterial productivity and diversity (285). The equilibrium of bacterioplankton productivity within a system is a result of a complex interplay of 'bottom-up' and 'top-down' control factors (340). In oligotrophic systems, selective grazing is one of the key 'top-down' control factors determining bacterial community composition (295). In a resource limited system, the selective feeding habits of these grazers have the potential to strongly alter population stability causing population, taxonomical and morphological shifts in a bacterial community (296, 311). On the contrary, in eutrophic systems, inorganic and organic nutrients are the main 'bottom-up' factors controlling abundance, biomass and production in planktonic bacterial

communities (295). Although, significant increases in bacterial productivity are observed in eutrophic environments, these are accompanied by increases in viral and protozoan abundance and related mortalities with viruses exerting stronger selective pressure (340). Thus, protistan grazing plays less of a role in shaping bacterial communities in eutrophic systems (295).

Protistan bacteriovory can also exert an indirect effect on bacterioplankton biomass. Protozoan grazing activities contribute to nutrient remineralization and recycling and stimulates bacterial growth, returning more carbon, nitrogen and phosphorous to the nutrient cycle (285-286). Protozoan excretions also provide a valuable source of organic compounds and trace metals for bacterial uptake (288). A positive feedback in the relationship between predators and prey occurs as grazing controls bacterial productivity but at the same time supports and stimulates bacterial growth (285).

In many systems, predation not only controls biomass, but also assumes a more significant ecological role in shaping bacteria communities, determining the taxonomic and morphological compositions that remain. Grazing events can induce morphological and taxonomical changes in model bacterial communities. For example, community composition in a model community shifted from predominantly edible medium-sized cells consisting of β-Proteobacteria to a community dominated by inedible Cytophaga/Flavobacterium filaments and γ-Proteobacteria (296-298). In a separate study, a community consisting of toxic *Pseudomonas fluorescens* and non-toxic cheaters, the latter benefited from by-products of the wildtype strain without having to produce the by-products, allowing it to dominate in a mixed community. The introduction of a grazer, with a preference for the cheater, alters and shifts the community to the advantage of the wildtype strain by decreasing the fitness of the cheater (153).

The impact of grazing on the trophic cascade and community structure was further elaborated by Wey *et al.* (345), who showed that the introduction of HNFs led to morphological shifts from a bacterial community characterized by single cells to one dominated by microcolonies. The introduction of ciliates, which grazed on HNFs, relieved grazing pressure to a certain extent, allowing the population of single cells to increase (345). In a separate study, Beardsley *et al.* (14) observed an increasing proportion of larger cells at reduced HNFs densities. However, once

regrowth of HNFs occurred, a decline in bacterial cell numbers also occurred, coinciding with an increase in bacterial size. Similarly, Hahn and Hofle (109) observed taxonomical changes accompanied by bidirectional shifts in bacterial cell size from single cells to aggregates of *Pseudomonas* sp. within the complex community being grazed. The shift to microcolonies and aggregate formation was stimulated under intense grazing pressure. Amoeboid and ciliated grazers had the ability to become integrated within the biofilm itself, causing sloughing and reductions in biofilm biomass by their movement and grazing activity (136). Cyclical shifts in size obviously contribute to predator/prey dynamics in aquatic systems (14). The morphological and taxonomical alterations induced in the community as a response to grazing is the result of the interactions between a variety of factors involving the grazer's discriminating feeding behavior, size distribution of prey and the response and growth of prey in the presence of grazers (109).

Obviously, protozoan grazing has profound ecological implications which act to stabilize or destabilize a bacterial community (234, 334). Size-selective grazing is destabilizing when protozoa constantly graze on their desired prey size and do not alter their behavior at low prey concentrations by switching to a more abundant prey (311). This constant selection can ultimately lead to the elimination of the preferred prey and hence affect higher and lower trophic levels (273). In contrast, other types of discriminatory feeding behavior such as prey ratio-based selection and abundance-based selection have stabilizing effects on prey abundances. In both cases, the predator undergoes behavioral changes to exercise prey switching to alternative prey, whenever the concentrations of preferred prey are low, thus preventing complete elimination of any prey species (311). Prey switching is dependent on prey densities, prey preference such as size and energy input and prey handling time of the predator (313, 334). This optimal foraging strategy, adopted by opportunistic predators for exploitation of the most abundant prey species whenever preferred prey densities drops below a threshold level (334), is fundamental in promoting species diversity in a community, allowing less superior competitors to grow (209, 327).

1.5.3 Application of eukaryotic ecological theory as a conceptual framework for microbial ecology

Microbial ecology and eukaryotic ecology have largely progressed as two separate entities. In eukaryote ecology, there is a rich understanding of ecology based on predator-prey interactions. For example, eukaryotic defense theories describing plant-herbivore interactions are well developed (120, 303). In recent years, the use of eukaryotic ecological theories for investigation of microbial systems has been introduced to improve our ecological understanding of how bacterial communities respond to grazing (155, 206-208). Plant defense theories, as conceptual frameworks for predicting biofilm defense strategies, have proven particularly relevant in this regard. Like plants, biofilms have a sessile mode of life where there is constant exposure to predatory grazers, similar to exposure of plants to herbivory (273, 308). Expression of morphological, structural or chemical defense (allelopathy) is hence crucial for inhibiting or reducing the impact of grazing in both systems (see below) (273). Terrestrial plants release volatiles in response to herbivory, that lead to the recruitment of co-evolved herbivore predators (75). Similarly, bacterial communities also produce chemoattractants. For example, the production of a thermostable compound by *Escherichia coli* induces chemotaxis in a range of protozoan predators (76). These chemotactic compounds also attracted an influx of predators targeting protozoa (6). Evolution among plant communities, has also led to the occurrence of signal cheaters, which exploit the benefits of signal production without paying any production costs. This behavior is homologous to the social cheaters observed in *P. aeruginosa* biofilm communities (75, 282).

The production of defensive allelochemicals in plants is an evolutionary consequence of maximizing fitness in the presence of grazers and requires efficient resource partitioning (120). As most defensive responses are dependent on environmental nutrient availability, numerous models have been proposed in an effort to predict the defense response expressed. One of the more fundamental theoretical models is the growth-differentiation balance (GDB) hypothesis (190), which incorporates other ecological theories such as the carbon: nutrient balance (CNB) hypothesis (43) and the growth rate hypothesis (59, 120). The GDB hypothesis proposes a negative correlation between competitive growth (e.g. cell division) and differentiation processes (e.g. chemical defense production), as both

processes share common precursors and intermediates (59, 181). Under low to moderate nutrient limitation, demands for growth and defense processes cannot be met simultaneously. As a result of the interactions between antagonistic species (e.g. grazing and competition) and growth differentiation processes, trade-offs are made and secondary metabolism for increased resistance and fitness, receives priority over competitive growth (303). Energy and metabolic resources are diverted from growth-related processes and allocated to the production of defensive metabolites (allelopathy).

In contrast, in nutrient replete environments, the growth rate hypothesis, also known as the resource availability hypothesis, predicts that production of defense metabolites is inversely correlated with growth rate and nutrient availability (59). Resource availability is a limiting factor determining the inherent growth rate in organisms and therefore also affects the production of chemical defenses. Where competitive growth is prioritized, fast growers are capable of compensating for grazing losses by exploiting nutrient availability and maintaining low levels of allelochemicals (120). As nutrient levels decrease, growth compensated resistance becomes too costly and chemical defenses are preferentially selected. However, in nutrient limited environments, growth-compensated resistance is inhibited and slow-growers, which are more efficient at maximizing the use of limited nutrients for production of chemical defenses are favoured (59). The GDB and the growth rate hypotheses are inter-related and depending on nutrient conditions, plants can either compensate for losses due to predation by rapidly growing or by the continual production of secondary metabolites (120, 128). As both growth-compensated resistance and allelopathy have also been demonstrated in bacterial models, in response to predation by protozoa or interspecies competition, these ecological theories provide an ideal basis for understanding complex interactions (explored in Chapters 3 and 4) (205).

The environmental constraint (or CNB) hypothesis, proposes that production of secondary metabolites is dependent on the environmental availability of carbon and nitrogen, which affects resource allocation to either carbon- or nitrogen-based defenses (114). On the assumption that competitive growth is prioritized, allelochemical production will only occur once growth requirements are achieved (114). In nitrogen depleted plants, for example, the accumulation of carbon in excess of growth requirement relative to other nutrients, diverts excess carbon to

the production of C-based defenses such as terpenes and phenolics; i.e. carbon-based defenses are upregulated (128). Conversely, the introduction of nitrogen means that competitive growth receives preferential resource allocation; there is a drop in C-based defenses. As growth proceeds, other growth factors become limiting, resulting in the excess nitrogen assimilated being allocated to the production of N-based defenses; i.e. N-based defenses are upregulated (120, 303).

Other ecological concepts such as keystone species can also be applied to study predator-prey interactions. A predator that prevents complete domination of a system by a specific prey is termed a keystone predator (215). For example, the presence of keystone species in predator-prey interactions can have far-reaching ecological effects on a community. Removal of these keystone predator species can lead to competitive exclusion, disrupting the community organization and decreasing species richness and diversity (215). In a study done by Paine (273) on predation in an intertidal system, the removal of a predator drastically altered the community composition at lower trophic levels as preferential grazing pressure on the competitively superior prey species was relieved, allowing them to outgrow and exclude their inferior competitors (249, 273). Similarly, a keystone prey is one, which is not necessarily the preferred prey but is resilient to predation via growth-compensated resistance thus sustaining growth of the predator, leading to increased grazing rates on other prey (129). A keystone prey is therefore, indirectly capable of restricting growth parameters available to other prey, limiting their abundances, thus influencing the community composition (129). The removal of a keystone prey can increase or decrease species diversity depending on grazer prey preference. If the grazer exercises prey switching by grazing on the keystone prey when preferred prey densities are low, species local extinction is averted as numbers of preferred prey are able to recover (228). On the contrary, if prey switching is not practiced and preferred prey densities are low, prolonged grazing will lead to species local extinction and hence a drop in species richness and diversity for that community (228).

Clearly, the structure and composition of bacterial communities in the environment are not solely dependent on protozoan selective grazing. Instead, interspecies and intraspecies interactions within the community in response to environmental factors such as nutrients and temperature are equally as crucial (136). Overall, the coexistence and interactions of both predator and prey in an

environment that arises from chemical defense and prey discrimination determines the predator/prey dynamics occurring and subsequently the bacterial communities that will persist (136).

1.5.4 Grazing resistance mechanisms

Phenotypic plasticity and genetic diversity, coupled with rapid growth rate, have allowed bacteria to adapt rapidly to intense grazing pressure by evolving a diverse array of behavioral, morphological and chemical modifications (154). These defense strategies can protect bacteria before or after ingestion, as a response to changes in the surrounding environment (Figure 1.4) (154, 204). Pre-ingestional defense adaptations can either involve a single-cell bacterium preventing capture (Figure 1.4 a to c) or production of cell aggregates and coordinate toxin production (Figure 1.4 d to e). Post-ingestional defense strategies involve inhibition of digestion within protozoan food vacuoles (Figure 1-4f to h). The resultant impact of these adaptive responses effectively shifts the microbial community to a grazing-resistant population and has a significant role in the transmission and persistence of many environmental opportunistic pathogens (205).

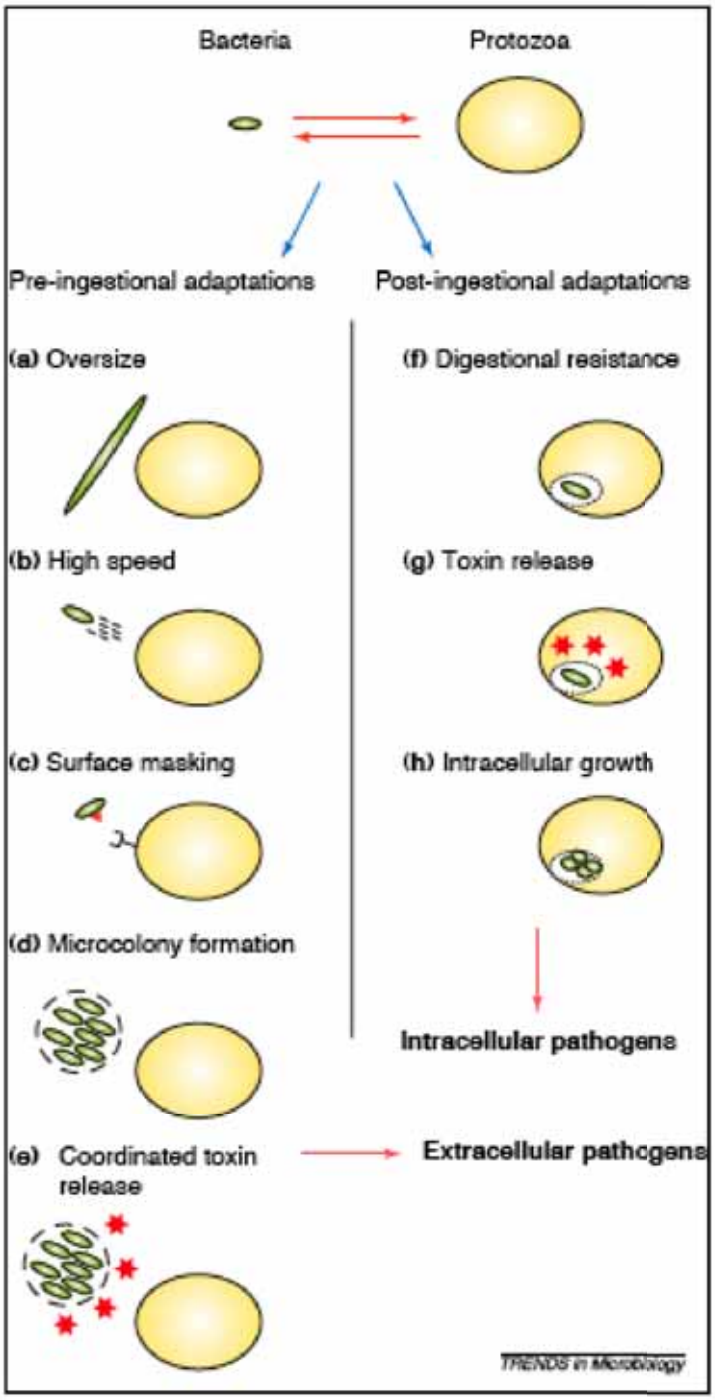


Figure 1.4: Diagrammatic illustration of the potential defense adaptation responses that can be utilized by bacteria to prevent grazing. Blue arrows indicate the pre-ingestional (a-e) and post-ingestional (f-h) adaptations adopted as a result of prey-predator interactions. These defense strategies become more complex when bacteria undergo coordinated behaviour to form multicellular biofilms (d, e and h). Red arrows illustrate the potential routes for contribution to pathogenesis (adapted from (205)).

1.5.4.1 Planktonic-based resistance adaptations

Expression of grazing defense mechanisms by bacteria in aquatic systems can result in an increase in inedible morphotypes. These defense strategies can be classified in three major groups: behavioural, morphological and chemical defense (205). The formation of complex inedible morphotypes such as filaments and spirals is an effective morphological defense against protozoan grazers (154). Chemical cues released from grazing activities of the flagellate *Orchomonas* sp., indirectly stimulate filamentous formation in β-Proteobacteria (111). However, not all classes of bacteria undergo morphological changes that result in inefficient capture and ingestion by their predators. For example, contrasting bacterial defense strategies exist among different classes of bacteria within a microbial assemblage of α- and β-Proteobacteria (248). In the presence of grazers, β-Proteobacteria form inedible filaments, whereas the α-Proteobacteria experience growth stimulation, allowing them to outgrow grazing pressure (248).

Defense mechanisms can also be regulated by 'bottom-up' factors like carbon (C) and phosphate (P). Matz and Jürgens (204) found that the effects of nutrient limitation and protozoan grazing were inter-related as nutrient starvation induced behavioral and morphological changes (204). Under P limitations, an increase in encapsulated filaments, induced by protozoan excretory products, was observed (60, 204, 248). Even though filament formation leads to increased prey capturing efficiency, it also results in handling difficulties by the protozoa, effectively conferring protection (204). Conversely, C starvation promotes cell size reduction (ultramicrobacteria) and higher swimming motility which increase overall fitness. Despite the increase in prey-predator contact rates, Matz and Jürgens (203) witnessed a simultaneous increase in escape rates due to the slow reaction time of flagellates. In a study of the effects of nutrients on the abundance of grazing resistant bacteria, Thelaus *et al.* (318) showed a positive correlation between productivity of an ecosystem and the amount of active bacteria grazed. Hence, the transition from oligotrophic to eutrophic and highly productive environments can potentially increase bacterial susceptibility to grazing by reducing the formation of inedible grazing resistant morphotypes (318).

Chemical defense involving the production of inhibitory compounds or the alteration of prey surface ligands, is another form of defense strategy that can be

adopted by planktonic prey. Inhibitory metabolite production by microorganisms can have broad-spectrum activity against predators and competitors. For example, a membrane-bound, antimicrobial compound with surfactant properties, viscosinamide, produced by *P. fluorescens* exhibits antagonistic effects through growth inhibition of amoebal and ciliated grazers (4).

Consequently, these changes can lead to prey-discrimination in predators due to receptor recognition failure or 'less palatable taste' (239). The majority of these single-cell based defense strategies appear to be expressed rapidly in response to the presence of the grazer. It is possible that a feedback mechanism exists in the form of chemical cues released by grazers and their grazing activity, to induce such defense mechanisms (248).

1.5.4.2 Biofilm-based resistance strategies

The co-existence and close proximity of bacterial prey and protozoan predators within a biofilm community facilitates more complex and intense interactions (e.g. coordinated behavior and cell-to-cell signaling) between biofilm members, thereby increasing biofilms dynamics (239). Hence, the grazing pressure exerted on a bacterial community is a major factor in determining genotypic and phenotypic characteristics of the resulting biofilm and a major selective force for the evolution of diverse defense adaptations to evade predation (205). Such bacteria-protozoa interactions are analogous to plant-herbivores interactions; observations from interactions of plants with their predators and their epiphytic communities are therefore appropriate for application to bacterial systems.

In plant ecology, the susceptibility of a plant to grazing is not only dependent on chemical and structural defenses *per se*, but also involves direct and indirect interactions where members exert influence over one another (329). These interactions result from responses relative to characteristics of other members in the community and can increase or decrease the susceptibility of the focal individual to being grazed, either associational susceptibility (AS) or associational resistance (AR) (12). For example, grazing-induced release of volatiles in neighbours can repel and deter grazers from the focal organism or lead to grazer dispersal, providing the focal organism with AR (12). AR can also occur when

high levels of chemical cues spill over onto the focal organism, providing it with a grazing defense (256). Similarly, in the attractant-decoy scenario, having palatable neighbours can function as a ‘sink’, offering AR to unpalatable focal plants by diverting grazer attention. However, spillover effects can also occur when grazers migrate away from a palatable sink, leading to AS (12). Alternatively, if a grazer demonstrates discriminatory feeding preferences, association of palatable focal organisms with unpalatable neighbors can lead to AS due to avoidance of toxic prey by the grazer and preferential feeding (256). AS and AR can also be mediated by physical traits and structural properties which limit grazer access (12). These mechanisms are comparable to the biofilm architecture, EPS matrix and production of secondary metabolites for defense by bacteria, which can potentially contribute to AS and AR of members in a biofilm community.

The association of bacteria predominantly as biofilms has significant ecological and medical consequences. Both environmental and clinically relevant biofilms are increasingly resistant to physical and chemical stresses (343). The formation of such complex three-dimensional structures is an important strategy for survival and persistence of bacteria and is one of the major defense mechanisms utilized by bacteria for protection against grazers (205-206). Studies done by various groups have shown that a majority of single-celled bacteria adopt such morphological defenses, favouring co-aggregation, flocculation and also the formation of complex microcolonies in the presence of grazers (154, 198, 205). For example, *Serratia marcescens*, an environmental opportunistic pathogen, forms different types of biofilms under different growth conditions. The microcolony-type biofilm formed under batch conditions is resistant to flagellate grazing but sensitive to predation by amoebae. However, under flow conditions, the biofilm undergoes QS-controlled morphological differentiation to form filamentous biofilms that becomes resistant to grazing by amoebae (264). Due to size-selective feeding of predators, these complex structures exceed the threshold required for efficient capturing and handling of the prey (205), effectively serving as a spatial refuge for bacteria, protecting them against predators and mediating AR (154).

Besides providing morphological defense, biofilms produce EPS, a major matrix component. EPS functions as a diffusion barrier for nutrients and enzymes as well as a protective barrier against antimicrobials, enhancing survival and competitive

success of bacteria in the environment. Jürgens and Güde (154) postulated that EPS can potentially function as an ingestion barrier by inhibiting penetration of the predators' digestive enzymes. For example, in *P. aeruginosa*, the EPS components consist of polysaccharides and alginate. Overproducers of alginate appear mucoid and form larger, inert microcolonies in response to grazing compared to wildtype strains, with the former exhibiting better survival rates (198, 201). Grazing resistance of *P. aeruginosa* also dependents on the biofilm developmental stage and the type of grazer (343). Biofilms in the environment are exposed to a succession of grazers and in order to cope with different forms of grazing stresses, different biofilm architectures are formed. The alginate mediated microcolony fomation is an example of an effective deterrent against early stage biofilm grazers but the biofilm is still vulnerable to amoebae, a specialist adept at grazing on biofilms (343). In order to inhibit late stage biofilm grazers, late *P. aeruginosa* biofilms also produce a wide array of QS regulated virulence factors with antiprotozoal activity against flagellates as a supplementary defense mechanism (198). Similarly, grazing resistance in *V. cholerae*, is enhanced by a combination of EPS production and QS-regulated toxicgenic compounds which in turn promotes environmetal persistence (206).

Many bacteria express QS-controlled secondary metabolites which function as predation defenses (207-208). In *Chromobacterium violaceum*, altruistic behavior is displayed when a sub-population of cells is sacrificed, which results in protection of the remaining viable clonal population (200). The biofilm-specific, QS-regulated toxic alkanoid, violacein, is released when the ingested cells are digested within the protozoan grazer, inhibiting the grazer's feeding activity (200, 208). Subsequently, the violacein induces apoptosis in the predator, resulting in its death and the survival of the remaining bacterial cells (208). Violacein is also produced by other marine bacteria such as *P. tunicata*, *P. ulvae* and *P. luteoviolacea* and is an effective form of chemical defense against protozoa and other types of predators (e.g. nematodes) (208).

In addition to synchronized chemical synthesis, bacterial biofilms are also capable of active defense against protozoan grazers. The close spatial coexistence of predators and bacterial biofilms allows bacterial colonization of the predator through the induction and upregulation of Type III secretion system-related (T3SS) genes (207). For example, *P. aeruginosa* biofilms utilize the T3SS-

mediated cytotoxicity against amoebal colonizers, whereby motile bacteria actively and rapidly colonized invading protozoa, thus displaying a coordinated behaviour of prey turning on predators (207). This process of active defense is facilitated by a combination of four effector proteins, the cytotoxin ExoU, bifunctional digestive and inhibitory enzymes ExoS and ExoT that interfere with phagocytosis and protozoan reproduction and the regulatory enzyme ExoY that controls the cells cAMP levels (207). Cell-to-cell contact and injection of potent cytotoxin into the protozoan cytoplasm are also required for effective exploitation and killing of the protozoan predator (207).

1.5.5 Protozoa as microbial Trojan horses: evolutionary link between environmental persistence to intracellular pathogens

Many of the survival strategies of bacterial biofilms described above pertain to diseases in humans. Over the past few decades, there has been an increase in reports on intracellular infections of macrophages and alveolar epithelial cells in both healthy and immuno-compromised individuals by amoebae resistant bacteria (ARB) that are phylogenetically evenly distributed, ranging from Proteobacteria to Chlamydiae, Flavobacteria, Bacilli and the Actinobacteria (107). The origin of invasion phenotypes and adaptation to intracellular survival within complex eukaryotic hosts like nematodes and humans by these virulent pathogens, are thought to have evolved from bacterial post-ingestional defenses used to evade and prevent digestion by protozoan predators such as amoebae and ciliates (42). Due to the similarities in the invasion, exploitation, survival and exit strategies utilized by bacteria within macrophages and amoebae, the potential for using protozoa as a model for understanding the basis of such bacteria-macrophage interactions is apparent (107, 164).

Some bacteria have evolved the ability to develop transient or stable interactions with their protozoan hosts as either a facultative or obligate intracellular pathogens or endosymbionts (13, 131). As a result of these close interactions, protozoans are viewed as training grounds for the selection of intracellular pathogens adapted to life within eukaryotic hosts (*Legionella* sp. and *Chlamydia* sp.) (217). The process of infecting a protozoan predator is initiated when a bacterium gains entry into the host usually through phagocytosis (receptor

mediated endocytosis or coiling phagocytosis) (Figure 1.5) (138, 217). This entry route is crucial in preventing lysosomal fusion by directing food vacuoles away from lysosomes to other sites like the nucleus, the endoplasmic reticulum, the cytoplasmic space or the space between the double-walled cysts of amoebae used for successful invasion and multiplication (13, 42, 154, 164). Subsequently, engulfed bacteria may utilize three different strategies for survival; exploitation of the host cell metabolic and genetic machinery for multiplication followed by lytic dispersal; multiplication and exit by exocytosis of pathogen filled vesicles; or remaining dormant within the host and thus avoiding digestion (13, 107). Hence, the bacteria-protozoa relationship that develops is highly dependent on the survival strategies adopted and can either be parasitic or endosymbiotic.

A well-characterized interspecies interaction is that between the causative agent of Legionnaires' disease, *Legionella pneumophila*, and its protozoan host, *Acanthamoeba* sp.. The ability of *L. pneumophila* to persist in the environment is attributed to its primary mode of propagation, which requires exploitation and growth within an amoebal or ciliated host (131). The entry mechanism of *L. pneumophila* is dependent on the species of amoebae. For example, coiling phagocytosis is required for uptake by *A. castellanii*. However, in the case of *Hartmanella vermiformis*, protozoan receptor (macrophage infectivity factor or Mip) mediated attachment is required before invagination of the amoebal cell surface occurs (42). Blockage of lysosomal fusion is also essential and this is achieved through the secretion of signal proteins across pores inserted into the host membrane by the Dot/ICM type *IV* secretion system (217). This is followed by migration of the bacterial phagosome to the rough endoplasmic reticulum (RER) for the recruitment of host cell machinery (42). Subsequently, lysis of the phagosomal membranes occurs, releasing *L. pneumophila* into the cytoplasm for replication. In the late stages of the infection process, a combination of Mip and temperature-dependent necrosis of the amoebal host occurs through the insertion of a pore within the host cell membrane, for the dispersal of motile cells to colonize new niches (107, 194).

Adaptation and growth within the intracellular environment serves as an evolutionary selection for a variety of invasive bacterial phenotypes (13, 42, 131). *Legionella* sp. grown within protozoa is more invasive for macrophages and epithelial cells and displayed increased resistance against anti-microbials

compared to those cultured on agar plates (13, 42, 194). The ability of amoeba to encyst provides an additional level of protection to intracellular pathogens against environmental stresses and high concentrations of biocides (131, 188). Amoebal cysts exhibit a higher level of resilience compared to their trophozoite counterparts and can also be easily transmitted via aerosols, causing disease in humans and animals (42, 107). The unique ability of *L. pneumophila* to survive within cysts is a major factor in preventing eradication and the host/parasite interaction promotes adaptation and increases phenotypic diversity, thus contributing to its persistence and infectivity in humans (13, 163, 217). Clearly, environmental protozoa are not only important microbial reservoirs for evolutionary adaptations but they also serve as transmission vectors of pathogenic bacteria with the potential of causing health problems.

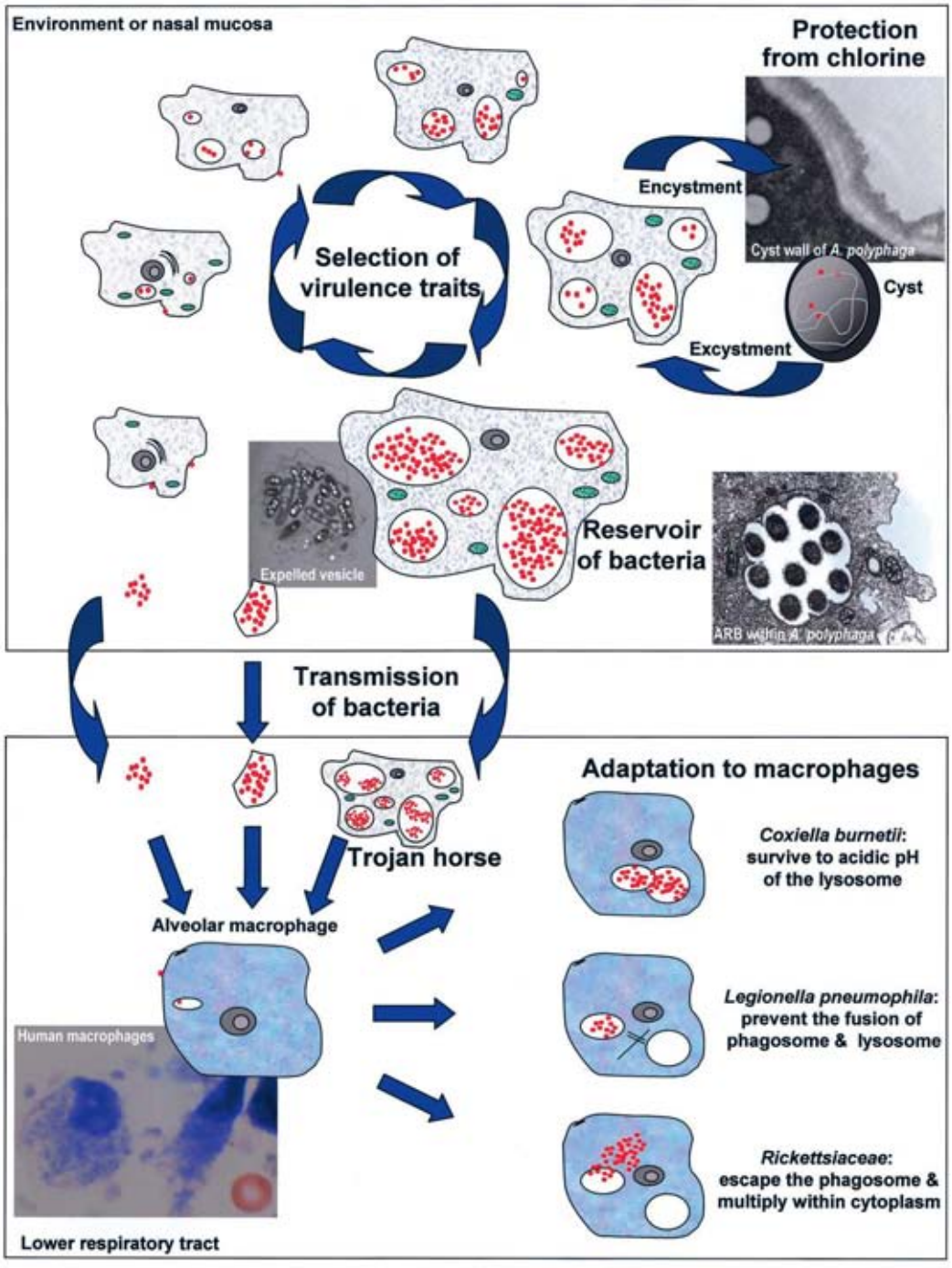


Figure 1.5: A simplified diagram illustrating the roles of amoebae (blue) as a spatial refuge for intracellular facultative and obligate pathogens (red) to the transmission of these pathogens from the environment to the nasal mucosa (107).

1.6 Eukaryotic models for screening of bacterial anti-protozoal and anti-nematode activities

Life on Earth originated in aquatic environments, so it is not surprising that aquatic environments host a huge diversity of eukaryotes, archaea and bacteria (221). As a result of the unpredictable environmental conditions, all domains of life have evolved adaptations for survival within their habitats, by forming close associations that may involve commensalism, parasitism, mutualism and symbiosis. These associations, which are generally multi-factorial, require specific interactions between predator-prey or parasite-host (270). There is clearly a need to elucidate the underlying mechanisms of the complex strategies used by these bacterial opportunistic pathogens for persistence in the environment.

The two eukaryotic model systems selected here for screening of biofilm-derived bioactives were *Caenorhabditis elegans* and *A. castellanii*, representatives of helminths and protozoa, respectively. To gain an understanding of the impact of such grazing activities on a community of bacteria, a range of different protozoan grazers were investigated.

1.6.1 Protozoan models for testing parasite-host and predator-prey interactions

Protists are the most cosmopolitan, simple unicellular eukaryotes (163). They appear in a variety of different forms and have evolved complex membranes and organelles to adapt to different environmental conditions. As a result of their bacterivorous activities, protozoan predators can appear as free-living organisms or commensals, mutualists, or even parasites to higher eukaryotic hosts (270). Predation by free-living protozoa emulates fundamental predator-prey interactions. Due to the selective feeding habits of protozoa, coupled with the inter- and intraspecies interactions within a consortia, predation is important in structuring bacterial community composition (296-297). In addition, protozoan predators also play a significant role in nutrient regeneration, biogeochemical cycles and microbial food webs (287-288). In order to understand the complexity of the predator-prey interactions, a variety of protozoa with different feeding

strategies were selected to determine the impact of grazing on a mixed-species consortium.

Predator-prey interactions can be detrimental when the predator abandons it complex sensory and feeding mechanisms to evolve into an ecto- (living on host's surface) or endo-parasite (living within host), both dependent on the host for survival (270). Heterotrophic protozoa are generally commensal. However, they may become opportunistic pathogens capable of causing mortality in their host when they exploit changes in environmental conditions such as salinity and temperature beyond the hosts' optimum, for their own growth benefit (270). For example, the free-living amoebae, *Neoparamoeba pemaquidensis*, the causative agent of amoebic gill disease, becomes an opportunistic pathogen when it attacks the gills of salmonid fish at high temperature and salinity (causing significant economic damage) (270). In addition to aquaculture, amoebic related medical infections such as *Acanthamoeba* keratitis, *Acanthamoeba* granulomatous encephalitis and cutaneous Acanthamoebiasis are products of the opportunistic nature of protozoa (163, 194).

The above mentioned observations reinforce the need for novel bioactives that exhibit anti-protozoa activity for aquaculture, livestock and medical use (163). The marine environment, with its huge diversity of microbial communities and untapped resources provides a reservoir for the discovery of potential bioactives (233). *A. castellanii*, which is resistant to a range of drugs and disinfectants, was considered to be an appropriate model for screening for potential inhibitors.

1.6.2 *Caenorhabditis elegans* as a model organism for screening of anti-helminths bioactives

Caenorhabditis elegans is a free-living nematode, ubiquitous in the soil environment, usually found in association with microbial blooms in compost heaps. Due to its small size (1.5 mm adults), rapid growth rate (three day generation time), reproductive capability (300 to 350 progeny per hermaphrodite), voracious feeding habits and ease of laboratory cultivation, *C. elegans* has significant potential as a model organism for the screening of anthelminthic bioactives (274). After hatching, *C. elegans* undergoes four postembryonic larval

stages of development (L1 to L4) (274). The average life span of a nematode is approximately three weeks under non-limiting substrate conditions. However, due to its feast and famine survival strategy, when depletion of microbial food sources occurs, *C. elegans* undergoes growth arrest and rearranges its metabolic activities in preparation for a stage of diapause (dauer larvae) (274). The formation of dauer larvae in *C. elegans* is a survival adaptation facilitating dispersal in new environmental niches (274).

The phylum Nematoda is composed of numerous families and species, well adapted to terrestrial and aquatic environments. Although the majority of nematodes are free-living, parasitic members exist which cause disease in marine, aquaculture and mammalian hosts (270). The diseases arising from nematode related infections are detrimental to the aquaculture and livestock industries (270). Parasitic nematodes have similar life cycles, survival strategies and basic anatomical structure with their non-parasitic counterparts (274). In addition, parasitic gene homologs discovered in *C. elegans* have been used as drug targets to obtain positive hits, which can be further tested on parasitic nematodes for anthelmintic compounds research (274). Therefore, the free-living *C. elegans* is an appropriate model system for the screening of anti-helminths compounds.

1.7 Aims of this thesis

In the recent years, synergism and antagonism have both emerged as recurring themes in mixed-species biofilm research. Such intra- and interspecies interactions, have been commonly studied in laboratory mixed-species biofilms, constructed to resemble environmental biofilms (5, 67, 299). The community composition of a mixed-species biofilm is dependent on the outcome of these interactions, which can potentially affect the response against environmental perturbations such as grazing.

The model mixed-species community comprised epiphytic isolates harvested from the green alga *Ulva australis: Microbacterium phyllosphaerae, Shewanella japonica, Dokdonia donghaensis* and *Acinetobacter lwoffii* (44). This four-species community interacts synergistically to exhibit higher levels of resistance against chemical stresses and invasion by *Pseudoalteromonas tunicata,* compared to

single-species biofilms (44). The diversity-functional stability displayed here against *P. tunicata* invasions indicates that the four-species model provides excellent opportunities for testing other eukaryotic ecological models and for investigating biofilm response to protozoan grazing.

The aim of this study was to understand the complex inter- and intraspecies interactions occurring in the model community, for an insight into how environmental biofilms function. Using that as a basis, environmental biofilms were screened for antagonistic activities against eukaryotic grazers to identify producers of inhibitory metabolites for potential industrial application. This work was further extended to determine the type of response between members in the mixed-species biofilms, when exposed to different-stage protozoan grazers and under varying nutrient conditions. As biofilms are complex communities with diverse functional responses, they serve as ideal candidates for testing and addressing community ecology and ecological defense theories.

1.7.1 Chapter synopses

Chapter 2 describes the method development, optimization and also validation of the amoeba- (*Tetrahymena* sp.) and the nematode- (*C. elegans*) based high throughput screens used for the identification of bacterial strains exhibiting biological activity. Biofilm communities in the environment that are dynamic in both synergistic and antagonistic interactions were screened for the production of inhibitory secondary metabolites. Evidence demonstrating higher levels of biological activity in biofilms is presented. Some of the active strains have been identified and sent to collaborators at Murdoch University for tests against parasitic protozoa.

Chapter 3 describes the complex inter- and intraspecies interactions occurring in a defined marine mixed community and the impact of grazing by different niche specific grazers on the community composition, diversity and ecological niche preferences of each species in the community. Evidence showing that protozoans do exhibit selective feeding preferences and that different grazers are more adapted to grazing on different stages of biofilms is presented. Antagonistic interactions are also shown to occur between members in mixed-species biofilms,

as a result of competition over space and nutrients. However, synergism is also present through commensal interactions. *M. phyllosphaerae* was demonstrated as a superior competitor sustaining the predator population and suppressing growth of all other members. *A. lwoffii*, on the other hand, is a poor competitor that does not appear to contribute to the mixed-species biofilm consortia.

Chapter 4 outlines the consequences of low nutrient conditions on the resistance of mixed- and single-species biofilms against *A. castellanii* grazing. Under low nutrient conditions, both mixed- and single-species biofilms were susceptible to *A. castellanii* grazing. The CNB hypothesis eukaryote-formulated defense theory , was applied to single-species biofilms of *M. phyllosphaerae* and *S. japonica* to predict the type of defense adopted against *A. castellanii* grazing, when CNP nutrient conditions were varied. *M. phyllosphaerae* was resistant to grazing under all nutrient variations. Whereas, in *S. japonica*, the decreased grazing resistance observed under low carbon concentrations, suggests that the grazing defense expressed was carbon-based.

Chapter 5 provides a summary and overview of discussion of the work present in the previous chapters. Possible directions for future research are also suggested.

Chapter 2 : Screening for novel anthelminthic and anti-protozoa compounds produced by bacteria

2.1 Introduction

Novel bioactives for the treatment of microbial and parasitic diseases are in constant demand due to the inevitable development of multi-drug resistance to compounds in current use, which causes significant economic burden to the livestock industries and human health (53, 187). For example, parasitic nematodes that commonly affect sheep, goats and cattle are now found to be resistant to commercial drugs such as benzimidazoles, levamisole, ivermectin and moxidectin (159). Even in domestic pets where anthelminthic resistance was previously not considered a serious issue, there are indications of drug resistance (e.g pyrantel resistance in canine hookworm) (173). Current drug treatments for parasitic diseases are based on drugs discovered decades ago, often used in combination (253). In the recent years, despite the alarming emergence of drug resistance, only three classes of anthelminthics (emodepside and welpan for domestic felines and canines and baymec for cattles) and one anti-protozoal (ponozuril for equine) drugs have been successfully developed and marketed commercially (108, 117). Therefore, with the increase in cases of multi-drug resistance parasitic diseases, there is increased need for development for new bioactives (253).

The most promising approach for the identification of novel antimicrobials and anti-parasitic drugs is to screen microorganisms for the production of natural bioactives, and to use synthetic chemistry to optimize activity (58, 331). Competitive amensal interactions between organisms in environmental communities are widespread and select for the production of inhibitory secondary metabolites to maximize individual fitness (81, 258). The diversity and range of inhibitory metabolites produced by the target organisms usually correlate with the microbial diversity of their environmental niche. For example, a common broad range antibiotic, streptomycin, is isolated from *Streptomyces* spp., a soil inhabitant which is well adapted to an environment rich in species diversity (121,

330). In contrast, agrocin 84, a narrow spectrum antibiotic, which specifically targets *Agrobacterium tumefaciens*, is produced by *Agrobacterium radiobacter* K84 for the removal of *A. tumefaciens* from its desired niche (121). This form of chemical defense which involves the production of inhibitory metabolites, has been observed in a variety of eukaryotes and prokaryotes and has been exploited for the isolation of bioactives during natural product discovery (99, 233). The discovery of the first antimicrobial, penicillin B, produced under stress by *Penicillium notatum*, by Alexander Fleming and Howard Florey, followed by Selman Waksman's discovery of streptomycin in 1952, are examples of successful isolation and use of natural bioactives for medical purposes (91, 276, 330).

Microbial biofilm communities, potentially provide a reservoir for the isolation of inhibitory metabolites. These epibiotic microbial communities produce allelochemicals to prevent colonization by other species, thereby preventing competition and benefiting the host by inhibiting fouling. One such example is the genus *Pseudoalteromonas*, whose members are common epiphytes of algae and are well known for the production of bioactives with antibacterial, antifungal and algicidal properties (127). Some species of bacteria that commonly form biofilms also exhibit cell to cell communication that allows the regulation of biofilm-based chemical defenses such as the production of virulence factors (197). *Vibrio cholerae* biofilms have been shown to produce a QS regulated antiprotozoal compound responsible for inducing cell lysis in flagellates (197, 206). Similarly, the production of QS regulated inhibitors by *Pseudomonas aeruginosa* mature biofilms also contributes to cytotoxic effects on protozoan predators (198, 343). Moreover, the semi-diffusible nature of biofilms can potentially hinder the diffusion of metabolites into the surrounding environment, serving to concentrate these allelochemicals to sufficiently high levels to be detected and harvested (197). Improving current conventional culturing techniques and exploring new environments may help to identify a large diversity of microorganisms with the potential to yield novel bioactives (58).

This chapter describes the methodologies used for the screening of bacterial biofilms for the identification of potential producers of inhibitory compounds active against helminths and protozoa. The model organisms selected as target organisms for bioactives produced by bacterial biofilms are the nematode,

Caenorhabditis elegans, and the free-living protozoans, *Tetrahymena* sp. and *Acanthamoeba castellanii*. Virulence factors of well known opportunistic pathogenic bacteria like *P. aeruginosa*, *Salmonella enterica*, *V. cholerae* and *Serratia marcescens* have been successfully identified using the *C. elegans* model. For example, the production and accumulation of hydrogen cyanide (HCN) by *P. aeruginosa* during late exponential and stationary phase is positively correlated with worm mortalities (96, 325). *C. elegans*, the closest free-living relative of parasitic helminths, provides a feasible, cost-effective model for high-throughput bioactives screening due to its ease of maintenance and cultivation (274, 300). In contrast, parasitic helminths require an animal host, which prevents development of a high-throughput screen (270, 300). Moreover, homologs and paralogs of anthelminthic targets in parasitic nematodes can be found in *C. elegans* (274, 300). These factors make *C. elegans* an appropriate model for the screening of potential bioactives with anthelminthic activity.

Flagellates, ciliates and amoebae may be free-living, facultatively or obligately parasitic with the potential to cause significant health and economic damage (270). Due to the wide range of adaptations displayed by ciliates, *Tetrahymena* sp. was selected as a model ciliate for screening purposes (270). Despite being a free-living protozoan, *Tetrahymena* sp. is capable of switching to a pathogenic lifestyle, causing infections in the skin, fins and internal organs of its fish host (179). The amoebal grazer, *A. castellanii* represents an excellent model for bioactive screening due to its ability to survive adverse conditions, invade and overcome the host immune system and to establish infection (163). In addition, secondary metabolites produced by bacteria have been shown to have toxic effects against these grazers. Matz *et al.* demonstrated that violacein producing epiphytic bacteria were capable of causing high mortality in both *Tetrahymena* sp. and *A. castellanii* (208). The close relationship between these model protozoa and their parasitic relatives as well as their sensitivity towards allelochemicals produced by epiphytic bacteria suggests that both *Tetrahymena* sp. and *A. castellanii* are suitable models for bioactives screens.

2.2 Materials and method development

2.2.1 Strains and culturing conditions

2.2.1.1 Bacterial isolates

Epiphytic isolates of the coralline red algae, *Corallina officinalis* and *Amphiroa anceps* (134) and *Pseudoalteromonas* strains were routinely maintained in Marine 2216 broth (Difco) or Väätänen nine salts solution (VNSS, pH 7.5) (195). The components of VNSS are 1 g l^{-1} bacteriological peptone, 0.5 g l^{-1} yeast extract, 0.5 g l^{-1} glucose, 0.01 g l^{-1} $FeSO_4.7H_2O$ and 0.01 g l^{-1} Na_2HPO_4 in 0.5 x artificial seawater, also known as nine-salt solution (0.5 x NSS), containing 8.8 g l^{-1} NaCl, 0.735 g l^{-1} Na_2SO_4, 0.04 g l^{-1} $NaHCO_3$, 0.125 g l^{-1} KCl, 0.02 g l^{-1} KBr, 0.935 g l^{-1} $MgCl_2.6H_2O$, 0.205 g l^{-1} $CaCl_2.2H_2O$, 0.004 g l^{-1} $SrCl_2.6H_2O$ and 0.004 g l^{-1} H_3BO_3, (195). Marine and VNSS agar were made by addition of 15 g l^{-1} of agar to the broth. Inoculated plates were incubated for 3 d and liquid cultures were incubated overnight with constant agitation at 200 rpm at room temp (ca. 22°C).

Bacteria isolated from activated sludge isolates from the Rouse Hill Sewage Treatment plant (NSW) were grown on R2A agar (Oxoid) at room temperature (ca. 22°C). Overnight cultures were grown in R2A broth consisting of 0.5 g l^{-1} protease peptone, 0.5 g l^{-1} casamino acids, 0.5 g l^{-1} yeast extract, 0.5 g l^{-1} glucose, 0.5 g l^{-1} soluble starch, 0.3 g l^{-1} sodium pyruvate, 0.3 g l^{-1} K_2HPO_4, 0.05 g l^{-1} $MgSO_4.7H_2O$ (pH 7.2) with constant agitation at 200 rpm.

V. cholerae, *Vibrio vulnificus* and *P. aeruginosa* strains were grown as overnight cultures in Luria-Bertani medium (21) supplemented with 10 g l^{-1} NaCl (LB10) at 37°C with constant agitation at 200 rpm. *S. marcescens* strains were also grown in LB10 at 30°C with shaking at 200 rpm.

Table 2.1: List of bacterial strains used.

Strain	Description (genotypic and/or phenotypic characteristics)	Reference
Vibrio cholerae		

A1552 Smooth	El Tor, Inaba, Amp^R, Kan^R, Wildtype, flat, smooth	(354)
A1552 S*hapR*	A1552 Smooth, *hapR*$^-$	(353)
Vibrio vulnificus		
L180	High hemolytic production	(357)
CVD707	Low hemolytic activity	(350)
I-21	Environmental isolate	(212)
Vibrio angustum		
S14	Model copiotroph, not known to produce bioactives	(135)
Serratia marcescens		
MG1	Wild type (Amp^R) (Tet^R), prodigiosin production	(101)
MG44	MG1; *swrI* gene disrupted with a streptomycin cassette (Str^R); AHL synthase mutant	(79)
BMG	MG1; Wild type biofilm derivative	(170)
SSV	MG1; biofilm variant that is smooth and sticky	(170)
SRUV	MG1; biofilm variant that is rough, umbonated and sticky	(170)
SRV	MG1; biofilm variant that is rough and sticky	(170)
SUMV	MG1; biofilm variant that is smooth, mucoid and sticky	(170)
NSV	MG1; biofilm variant that is smooth and non-sticky	(170)
NSCV	MG1; biofilm variant that appear as small colony and is non-sticky	(170)
Pseudomonas aeruginosa		
PAO1 ATCC	Wildtype, ATCC 15692, produces a range of QS-regulated phenazines	(123)
QS –(R/R)	QS mutant (PAO1::*las*R, *rhl*R), non-pigmented	(15)

Pseudoalteromonas		
ulvae	Dark purple, violacein producer, gelatinase activity (Pen G^R)	(80)
tunicata D2	Dark green, violacein and tambjamine producer, gelatinase activity	(126)
luteoviolacea	Purple, violacein producer	(127)
rubra	Red, cycloprodigiosin HCl producer	(37)
piscicida	Yellow, produces neuromuscular toxin that results in fish mortality	(37)
aurantia	Yellow	

Abbreviations: Amp^R – Ampicillin resistance, Kan^R – Kanamycin resistance, Cm^R – Chloramphenicol resistance, Tet^R – Tetracycline resistance, Pen G^R – Penicillin G resistance

2.2.1.2 Protozoan cultures

Acanthamoeba castellanii ATCC 30234 was obtained from the American Type Culture Collection (Manassas, V.A.) as an axenic culture and was routinely passaged in 60 ml growth medium containing protease-yeast-glucose (PYG) supplemented with 0.1 X M9 minimal medium (4.78 mM NaH_2PO_4, 2.2 mM K_2PO_4, 0.86 mM NaCl, 1.87 mM NH_4Cl, 0.2 mM $MgSO_4$ and 0.01 mM $CaCl_2$) and 0.1 M sterile-filtered glucose in tissue culture flasks with ventilated caps (Sarstedt Inc.). Three-day-old protozoan cultures were used for the experiments and were prepared as follows. Prior to harvesting, *A. castellanii* were grown aerobically for 3 d at 30°C in a static incubator. Tissue culture flasks containing three-day-old amoebae cultures were incubated on ice for 15 min to completely dislodge all surface-adhered trophozoites before harvesting by centrifugation at 800 X *g* for 5 min at 4°C. The trophozoites were concentrated by discarding the spent PYG media, and resuspended in fresh PYG and allowed to adjust to the new medium overnight. This amoeba concentrate was enumerated and adjusted to the desired working concentration in PYG by dilution, for use in the high-throughput grazing experiments.

The ciliate *Tetrahymena* sp. was isolated from a pond located at the University of New South Wales (Sydney, NSW) (343) and maintained in 15 ml of 0.1 X M9 minimal media supplemented with heat-killed *P. aeruginosa* in ventilated tissue culture flasks at room temperature without shaking. *Tetrahymena* sp. were treated with 100 μg ml^{-1} of ampicillin and 20 μg ml^{-1} of chloramphenicol in order to remove associated bacteria to obtain axenic ciliate cultures (206). The axenic ciliates were adapted to higher salt concentrations in the bacterial growth media by diluting with 0.1 X M9 minimal media to 30% the original concentration. The culture was grown for three days on heat-killed bacteria (HKB). *Tetrahymena* sp. was enumerated by counting 5 μl of culture fixed in equal volumes of Lugol's solution (Merck & Co., Inc.).

2.2.1.3 Nematode cultures

Caenorhabditis elegans Bristol N2 (39) was cultured as previously described (312). Nematodes were maintained and routinely sub-cultured at room temperature in petri dishes (Sarstedt Inc.) on nematode growth media (NGM), containing an overnight lawn of *E. coli* OP50 as the food source. The NGM agar contained 50 mM NaCl, 25 mM KPO_4, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.0005% (wt vol^{-1}) cholesterol, 0.75% (wt vol^{-1}) peptone and 1.7% (wt vol^{-1}) agar. It was supplemented with 0.0002% (wt vol^{-1}) uracil to sustain growth of the uracil auxotroph, *Escherichia coli* OP50. Both the nematode *C. elegans* Bristol N2 and *E. coli* OP50 were gifts from the *Caenorhabditis* Genetics Centre at the University of Minnesota (USA).

2.2.2 Optimization of screening conditions

A range of screening and culturing conditions were tested to determine the most appropriate method for the screening assays. Firstly, three different temperatures, 37°C, 30°C and room temperature were tested to determine the optimal temperature for culturing amoebal trophozoites and for the agar drop plate assay (as described below). The grazing efficiencies of *A. castellanii* were tested with

and without overnight incubation of amoeba in fresh PYG before the preparation of the amoebal lawn in the amoebae-based assays.

Bacteria strains listed in Table 2.1 were selected for the validation of the protozoan and helminth grazing assays due to their known cytotoxic activities. A pure extract of violacein resuspended in butanol was included in the optimization as a positive control.

2.2.3 *Acanthamoeba castellanii* based screens

2.2.3.1 Agar drop plate assay (APA)

The high-throughput agar drop plate assay, adopted from Albers *et al.* (1) was used for the screening of marine epiphytic and activated sludge bacterial libraries for identification of producers of bioactives. Serial dilutions of bacterial cultures were drop plated onto a lawn of *A. castellanii* and the growth of each strain monitored under grazing pressure.

Axenic cultures of amoeba were adjusted to a desired working concentration of approximately 1.7 X 10^6 amoebae ml^{-1}. Two different protozoan concentrations were used for screening: undiluted amoebae (2.5 X 10^6 amoebae), 1:10 diluted (2.5 X 10^5 amoebae) and controls without amoebae. One and a half ml of undiluted or 1:10 diluted amoebae concentrate was seeded onto VNSS, LB10 or R2A agar plates, swirled for even distribution and allowed to adhere and dry in a laminar flow cabinet (Email Air Handling, NSW Australia) for 2 hrs. On the non-grazed control, the amoebae concentrate was replaced with 1.5 ml of PYG media. Overnight cultures of bacterial isolates grown in the appropriate media were adjusted to an optical density of 0.7 at OD_{600nm} (NovaSpec® spectrophotometer Pharma Biotech). Serial dilutions of bacterial cultures (200 x, 400 x, 4000 x, 8000 x, 80000 x, 160000 x) were performed and 2 µl of each was spotted onto the agar plates (Figure 2.1). Inoculated plates were incubated at room temperature for 24 hrs before the number of bacterial colonies for each dilution was determined. To calculate the percentage survival of each strain, the formula: [(total colony forming units in grazed treatment)/ (total colony forming units in ungrazed

treatment)] X 100% was used. Graphs of percentage survival of each strain were plotted using Prism ® version 5 for Windows (GraphPad Software Inc.).

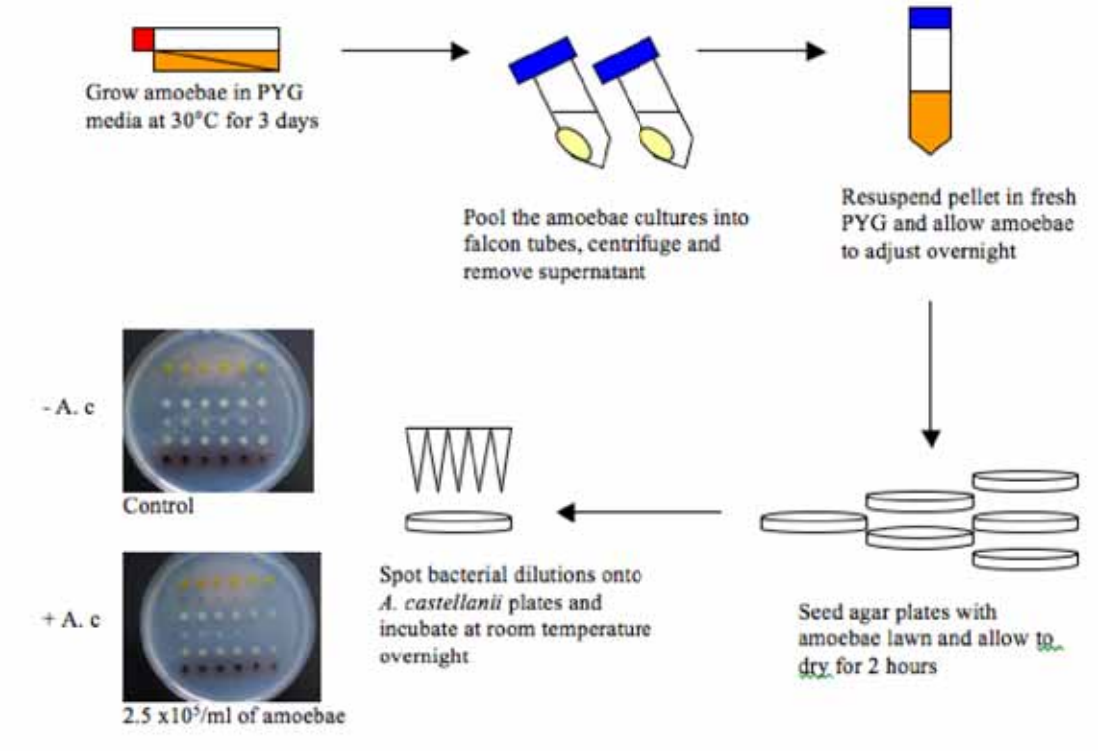


Figure 2.1: A schematic flow diagram illustrating the steps involved in the *A. castellanii* agar drop plate assay for screening of bacterial libraries. –A. c denotes non-grazed control and +A. c shows the differential growth of each strain under grazing pressure.

2.2.3.2 Selective grazing assay

The selective grazing assay was adopted from Wildschutte *et al.* (346). Briefly, a drop of *A. castellanii* concentrate was inoculated at the centre of the agar plate at equal distances away from lines of bacterial inoculum (Figure 2.2). The grazing preference of the amoebae could be determined based on the clearance zone of each strain, which directly correlated to the sensitivity or resistance of the bacterial isolate.

Bacterial overnight cultures were adjusted to an optical density of 0.7 at OD_{600nm} before inoculating as 33 mm lines onto petri dishes (Sarstedt Inc.) (maximum 8 strains) containing appropriate agar for growth. For grazed treatments, 15 µl of the amoebae concentrate containing 5.1 X 10^4 trophozoites was spotted onto the center of the agar plates. For ungrazed controls, the amoebae concentrate was

replaced with PYG media. Agar plates were sealed with parafilm to prevent drying and incubated at room temperature for approximately 30 days. Routine measurements of clearance zones of the bacterial streaks were taken and the resistance or sensitivity of each bacterial strain to grazing was determined by the distance of the amoebae-grazing front, relative to the original distance in the ungrazed treatments. At least four replicates were prepared and bacterial isolates were inoculated randomly in each replicate to avoid bias in the experimental setup. Graphs were plotted based on the distance grazed by amoebae using Prism ® version 5 for Windows (GraphPad Software Inc.).

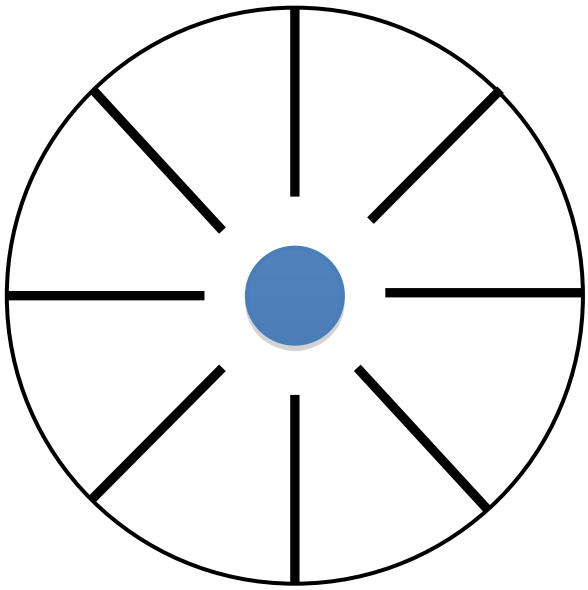


Figure 2.2: A simplified diagram of the selective grazing set-up. For the grazed treatments, *A. castellanii* was inoculated at the centre of the agar plate (shaded region) and streaks of different bacterial isolates were inoculated at equal distances away from the centre. In non-grazed treatments, amoebae were replaced with PYG media.

2.2.4 *Tetrahymena* sp. based screening

2.2.4.1 *Tetrahymena* sp. motility assay (TMA)

Tetrahymena sp. motility and activity in the presence of bacterial isolates were used as criteria to identify strains having anti-protozoal activity. Overnight cultures of bacteria were adjusted to an optical density of 0.7 at OD_{600nm} before a 100-fold dilution was performed. MBEC™ plates (Innovotech Inc.) were used for

the growth of the biofilms, except that the troughs were replaced with 96-well round bottom tissue culture plates (Sarstedt Inc.). An inoculum of 150 µl of 1:100 diluted bacterial cultures was added to each well and biofilms were allowed to form for three days at room temperature with shaking at 60 rpm. For ungrazed control treatments, 150 µl of HKB was added to the biofilms formed on the pegs. Ciliate cultures were prepared as described above and enumerated before 10 ciliates μl^{-1} were added to each well. The activity of *Tetrahymena* sp. was monitored over time using an inverted light microscope (Olympus INVT200, Olympus Optical Co. Ltd., Tokyo, Japan) at 10 X magnification with 1.5 X intermediate magnification. Four replicates of grazed and ungrazed treatments were prepared.

2.2.5 *Caenorhabditis elegans* based screening

2.2.5.1 Synchronization of *Caenorhabditis elegans* for stage 4 larvae

C. elegans were cultured by seeding 1 cm^3 squares from the subcultures onto fresh NGM plates containing a lawn of *E. coli* OP50. After 4 d, gravid adults were harvested for isolation of eggs and worm synchronization (310). Briefly, gravid adults were collected by rinsing NGM agar plates with 10 ml of sterile water. The nematodes were washed three times with sterile water and centrifuged at 2566 X *g* at room temperature in a benchtop Hettich centrifuge (Andreas Hettich GmbH & Co. KG, Germany) for 3 min after washing to remove any attached *E. coli* OP50. The clean nematodes were lysed with 3 ml of 5% (vol vol^{-1}) sodium hypochlorite solution and 1 ml of 5M NaOH with gentle shaking for 5 min. The lysis reaction was stopped by the addition of egg buffer (118 mM NaCl, 48 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES (pH7.4)) and the eggs were washed 3 times with egg buffer and the presence of eggs confirmed after each wash with a dissecting microscope (Leica® ZOOM 2000™) to minimize egg loss. The harvested eggs were allowed to hatch in 100 ml of M9 minimal media without glucose in ventilated cap tissue culture flasks at room temperature with shaking at 110 rpm. After 12 to 16 hrs, L1 worms were fed with *E. coli* OP50, incubated for another 30 to 36 hrs until they had reached the L4 stage, at which time they were

washed three times in M9 minimal medium to remove *E. coli* OP50 and concentrated for use in the screening assays.

2.2.5.2 Biofilm cell-free supernatant bioassay with *Caenorhabditis elegans*

The biofilm cell-free supernatants harvested from the bacterial libraries were screened against *C. elegans* for potential producers of anti-helminthic compounds. Bacterial isolates were grown as overnight cultures and adjusted to an optical density of 0.7 at OD_{600nm}. The OD_{600nm}-adjusted, 1:100 diluted inoculums were grown as three day-old biofilms at room temperature with shaking at 60 rpm in 24-well tissue culture treated microtitre plates (Sarstedt Inc.). Supernatants from each well were harvested and filtered through a Millex® syringe filter with a pore size of 0.22 µm (Millipore Corp.). The synchronized *C. elegans* L4 concentrate was enumerated and diluted to 500 worms ml^{-1} before 50 µl aliquots containing approximately 25 worms, were added to each well of a 96-well tissue culture treated microtitre plates (Sarstedt Inc.). An equal volume of cell-free supernatant was added to the nematodes in each well and the plates incubated overnight at room temperature before scoring for live (moving and sinusoidal in shape) and dead (rigid and straight) worms. Four replicates of each bacterial isolate were tested. The controls were M9 minimal media, 50% (vol vol^{-1}) Marine 2216 broth, 50% (vol vol^{-1}) LB10, 10% (vol vol^{-1}) butanol and 10% (vol vol^{-1}) ethanol which were used for dilutions of test samples. Positive controls included 5 µg ml^{-1} cyclohexamide, 1 µg ml^{-1} of nystatin and 200 µmol of Ivermectin, which are commonly used as anti-helminthic compounds. The percentage survival of *C. elegans* in cell-free supernatants was calculated using the formula: [(total number of live worms)/ (total number of worms)] X 100%.

2.2.5.3 Agar-based nematode paralysis assay

The nematode paralytic assay for high-throughput screening of potential producers of bioactives with the following modifications was adapted from Gallagher and Manoil (96). Bacterial lawns from test libraries were prepared by inoculating 50 µl of overnight cultures onto 55 mm diameter petri dishes (Sarstedt

Inc.) containing appropriate agar and incubating at their optimal temperature for growth. A drop of nematode concentrate containing 10 to 20 worms was added to the bacterial lawns. Live and dead worms were enumerated immediately after inoculation and after 24 hrs incubation (Figure 2.3). The positive controls for this assay were *P. aeruginosa*, a pyocyanin overproducer and *P. tunicata* D2, a violacein producer, while *E. coli* OP50 was used as a negative control. Four replicates of each strain were prepared and the percentage survival of *C. elegans* on each set of bacterial lawns determined.

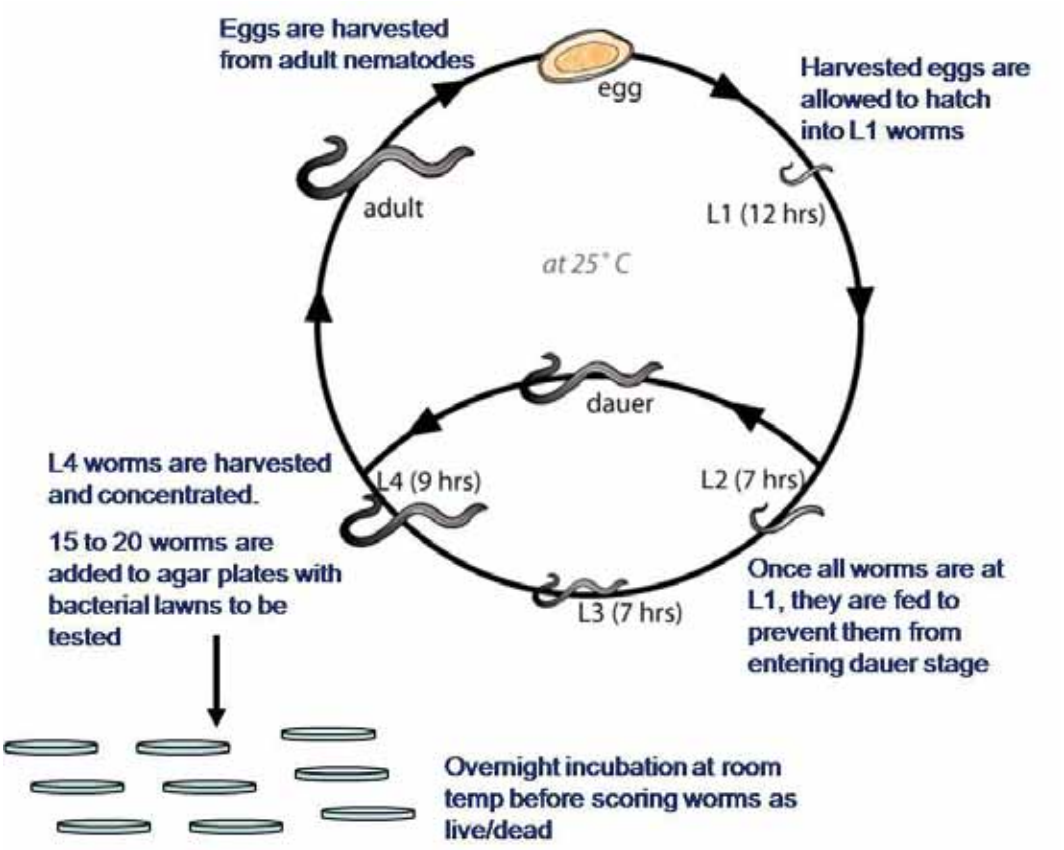


Figure 2.3: A schematic illustration of the steps involved in the preparation of the agar-based nematode paralysis assay used for screening the bacterial libraries.

2.2.6 Identification of positive strains using the 16S rRNA gene

The identity of strains that displayed inhibitory activities against predators in both the protozoan and helminthic screens was determined by PCR amplification of the 16S rRNA gene (341), using universal 16S primers; 1492R (5’ -

ACGGTTACCTTGTTACGACTT) and 27F (5' - AGAGTTTGATCCTGGCTCAG). After subjecting the PCR mixture to an initial 5 min of denaturation, 25 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 2 min was carried out for the exponential increase in the PCR product; followed by a final extension period of 10 min at 72°C and refrigeration at 4°C in a PCR Sprint Thermal Cycler (Hybaid Ltd.). The 1.5 kb PCR products were confirmed on a 0.8% agarose gel before purification steps with a QIAquick® PCR purification kit (QIAGEN Pty. Ltd.) according to the manufacturer's instructions, followed by resuspension in 50 µl of sterile molecular grade water.

A 20 µl 16s rRNA sequencing mix was prepared by combining 200 ng of purified DNA template, 160 nM of primer (either 1492R or 27F primer per reaction) with BigDye terminator™ version 3.1 (Applied BioSystems) in a thermal cycler. The sequencing products from the thermocycling reactions were purified using the butanol purification protocol described by Tillett *et al.* (322), before analysis at the Ramaciotti Centre for sequencing analysis on an ABI 377 automated DNA sequencer (Applied BioSystems). The ends of the 16s forward and reverse DNA sequences were trimmed using 4Peaks (http://mekentosj.com/science/4peaks/). The forward and reverse complemented sequences were assembled and aligned using Lasergene DNA & Protein analysis software version 6 (DNAStar, Inc.). The closest identity for each strain was determined by performing searches based on sequence similarities using the Basic Local Alignment Search Tool for nucleotides (BLASTn) at the National Center for Biotechnology Information database (http://www.ncbi.nlm.nih.gov/BLAST/).

2.2.7 Statistical analysis

The data were analysed by one-way ANOVA using Minitab 15 software for Windows (Minitab Inc., State College, PA, USA) followed by Tukey's posthoc tests.

2.3 Results

2.3.1 Optimal growth and grazing efficiencies of *Acanthamoeba castellanii* are affected by incubation, adjustment period and temperature

Amoebal growth rate and grazing efficiencies have been shown to be constrained by a number of factors such as availability of substratum for attachment and the state of satiation of the protozoan predator (28, 239). The optimal growth temperature and period for harvesting amoebae with the highest grazing efficiencies for carrying out the amoebal-based screening assays was investigated. Also, the ideal predator concentration of the amoebal lawn for significant differences in the prey grazing resistance in the APA was assessed.

The amoebae used in this assay were allowed to grow in PYG media for 3 and 7 days before harvesting for the amoebae lawn. The percentage survival of the control *Vibrio* strains were determined by comparing the colony-forming units of the grazed (with *A. castellanii*) with that of ungrazed (without *A. castellanii*) treatments and is reflective of the individual resistance of each strain. Resistance of prey on a lawn of 3 day-old amoebae (Figure 2.4A) was significantly lower as determined by analysis of variance ($P < 0.01$), compared to the resistance of prey on a 7 day-old amoebae lawn (Figure 2.4B).); i.e. the grazing efficiency of the 3 day-old amoeba was significantly higher than the 7 day-old amoeba.

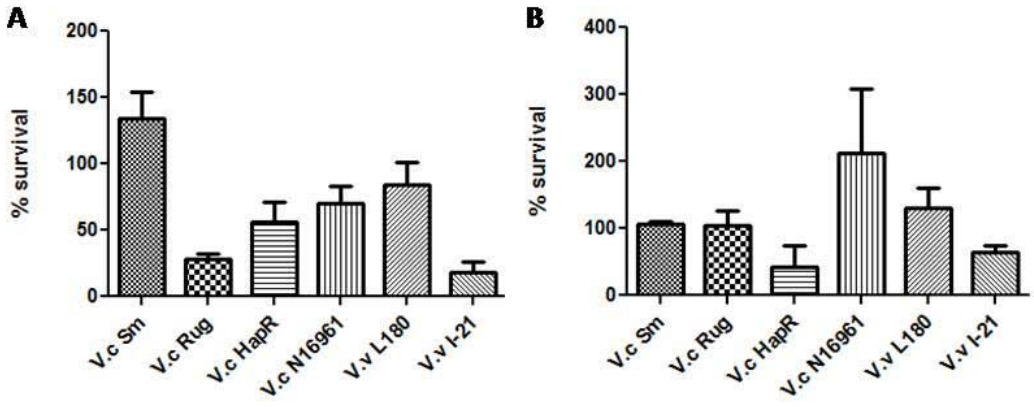


Figure 2.4: Amoeba plate assay demonstrating the grazing efficiencies of *A. castellanii* grown for 3 and 7 days on *V. cholerae* and *V. vulnificus* strains. The percentage survival of each prey was determined by enumeration of colonies formed in the presence of

grazing over that in the absence of amoeba. The resistance of prey on a lawn of 3 day-old amoebae lawn (A) was significantly lower than on a 7 day-old amoebae lawn (B) ($P<0.01$).

The effect of overnight adaption of amoebae to the plating medium on grazing efficiency of amoeba on *Vibrio* prey was investigated. A similar trend in grazing resistance was observed in both treatments (without and with adapting amoebae to new media) with *V. vulnificus* strains exhibiting higher survival rates compared to *V. cholerae* strains (Figures 2.5A and B). However, the grazing efficiencies of *A. castellanii* were more pronounced when allowed to acclimate to new media. A comparison between both treatments showed an increased susceptibility of prey to amoebal grazing after adaptation (percentage survival of *V. cholerae* strains were below 10% and *V. vulnificus* strains were below 50% (Figure 2.5B) compared to 30% and 500% respectively (Figure 2.5A).

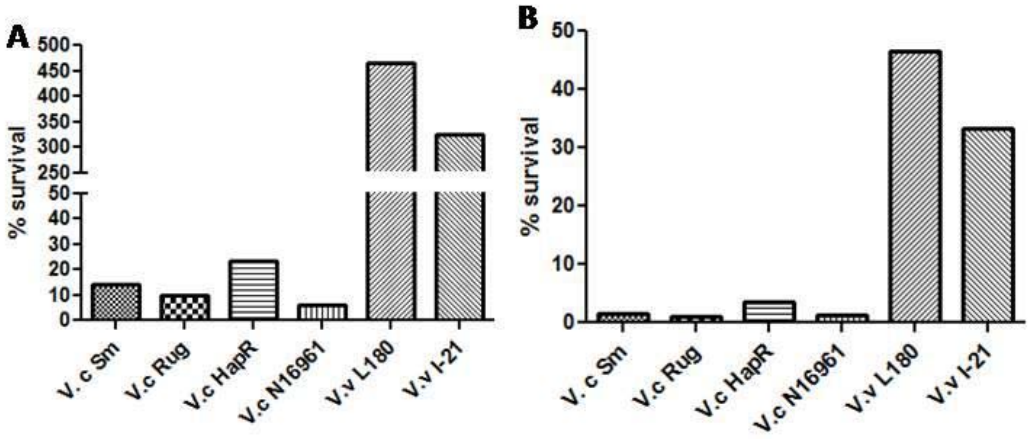


Figure 2.5: Comparison of the grazing efficiencies of *A. castellanii* on *V. cholerae* and *V. vulnificus* strains in the APA performed at 30°C without (A) and with (B) overnight adjustment of amoebae to new growth media.

The APA was repeated to determine the incubation temperature that gave the highest amoebal grazing efficacies. *Vibrio* prey exhibited variations in growth rate under the 3 different temperatures tested (37°C, 30°C and room temperature) (data not shown). A comparison of the APAs using both undiluted and 1: 10

diluted amoebal lawns, confirmed that room temperature was the best due to the slower bacterial growth rate, which encouraged amoebal grazing (data not shown). At room temperature, the trend of percentage survival of the *Vibrio* strains is generally similar, however, the grazing pressure exerted by the undiluted amoebal lawn was 10 fold higher compared to that with the 1: 10 diluted amoebal lawn (Figure 2.6A and B). Significant differences in the percentage survival of different *Vibrio* preys were observed, with *V. cholerae* Rugose exhibiting the highest grazing resistance (7.74 % in undiluted amoebae and 86.08 % in 1: 10 diluted amoebae) compared to other *V. cholerae* strains (2.24 % and 46.67 % for Smooth, 0.06 % and 34.47 % for HapR mutant and 5.32 % and 42.58 % for N16961 on undiluted and 1: 10 diluted amoebal lawns respectively) and *V. vulnificus* strains (1.01 % and 22.57 % for L180 and 0.54 % and 21.00 % for I-21) ($P < 0.01$ for both treatments, Tukeys Post hoc tests) (Figure 2.6A and B).

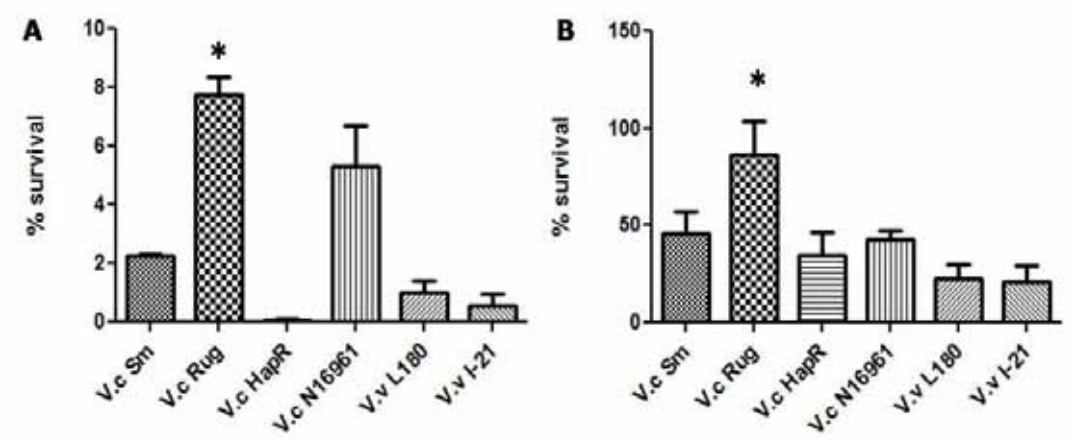


Figure 2.6: Grazing resistance of *V. cholerae* and *V. vulnificus* strains in the APA on 2 different amoeba lawn concentrations; (A) undiluted with 2.5 x 10^6 amoebae and (B) 1:10 dilution with 2.5 x 10^5 amoebae at room temperature. Statistical significance as determined by analysis of variance followed by Tukey's posthoc tests; *, $P < 0.01$, showed significant grazing effects with *V. cholerae* Rugose exhibiting the highest grazing resistance in both treatments.

2.3.2 APA validation using *Pseudoalteromonas* strains

As described in Chapter 1, *Pseudoalteromonas* strains are well known for their extensive production of inhibitory metabolites which provide them with a

competitive advantage in marine systems (127). As, not all *Pseudoalteromonas* strains exhibit such inhibitory activity (81), a variety of strains, both producers and non-producers of secondary metabolites, were selected to test the sensitivity of the APA.

P. aurantia exhibited the highest grazing resistance (30.17 % survival) under the strongest grazing pressure (undiluted treatment). In contrast, all other species of *Pseudoalteromonas* species were susceptible to *A. castellanii* grazing (survivals below 10.00 %) (Figure 2.7A). However, when the grazing pressure was relieved by dilution of the amoebal lawn, the grazing resistance profile changed, with the fish pathogen, *P. piscicida* exhibiting a significant increase in resistance (215.00 % survival) (Figure 2.6B). *P. aurantia, P. ulvae* and *P. tunicata* known to produce inhibitory metabolites were also resistant (70.20 %, 67.50 % and 73.10 % survival respectively), whereas non-producers like *P. citrea, P. undina, P. haloplanktis* and *P. nitrifaciens* were sensitive to amoebal grazing (37.10 %, 42.60 %, 5.00 % and 8.80 % survival respectively) (Figure 2.7B).

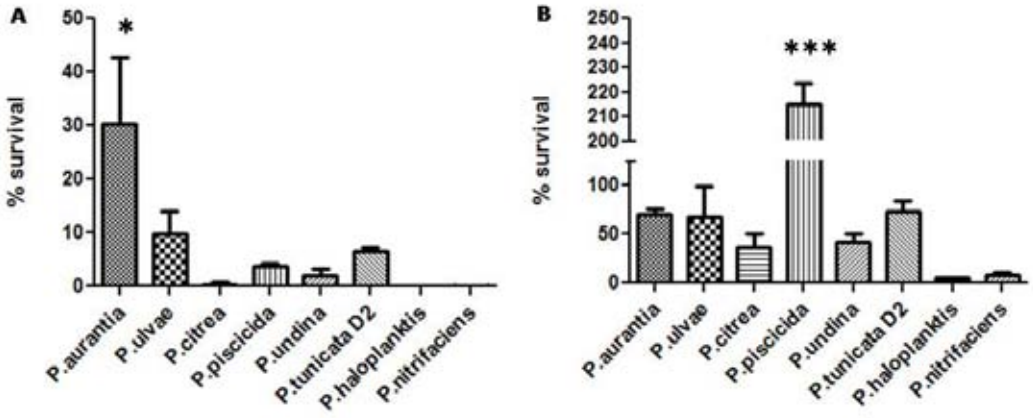


Figure 2.7: Validation of the APA using *Pseudoalteromonas* strains. The grazing resistance of each strain was expressed as percentage survival in the presence of an undiluted (A) or 1: 10 diluted (B) amoebal lawn. Significant grazing differences between strains in a single treatment as determined by analysis of variance followed by Tukey's posthoc tests; *, $P < 0.05$ and ***, $P < 0.0001$.

2.3.3 Identification of *Ulvae* epiphytic isolates with anti-protozoal activity

The green alga *U. australis* remains free from fouling despite the lack of secondary metabolites. Their anti-fouling property has been attributed to epibiosis (82, 125). A library of *Ulvae* epiphytic isolates was screened for inhibitory activity using the APA as a screen. The significant increase in percentage survival of isolates 33 and 85 (55.40 % and 100.00 % survival respectively) compared to all other strains in the library, identified them as positive strains resistant to amoebal grazing and hence actively producing of inhibitory compounds (Figure 2.8). In contrast, all the other epiphytic isolates were susceptible to *A. castellanii* grazing (survival less than 50.00 %).

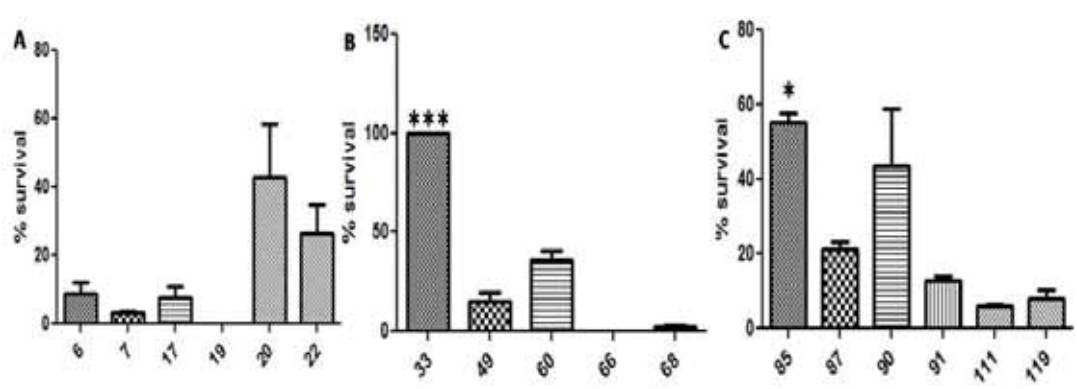


Figure 2.8: *Ulvae* epiphytic isolates percentage survival when grazed by *A. castellanii* in the APA (A, B and C are separate screens). Resistance of isolate 33 and isolate 85 were statistically significant as determined by analysis of variance; ***, $P < 0.0001$ and *, $P < 0.05$.

2.3.4 Screening and identification of active strains from bacterial libraries using the selective grazing assay

Another agar-based screening system, the selective grazing assay, was used to validate the results obtained with the APA. In this assay, a maximum of 8 different isolates from a collection of strains from *Ulva, Amphiroa, Corallina* epiphytes and activated sludge bacterial strain libraries were plated around a central drop of amoebae. The susceptibility of each strain to grazing and the

preferential grazing of *A. castellanii* was determined by the reduction in the bacterial inoculum (Figure 2.9). *P. luteoviolacea, P. ulvae* and *S. marcescen* which exhibited inhibitory activity in the APA also demonstrated grazing resistance to *A. castellanii* in this assay. Similarly, *V. vulnificus* I-21, which was non-toxic to amoebae in the APA, was also grazed (Figure 2.9A). A comparison of the consumption rates of *V. angustum* S14 and *V. vulnificus* I-21 showed preferential grazing of S14 by *A. castellanii*, as demonstrated by the higher rate of grazing. In addition to the control strains, a total of 66 strains from the Centre's culture collection were screened with 15 positive strains identified (22.00 %) (Table 2.3). This selective grazing assay was subsequently used for screening epiphytic isolates of *Ulvae*; of the 90 screened, 8 were found to exhibit inhibitory activity (8.90 %) (Figure 2.9A and Table 2.3). The activated sludge library of 91 isolates was screened for activity in a similar fashion and 5 strains were shown to be resistant to grazing (5.50 %). There was preferential amoebal grazing on activated sludge isolates, as indicated by the greater grazing distances which are reflective of higher grazing rates (Figure 2.9B to C). In addition, 134 *Amphiroa* and *Corallina* epiphytes were also screened in a similar manner with 17 epiphytic isolates identified as active strains (12.70 %) (Table 2.3). The identities of some of these active strains are listed in Table 2.4.

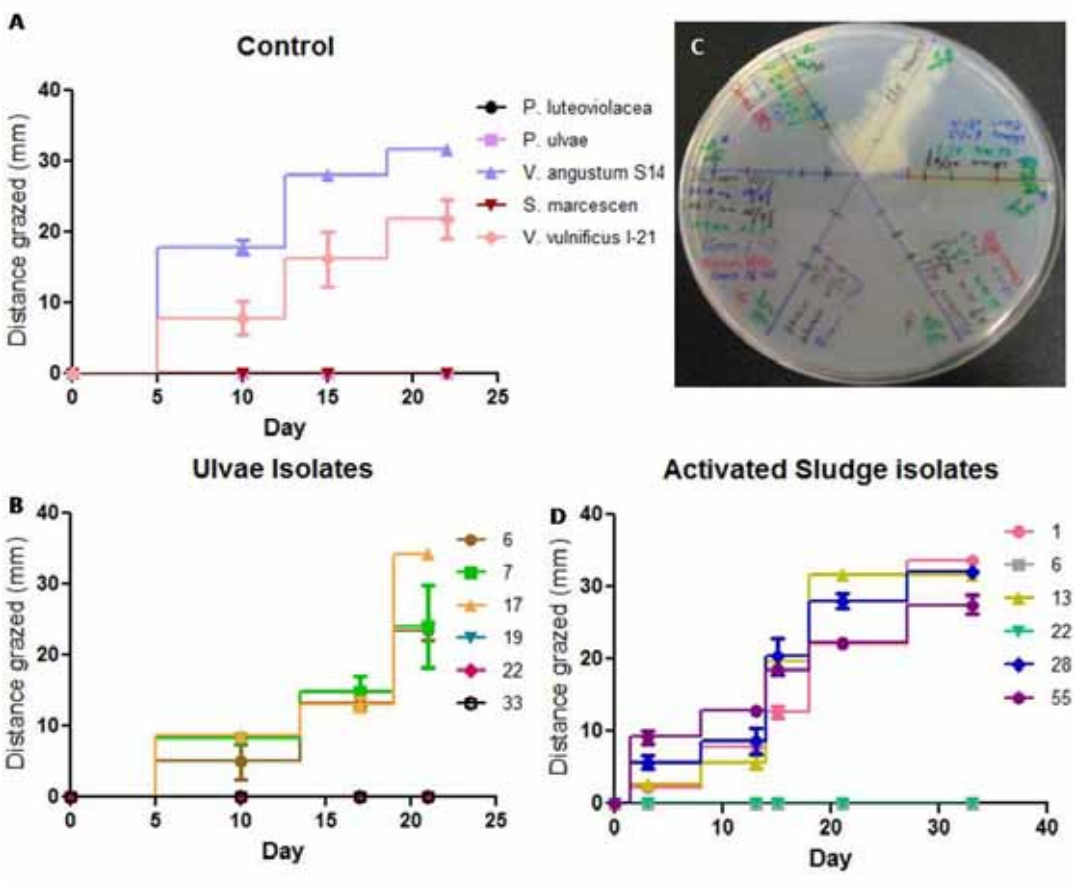


Figure 2.9: Preferential grazing of bacterial isolates from *Ulvae* epiphytic and activated sludge libraries. (A) Control strains with known activity were used to validate this selective grazing assay. The rate of predation was determined by measuring the distance of the amoebal grazing front of the *Ulvae* epiphytic (B) and the activated sludge (D) bacterial inocula. Error bars represent the SDs of four replicates. The grazing assay was carried out for more than a month and the remaining ungrazed streak represents an active strain (C).

2.3.5 Grazing resistance of bacterial strains is grazer dependent

Anti-microbials, have different specificities and hence different targets (254), with some being broad spectrum and others less so. In order to identify novel bioactives that are active against parasitic ciliates which pose a major problem in aquaculture, the feasibility and sensitivity of a *Tetrahymena* sp. screen for identifying active strains based on monitoring the protozoan motility and activity after 2, 4 and 6 hr incubations with bacterial cell-free supernatant was designed and tested.

In this assay, the grazing resistance of each bacterial strain was determined microscopically and a qualitative interpretation and assessment of the motility and activity of *Tetrahymena* sp. in test cell-free supernatants, relative to those in the media control, was made. Incubation with cell-free supernatants from *P. tunicata* D2 led to the most pronounced changes in *Tetrahymena* sp., causing loss in motility and death within 6 hrs (Table 2.2). Cell-free supernatants from *P. ulvae, P. aurantia, P. citrea, P. nitrifaciens* and *P. undina* also caused impaired movement in *Tetrahymena* sp. after 6 hrs. In contrast, *Tetrahymena* sp. incubated with *Vibrios* and the remaining *Pseudoalteromonas* strains showed either no change in activity or exhibited enhanced motility. Consistent with previous findings using flagellates as a target, the degree of toxicity displayed towards *Tetrahymena* sp. by the *Pseudoalteromonas* and *Vibrio* strains were also positively correlated with the production and secretion of pigments into the cell-free supernatants (data not shown).

Table 2.2: Varying degrees of the rate of *Tetrahymena* sp. motility after 2, 4 and 6 hrs incubation with bacterial cell-free supernatants

Strains	After 2 hrs	After 4 hrs	After 6 hrs
V. cholerae Sm	2	2	3
V. cholerae Rug	2	2	2 to 3
V. cholerae HapR	2	2	3
V. cholerae N16961	2	2	2
V. cholerae L180	2	3	3
V. vulnificus I-21	2	2	2
P. tunicata D2	1	1	0 to 1
P. ulvae	1 to 2	1 to 2	1 to 2
P. aurantia	1 to 2	1 to 2	1 to 2
P. citrea	1 to 2	1 to 2	1 to 2

P. nitrifaciens	2	1 to 2	1 to 2
P. undina	2	1 to 2	1 to 2
P. piscicida	2	2	2
P. haloplanktis	2	2	2
Media Control	2	2	2

Abbreviations: 0, protozoans inactive and dead; 1, protozoans are less active compared to control; 2, Motility and activity of protozoa in test supernatant is similar to control and 3, protozoa in test samples more active than control.

2.3.6 Identification of strains with anthelminthic activity

Environmental bacteria are known to secrete inhibitory metabolites at high cell densities in a biofilm to increase their fitness during competition and growth (121). These secondary metabolites can be secreted through passive diffusion or with the help of various specific types of secretion systems (172). For example, phenazines produced by *P. aeruginosa*, which have been shown to display antimicrobial properties, were in fact secreted QS signals involved in cell-cell communication (77, 261). Hence, in order to screen for secreted anthelminthic bioactives, cell-free supernatants from bacterial biofilms were harvested and incubated with *C. elegans* overnight and assessed for inhibitory activity.

The survival rate of *C. elegans* in the media control and on non-pathogenic *E. coli* OP50, was greater than 95.00 %. In contrast, incubation with the anthelminthic compound, Ivermectin, killed all the nematodes. In this assay, cell-free supernatants were harvested from the bacterial libraries and incubated with nematodes to test for growth inhibition or nematode death. Fifty percent survival (IC_{50}) was used as a cut-off point for identification of cell-free supernatants with inhibitory activity. Supernatants resulting in < 50.00 % worm survival were considered to be active against *C. elegans* (347). Amongst all the supernatants screened from the libraries, significant inhibition was observed only for the *Corallina* isolate 36 (C36), with a nematode survival of 7.14 % after 24 hrs. In the activated sludge libraries, moderate inhibition of nematode survival was also displayed by isolate 9 and 30 (nematode survival 66.00 % and 58.00 %,

respectively) (Figure 2.10B). The majority of the pathogenic *Vibrio* and *Pseudomonas* strains from the culture collection showed a lack of activity towards *C. elegans*, except for *V. harveyi* 642 (61.00 % nematode survival) (Figure 2.10A).

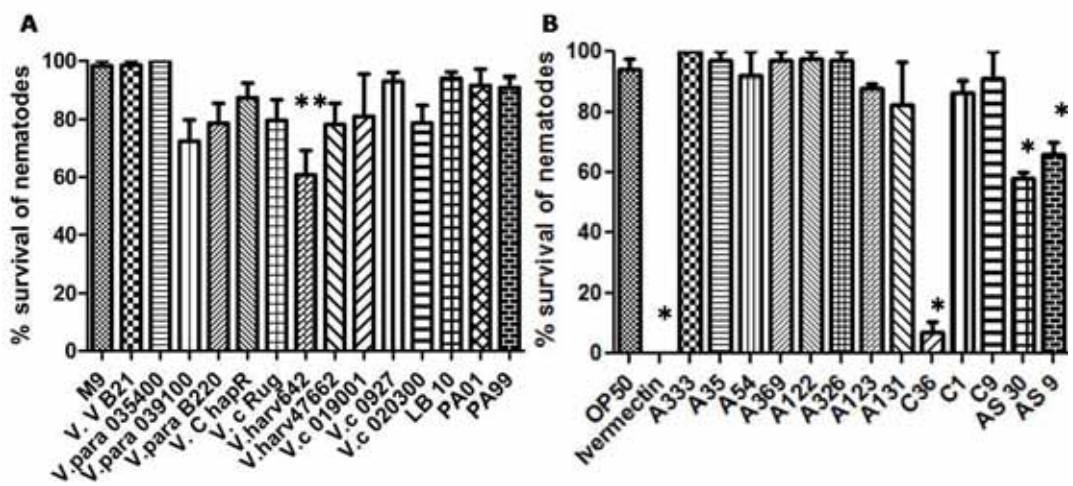


Figure 2.10: Screening of cell-free supernatants for activity against parasitic helminths from the culture collection of *Vibrios* and *Pseudomonas* (A) and from environmental libraries of *Amphiroa* and *Corallina* epiphytes represented by A and C respectively and activated sludge isolates, AS (B). *E. coli* OP50 served as a positive control and Ivermectin as a negative control. Error bars represent SDs of 4 replicates. Statistical analysis was determined by one-factor ANOVA; **, $P < 0.05$, *, $P < 0.0001$.

2.3.7 Bacterial biofilms tend to exhibit higher virulence against nematodes

Due to the lack of bioactives detected in the biofilm-cell free supernatants, the following approach was to assess bacterial biofilms for such inhibitory activity, using the *C. elegans* infection model, a method widely used to study bacterial virulence and host-pathogen interactions (97, 220). This infection model was adapted as a high-throughput screen for novel bioactives. According to Koh (170) who determined the virulence of his isolates based on the number of days required to kill 50.00 % of the worms (LT_{50}), strains with shorter LT_{50} were more virulent. Instead of having a variable LT_{50}, an incubation period of 24 hrs was chosen and a 50.00 % nematodes survival was used as a benchmark for assessing the toxicity of

each strain. Biofilms exhibiting > 50.00 % survival of nematodes were considered non-toxic, whereas biofilms resulting in < 50.00 % survival were treated as potential candidates for bioactive production (Figure 2.11). As 50.00 % nematode survival is not a stringent cut-off point, it ensured that strains with slight inhibitory activity were included.

The majority of biofilms from the culture collection of *Vibrios, Pseudomonas, Serratia* and *Pseudoalteromonas* species exhibited significantly higher levels of toxicity against *C. elegans* compared to isolates from the environmental libraries (Table 2.3) (Partial data shown in Figure 2.11). In contrast, only 12 out of the library of activated sludge isolates demonstrated inhibitory activity against nematode grazing (13.20 % positive in Table 2.3, $P < 0.0001$) (7 out of 12 positives represented in Figure 2.11 and identity of these strains in Table 2.4).

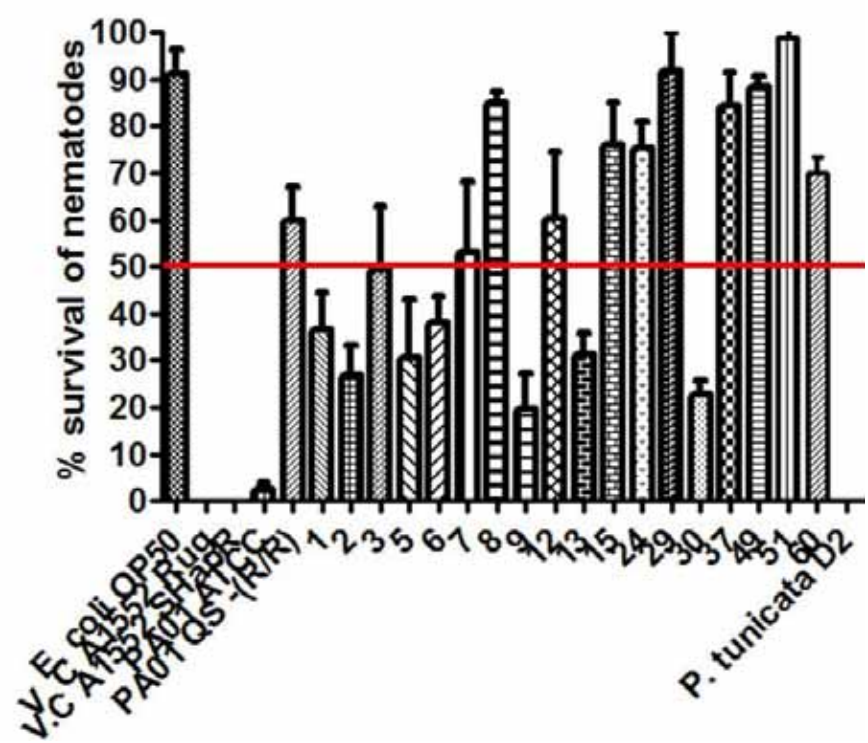


Figure 2.11: Biofilms from representatives of the culture collection and activated sludge libraries shown here demonstrate different levels of resistance against *C. elegans* after 24 hrs incubation. *E. coli* OP50 served as a positive control and *P. tunicata* D2 as a negative. Error bars represent SDs of four replicates. The red line represents the cut-off point for toxicity.

Ulvae epiphytic isolates had a slightly higher percentage of isolates with inhibitory activity (21.10 %) against nematodes compared to activated sludge isolates, whereas *Amphiroa* and *Corallina* isolates had a much lower percentage of positives (0.74 %) (Table 2.3). There were no *Amphiroa* isolates with inhibitory activity and *Micrococcus* sp. was the only strain from *Corallina* toxic to *C. elegans* (Table 2.4).

Table 2.3: Percentage of strains exhibiting activity against either nematodes or amoebae or both, calculated by dividing the number of active strains over the total number of isolates from the libraries screened.

Bacterial library	**Total isolates screened**	**Positive strains (amoeba)**	**Percentage positive hits**	**Positive strains (nematode)**	**Percentage positive hits**
Activated sludge	91	5	5.50 %	12	13.20 %
Ulvae epiphytes	90	8	8.90 %	19	21.10 %
Amphiroa and Corallina epiphytes	134	17	12.70 %	1	0.74 %
Culture Collection	66	15	22.00 %	64	96.00 %

Table 2.4: Identity of isolates positive for inhibitory activity in the amoeba and nematode screens based on 16s DNA sequence alignments

Isolate Number	**Blast closest match**	**% similarity**	**Accession number of closest match**	**Source**	**Activity against**
1	*Aeromonas hydrophila*	100	GU563995	Sludge	Nematode
2	*Acinetobacter* sp.	99	AY055373	Sludge	Nematode

5	*Acinetobacter johnsonii*	99	EU275352	Sludge	Nematode
9	*Raoultella* sp.	93	GU935765	Sludge	Nematode
13	Uncultured bacterium clone D15_1_SS_C_93	97	EU181859	Sludge	Nematode
22	*Microbacterium flavum*	99	AB286029	Sludge	Amoeba and nematode
26	*Acinetobacter johnsonii*	100	EU275352	Sludge	Nematode
28	Uncultured *Acinetobacter* sp.	98	FJ192868	Sludge	Nematode
30	*Klebsiella terrigena*	97	Y17670	Sludge	Nematode
54	*Klebsiella oxytoca*	99	AY150697	Sludge	Nematode
C36	*Micrococcus* sp.	96.9	AY258119	Corallina	Amoeba and nematode
A14	*Aestuariibacter halophilus*	94.9	AJ391191	Amphiroa	Amoeba
A360	*Pseudoalteromonas bacterolytica*	94.2	AF173962	Amphiroa	Amoeba

2.4 Discussion

Environmental bacteria have been demonstrated to associate as unique biofilm communities on animate surfaces such as sponges and algae which are habitats high in nutrient content, especially organic materials (9). For example, in marine environments, sponges of similar species within close proximities were found to harbor distinctly different bacterial communities (347). Improvements in molecular techniques have also led to the discovery of a significant proportion of previously unclassified and unculturable bacterial genera from such communities (132). The diversity of environmental habitats coupled with the metabolic plasticity and adaptive capabilities of bacteria have resulted in the realization of the extensive potential variety of bioactives available for harvesting from these relatively unexplored reservoirs such as biofilms (132, 347). As such, there has been an increase in the number of studies aimed at novel bioactive discovery from surface-attached bacteria to meet the critical shortage of drugs and increasing demand for novel classes (233, 347). Several studies have diverged from mainstream screening of antimicrobials to using live whole-animal models like *C. elegans* in an attempt to understand bacterial infection and pathogenesis and at the same time, screen for the production of novel bioactives (97, 220). In this chapter, both unicellular and multi-cellular organisms (*A. castellanii*, *Tetrahymena* sp. and *C. elegans*) were selected as ideal models for development and validation of these bioactive screens to account for the possibility of the three organisms having different targets for antibiotic control. Active strains were identified in all of the screens (Table 2.4). It was found that biofilms exhibit higher levels of anti-protozoal and anthelminthic activity, compared to their planktonic counterparts. These inhibitory activities (antagonism - allelopathy) reflect one of the many types of interactions occurring in surface-attached biofilm communities in the environment and hence provides a framework for understanding the inter- and intra-species interactions occurring in mixed species biofilm consortia (described in Chapters 3 and 4).

2.4.1 Protozoa exhibit variable susceptibility to different bacterial strains

Predators display a range of feeding efficiencies, dependent on a variety of factors such as temperature and nutrient availability. For the biofilm-feeding amoeba, *A. castellanii*, receptor recognition plays a crucial role in determining grazing efficiencies (Section 1.5.1) (34). In the optimization and validation assays, *A. castellanii* demonstrated the highest grazing efficiencies when 3 day-old amoebae were used as the target (analysis of variance, $P < 0.01$) (Figure 2.4). The growth cycle of amoebae includes a lag phase of around 8 hrs, followed by an exponential phase of approximately 40 hrs, doubling every 7-10 hrs in defined media (68, 306). Hence, 3 day-old amoebae inocula used correlated with the exponential phase, where the number of active trophozoites was at its maximum. In contrast, 7 day-old inocula coincided with the stationary phase, where nutrient depletion would have resulted in trophozoites becoming committed to undergoing cyst formation. It indicated that there were fewer trophozoites available to actively graze on *Vibrio* strains which could have accounted for the observed increased bacterial resistance (Figure 2.4). The effect of nutrient availability on amoebal grazing efficiency is further reinforced by the ten-fold difference in percentage survivals of *Vibrio* strains on a lawn consisting of amoeba exposed to new media, compared to those without exposure (Figure 2.5). Similarly, the significant difference in bacterial survival rates, demonstrated by the nutrient effect, is also observed at room temperature between undiluted and 1:10 diluted amoebal lawns ($P < 0.01$, analysis of variance) (Figure 2.6).

In order to test the validity of the amoebal screens, *Pseudoalteromonas* strains, common epibionts of marine eukaryotes, were selected (127). Due to their epiphytic lifestyle on animate surfaces, the intense competition for space and nutrients selects for diverse metabolic pathways, which is reflected in the variety of secondary metabolites produced (192). Egan *et al.* (81) observed a correlation between production of pigments in *Pseudoalteromonas* strains and their level of biological activity. A similar pattern was observed within the selected *Pseudoalteromonas* strains tested. Pigmented strains (*P. aurantia, P. ulvae, P. piscicida* and *P. tunicata*) and non-pigmented strains (*P. haloplanktis* and *P. nitrifaciens*) formed two distinct clades with and without activity respectively (Figure 2.7A). *P. piscicida* which produces only the yellow pigment, exhibited the

highest survival rate in 1:10 diluted amoeba, compared to *P. tunicata* which produces three pigments (green, yellow and purple) ($P < 0.0001$, analysis of variance) (Figure 2.7B) (37). The results suggest that there are other underlying factors contributing to its enhanced resistance. One possibility could be the different targets and functions of the yellow pigment, which enhanced its virulence. Zheng *et al.* (360) identified the yellow pigment in *P. piscicida* as β-carboline (norharman), a broad-spectrum bioactive, inhibitory against a range of Gram positive-, and Gram negative- bacteria and heterocyst forming cyanobacteria. The activity observed against heterocysts (non-vegetative cyanobacteria cell with three cell walls) could have accounted for the activity against amoebal trophozoites and cysts (double cell-wall) due to structural similarities. In contrast, the pigments produced by *P. tunicata* are used for target-specific chemical defense. For example, the yellow pigment, tambjamine, deters fungal infections whereas the purple pigment violacein confers antimicrobial properties and grazing resistance (208, 320).

Cell-free supernatants were harvested from 3 day-old biofilms from the same *Pseudoalteromonas* strains and incubated with *Tetrahymena* sp.. Strains producing and secreting the purple pigment, violacein, were shown to display acute toxicity. This is in agreement with a study done by Matz *et al.* (208), which demonstrated the ability of violacein to induce apoptosis in protozoan grazers. In contrast, supernatants from *P. piscicida* showed a lack of cytotoxic activity. Comparison of the results from the APA and TMA for this strain indicated, that biofilms were more toxic to protozoa than the cell-free supernatants. Despite the secretion of bioactives into the supernatant, the volumes used in the TMA were not sufficiently concentrated to induce the inhibitory activity observed by Zheng *et al.* (360) who harvested pure extracts from a 30 L fermenter. However, when grown as a biofilm, levels of bioactives produced by *P. piscicida* could be concentrated within the biofilm matrix to levels high enough to cause mortality. Overall, the data presented in the APA, TMA and selective grazing assays indicated that expression of inhibitory activity was greater in biofilms than in supernatants. The high quorum of cells within a biofilm community selects for novel metabolic pathways which are not fully understood and hence provides opportunities for the exploitation of undiscovered target-specific bioactives for novel drug development (192).

2.4.2 Nematodes drug targets differ to protozoan drug targets

A range of anthelminthics and anti-protozoal drugs are widely available commercially. However, drug discovery for these two categories have always progressed separately (108, 157). Despite each category consisting of both broad-spectrum and target-specific anthelminthics and anti-protozoal drugs, they can rarely be used interchangeably due to differences in targets (37, 145). For example, the most common anthelminthic drug known for its potency, Ivermectin, functions by paralyzing the nematode pharyngeal and body musculature because of its broad-spectrum and high affinity binding to a wide range of ligand-gated channels (122). Ivermectin would therefore be expected to not exhibit an equal level of potency in parasitic protozoa. In contrast, parasitic protozoal drugs such as Nifurtimox used for treatment of Chagas' disease caused by *Trypanosoma cruzi*, function by binding to free thiol groups in the protozoan, decreasing metallothionein (cysteine rich molecules that protect cell from oxidative stress) making it more susceptible to oxidative stress (210). Nevertheless, there is a rare group of broad-spectrum drugs such as Nitazoxanide, available that are effective against both kinds of parasites. Nitazoxanide is an anti-parasitic protozoal drug in current use that inhibits pyruvate ferredoxin oxidoreductase in protozoa. It has also been demonstrated to work on helminths, albeit at lower efficiencies by potentially targeting different enzymes like those in the nematode anaerobic electron transport chain (122).

Strains that were screened for toxicity against protozoan predators were also screened against *C. elegans* for such Ivermectin-like activity. Data from the cell-free supernatant and agar-based nematode paralysis assay indicated that nematode survival was lower when incubated with biofilms, which further supports the observation of inhibitory activity being more widespread in bacterial biofilms than in their supernatants (Figure 2.10 and Figure 2.11). A comparison between the proportions of positive hits with inhibitory activity from the culture collection of opportunistic pathogens indicated that the majority of strains exhibited anthelminthic activity (Table 2.3). Inhibitory activity observed in isolates from the remaining libraries was either against nematodes only or amoeba only. However, strains that exhibited activity against both were also occasionally observed (Table

2.4). Even though both models are Eukaryotic, the process of bacterial-induced mortality is slightly different. For example, nematode mortality is usually a result of a well-established infection by bacterial biofilms which leads to the expression of virulence factors such as the production of hydrogen cyanide (HCN) by *P. aeruginosa* and thus cyanide poisoning and eventually death in *C. elegans* (96). In contrast, the structural differences in amoeba such as the formation of double-wall cysts, enhances its resistance and persistence in the environment, possibly accounting for less positive (Table 2.3) (163). Hence, the broad spectrum HCN produced by *P. aeruginosa* did not result in *A. castellanii* mortality. Instead, QS-regulated bioactives like violacein in *Chromobacterium violaceum* (200, 208) and cell-cell contact dependent mechanisms such as the Type III secretion system in *P. aeruginosa* were required to cause death (207). In addition, the QS-regulated production of pyocyanin in *P. aeruginosa* has also been demonstrated in a separate study to impair macrophage clearance of apoptotic neutrophils in cystic fibrosis patients (23, 77). Therefore, the production of two different bioactives in *P. aeruginosa* suggests that positive strains like *Micrococcus* sp. (Table 2.4), which exhibit inhibitory activities in both protozoal and helminth screens could potentially express a range of bioactives with different drug targets which have activity against different types of organisms encountered in the environment.

2.4.3 Ecological significance of bioactive production

Bacterial diversity in the environment is extensive. The microbial communities formed are usually unique to their eukaryotic hosts and have the ability to enhance or inhibit further settlement of macro-foulers, through the production of secondary metabolites (8, 134). The production of secondary metabolites has been shown to be more common in biofilms which have exhibited inhibitory properties against a range of organisms (189). The diversity of surface-associated communities and their metabolic diversity, which is related to secondary metabolite production (347) is as one of the major focuses of this chapter.

The most significant ecological impact that arises as a result of inhibitory metabolite production is the effect on the distributions of bacteria and their community composition in that particular niche. In marine environments, the higher nutrition content on animate surfaces attracts bacteria (9). Due to

limitations in nutrient and space, the competition results in selection for inhibitor production in epibionts, as a form of deterrence against potential competitors, in order to prevent invasion and increase fitness (266). As a result, microbial epiphytic communities will consist of strains that are capable of expressing resistance against these inhibitors, and allows them to dominate. The data demonstrating that biofilms are more active than supernatants extends the observations of Long and Azam (189) who also reported that interspecies antagonism through inhibitory metabolite production were more common in biofilms than in free-living bacteria. In addition, it is shown that a majority of strains in the culture collection of *Vibrios* and *Pseudoalteromonas* are capable of infecting and causing mortality in *C. elegans* due to their opportunistic nature (Table 2.3). A small proportion of the culture collection expressed more than one inhibitory activity, and was also effective against *A. castellanii.* The ability of a strain to cause mortality in amoebae, suggests that it might also result in cytotoxic effects in macrophages. The co-evolution of such bacteria-protozoa interactions has been demonstrated by Cianciotto and Fields (57) in *L. pneumophila* to be responsible for priming for the bacterial surface protein, the macrophage infectivity potentiator (Mip), required for successful infections in macrophages, enhancing the virulence of the pathogen. Hence, these toxic strains can also be used to further understand how such bacteria-protozoa interactions evolve to infect higher organisms.

A comparison of the other environmental libraries shows that *Ulvae* epiphytes were the most active against *C. elegans*. In contrast, *Amphiroa* and *Corallina* epiphytes displayed the highest level of inhibitory activity against amoeba and the lowest against nematodes (Table 2.3). The different specificities of inhibition between *Ulvae* isolates and *Amphiroa* and *Corallina* isolates suggest that the epiphytic community between each alga is slightly different. The microbial diversity on these three algae and the amensal interactions between their epibionts and higher organisms could represent a selection factor for complex metabolic pathways, that produce broad range and target specific bioactives, which protects the algae from fouling (82, 125).

On the other hand, activated sludge communities expressed lower numbers of strains possessing inhibitory activity, than isolates from the *Ulvae* community (Table 2.3). It is possible that interactions occurring within activated sludge

communities are usually less intense, due to the limitless supply of nutrients (144). The constant introduction of nutrients from remineralization processes occurring as a result of high grazing pressure exerted by protozoan predators indicates that competition between community members are less common (156, 267). 16s rRNA gene sequencing results revealed that the positive strains consisted mainly of γ-Proteobacteria and can be classified into two clades; terrestrial and marine-derived organisms. The terrestrial group can be further classified into one group well known for mineralization of aromatics (*Acinetobacter*) (5, 54) and the other for being pathogenic (*Klebsiella*) (150). The identities of these strains and the clustering of closely related bacteria, provide a relatively good indication of the origin of the strain and its distinct environment. In addition, the high proportion of isolates identified belonging to *Acinetobacter* species despite being a random identification process, suggests that they are ubiquitous commensals in the environment (326). It is therefore not surprising that *Acinetobacter* species might be a core taxa responsible for carrying out crucial metabolic processes in activated sludge communities (243). However, in recent years, there has also been an increase in *Acinetobacter*-related nonsocomial infections, such as bacteremia caused by *Acinetobacter johnsonii* which provides a good indication of potential virulence factor production in this species (19). Vallenet *et al.* (326) demonstrated that virulence factors production which has been programmed into the genomes of *Acinetobacter* species are a result of selection by their ecological niches. Therefore, the production of inhibitory compounds observed here could be an environmental adaptation, resulting in diversification for development into a superior competitor with enhanced survival (317).

With the astounding diversity of Eukaryotic organisms in the environment, a proportion of bacteria symbionts and epibionts communities are still under-studied (132). This chapter has provided a fundamental basis for future projects aimed at screening other environmental libraries for bioactives (243). It also serves as a foundation for exploring other ecological interactions in microbial communities (as described in Chapter 3 and 4).

Chapter 3 : Synergism and antagonism within defined mixed-species biofilms

3.1 Introduction

Environmental ecosystems are highly diverse and unpredictable. Bacteria in such habitats have the versatility to survive and persist not only in hospitable but also under extreme conditions (70). This ability is in part due to their phenotypic and genotypic plasticity, which provides them with the ability to utilize a wide range of resources and to quickly adapt and survive when living as sessile surface-associated heterogeneous biofilms assemblages (70, 116).

Biofilms in environmental settings are usually comprised of multi-species consortia (62, 171). Highly stable communities have been suggested to be a consequence of interspecies communication and interactions, which encourages collective behaviour and coexistence among community members (144). The microbial interactions within multi-species consortia can be broadly grouped into synergistic or antagonistic interactions, both of which contribute to the spatial distribution of members in the biofilm. For example, the development of a commensal relationship involving the exchange of metabolites between *Burkholderia* sp. and *Pseudomonas* sp. encourages co-aggregation in a two-species biofilm (227). This form of synergistic interaction suggests that bacterial community composition can be manipulated to enhance complex processes such as biodegradation (171). In clinical settings, pathogenic strains susceptible to antibiotics are capable of causing persistent infections due to commensal associations with antibiotic resistant, low virulence, strains (230).

Bacteria also adjust interspecies interactions according to environmental conditions. For example, initial non-competitive interactions between *Pseudomonas putida* and an *Acinetobacter* sp. strain evolved into an exploitative one, in which *P. putida* formed intimate associations with the *Acinetobacter* strain to facilitate metabolite transfer (116). This eventually leads to an antagonistic interaction where both member compete for a common resource (116).

While most mixed-community studies have concentrated on addressing inter-species interactions in bacteria, our appreciation of inter-kingdom interactions such as bacterial-protozoal interactions is limited. Eukaryote ecology offers a large knowledge base and there is an increasing trend towards the application of eukaryote ecological theories to microbial systems to integrate both fields and understand the evolution of complex interactions. For example, the simplest Lotka-Volterra predator-prey model was commonly used to describe predation in terrestrial systems such as in the predatory relationship of the lynx and its prey, the hare (20). This model is characterized by a cyclical relationship between the predator and prey, where predator peak oscillations lag behind that of prey. This predator-prey model was tested in microbial systems using ciliates and bacterial prey and showed oscillations of predator and prey populations similar to those observed in eukaryote systems (16). Thus predation, one of the most fundamental interactions, plays a significant role in shaping community composition and structure in eukaryotes as well as microbes (52).

In eukaryote ecology, many biotic and abiotic factors contribute to the stability of a community, including species richness, resilience and resistance against environmental disturbances (141). The eukaryote-derived diversity-stability concept states that the stability of a community is directly proportional to its diversity and depends on the potential of community members to generate differential responses to various forms of perturbations (211). This study on diversity-stability hypothesis by McCann (211), highlights the importance of understanding the relationship between structural and functional diversity (i.e. for competition, predation and other microbial interactions) and community stability.

In marine microbial systems, protozoan predation contributes to environmental perturbations which affect bacterial communities, accounting for major bacterial mortalities and functioning as a selective force in controlling microbial food web dynamics (16). The selective grazing preferences of a predator have the potential to cause niche restriction of prey, competitive exclusion, and species extinction. These changes can disrupt the coexistence of members in the community, triggering a cascade of subsequent extinctions, which in turn affect the overall biological diversity, possibly resulting in community collapse (55, 129).

Microbial communities can be defined by their functional responses (196). A mixed-species bacterial consortium and protozoan grazers (an amoeba, a ciliate and a flagellate) were used as the model organisms in this investigation to further study the complex inter- and intra-species interactions and their effects on community dynamics. Bacterial biofilms and protozoan grazers offer experimental simplicity due to their short generation times, ease of genetic manipulation and small cell size. They provide the advantage of studying ecological interactions such as predation within a controlled laboratory system (16). For example, both synergism (e.g. cooperation) and antagonism (e.g. resource competition) can occur between members in a laboratory mixed-species biofilm generated from drinking water isolates (299).

For the work described in this chapter, a defined mixed community of four marine epiphytic bacteria (*Microbacterium phyllosphaerae* (2.04), *Shewanella japonica* (2.12), *Dokdonia donghaensis* (2.3), and *Acinetobacter lwoffii* (2.34)) from the surface of the green alga *Ulva australis* was selected to mimic natural environmental biofilms. The macroalgal host remains relatively free from fouling despite the lack of secondary metabolites. The anti-fouling properties of *U. australis* have been attributed to its epiphytic bacterial community (82). The four strains used in this current study occur synergistically as a stable, polymicrobial biofilm, capable of resisting invasion by the epiphytic bacterium *Pseudoalteromonas tunicata* and having increasing resistance to antimicrobials (44). Single-species biofilms on the other hand are susceptible to antimicrobial treatments and sensitive to *P. tunicata* invasion (44). This chapter describes an investigation of the increased resistance displayed by this four-species community extending to increased predation resistance.

Eukaryote ecological theories (Section 1.5.3) were applied to the bacterial-protozoal system in order to develop an improved understanding of predator-prey interactions within a complex community, and to determine the underlying mechanisms governing grazing resistance in biofilm communities. Niche-specific grazers, i.e. flagellates, ciliates and amoebae, were introduced to single- and mixed-species biofilms of various ages to determine the grazing impacts on community composition and to identify interspecies interactions within the biofilm community. This study investigated whether grazers display specialist or generalist feeding preferences (119) and if grazing results in prey niche

restriction. In addition to competition within a community, environmental disturbances also affect the biological diversity of a community and hence perturbation in the form of grazing was tested for effects on community stability (250). Finally the stability and response of biofilm communities to predation, for identification of keystone species and determination of the roles played by these species in the community was investigated with the aim of elucidating complex interactions and ecological dynamics in environmental biofilm communities.

3.2 Materials and methods

3.2.1 Strains and culturing conditions

3.2.1.1 Bacterial strains

Four epiphytic bacterial isolates obtained from the green macroalga *Ulva australis*, identified as *Microbacterium phyllosphaerae, Shewanella japonica, Dokdonia donghaensis* and *Acinetobacter lwoffii* (44) were used in the single- and mixed-species biofilms investigated in this chapter. These isolates were routinely maintained in Marine 2216 broth (Difco) or VNSS made with 1 × NSS which consisted of 17.6 g l^{-1} NaCl, 1.47 g l^{-1} Na_2SO_4, 0.08 g l^{-1} $NaHCO_3$, 0.25 g l^{-1} KCl, 0.04 g l^{-1} KBr, 1.87 g l^{-1} $MgCl_2.6H_2O$, 0.41 g l^{-1} $CaCl_2.2H_2O$, 0.008 g l^{-1} $SrCl_2.6H_2O$ and 0.008 g l^{-1} H_3BO_3, (195). Agar plates were made by addition of 15 g l^{-1} of agar to marine or VNSS broth. Plate cultures were incubated at room temperature (ca. 22°C) for 3 d and overnight liquid cultures were incubated with constant agitation at 200 rpm.

Table 3.1: Identities of the epiphytic isolates and their associated characteristics.

Strains	Characteristics (Phenotypic/Genotypic)	Reference
Microbacterium phyllosphaerae	Gram-positive aerobic rods, pale yellow small colonies, $Strep^R$, $Gent^R$, $PolyB^R$, Kan^R, Cm^R	(18, 44)
Shewanella japonica	Gram-negative flagellated facultative anaerobic rods, pinkish-orange shiny colonies, agar digesters, $Strep^R$, $Gent^R$, $PolyB^R$, Kan^R, Cm^R	(44, 140)
Dokdonia donghaensis	Gram-negative, non-motile, dark yellow shiny colonies, Amp^R, $Strep^R$, $Gent^R$, $PolyB^R$, Kan^R	(44, 356)
Acinetobacter lwoffii	Gram-negative, non-motile aerobe, white small colonies, $Strep^R$, $Gent^R$, Kan^R, Cm^R	(44)

Abbreviations: Amp^R – Ampicillin resistance, $Strep^R$ – Streptomycin resistance, $Gent^R$ – Gentamicin resistance, $PolyB^R$ – Polymycin B resistance, Kan^R – Kanamycin resistance, Cm^R – Chloramphenicol

3.2.1.2 Protozoan cultures

The amoeba, *Acanthamoeba castellanii* ATCC 30234, was obtained as an axenic culture from the American Type Culture Collection (Manassas, V.A.) and maintained as stock in 15 ml of protease-yeast-glucose (PYG) growth medium, supplemented with 0.1 × M9 (4.78 mM NaH_2PO_4, 2.2mM K_2PO_4, 0.86 mM NaCl, 1.87 mM NH_4Cl, 0.2 mM $MgSO_4$ and 0.01 mM $CaCl_2$) and 0.1 M glucose in tissue culture flasks with ventilated caps (Sarstedt Inc.) at room temperature. Prior to grazing experiments, 500 µl of the *A. castellanii* culture were passaged in 15 ml of 0.5 × NSS (nutrient-free, marine minimal media) (324) and fed 1 ml of heat-killed *Pseudomonas aeruginosa* (HKB) to acclimatize the amoebae to phagotrophic feeding and to the salinity and media used in the grazing assays. The amoeba cultures were incubated at 30°C for 3 d before harvesting. The concentration of amoebae was determined by visual enumeration of six 5 µl drops under 10 × magnification on a Leica DMLB epi-fluorescence microscope (Leica Microsystems, NSW, Australia).

The browsing ciliate, *Tetrahymena pyriformis* CCAP 1630/1W, was obtained from the Culture Collection 241 of Algae and Protozoa (Windmere, UK) and routinely passaged in PYG media. *T. pyriformis* was slowly acclimatized to the higher salinity of 0.5 × NSS by gradually increasing the ratio of NSS to PYG when passaged weekly over a period of five weeks. This process is essential for the complete removal of nutrients and to acclimatize the ciliate to phagotrophic feeding. Prior to experiments, 500 µl of *T. pyriformis* were passaged into 20 ml of 0.5 × NSS supplemented with 1 ml of HKB and grown at room temperature for 3 d before enumeration and use.

Rhynchomonas nasuta, a benthic grazing flagellate was isolated from Chowder Bay at Sydney Harbour Institute of Marine Sciences (Long. -33.83998, Lat. 151.255431) and maintained in 0.5 × NSS (324) supplemented with HKB. The culture was subjected to a gradual increase in the concentration of an antibiotic cocktail (50 g ml^{-1} ampicillin, 30 g ml^{-1} gentamycin, 30 g ml^{-1} kanamycin, 60 g ml^{-1} neomyxin, 30 g ml^{-1} polymyxin, 30 g ml^{-1} streptomycin, and 50 g ml^{-1} tobramycin) and diluted to extinction for over 1000 generations to obtain axenic cultures (206). Axenic *R. nasuta* for experimental use was passaged from

antibiotic stocks into 0.5 × NSS with 1 ml of HKB, one week prior to use and incubated at room temperature before enumeration.

3.2.2 Impact of grazing on *Ulva australis* epibionts as assessed by the amoebae plate assay

The four epiphytic isolates from *U. australis* were subjected to grazing as single- and mixed-species consortia in different combinations (two, three and four species, respectively) using the amoeba plate assay (APA) described in Section 2.2.3.1 and Figure 2.1. The mixed species combinations were prepared by mixing equal concentrations of each strain and serially diluting and inoculating (2 µl) them onto lawns of amoeba on agar plates. Enumeration and data presentation were as described in Section 2.2.3.1.

3.2.3 Selective feeding on biofilms of *Ulva australis* epiphytic isolates by *A. castellanii*

This grazing assay was performed as described above, to determine if *A. castellanii* displayed a selective feeding preference for any of the four epiphytic isolates from *U. australis*. The following changes were made to the experimental procedure. Plates contained only four isolates and the bacterial streaks were allowed to grow completely before the amoebae were added to the centre of the plate at equal distances from the bacterial isolates. Data obtained were analyzed and presented as described previously. To determine whether chemical cues are involved in the chemotaxis toward or deterrence of amoeba grazers to any particular strain, the distance moved by the grazer away from the centre of the initial drop and the number of amoebae were counted at different time intervals with a Leica DMLB epi-fluorescence microscope (Leica Microsystems, NSW, Australia) using 10 × magnification. Photographs were taken with a Leica DC100 digital camera using the Leica IM50 imaging software system.

3.2.4 Impact of grazers and their associated feeding mechanisms on single and defined mixed-species consortia in a semi-continuous system

3.2.4.1 Bacterial growth conditions

Serial dilutions were performed on the overnight cultures of four epiphytic isolates. Five µl drops of dilutions (10^{-1} to 10^{-9}) were inoculated onto Marine 2216 agar and VNSS agar and incubated at room temperature for 3 - 4 d. Colony growth was observed to determine the appropriate dilutions required for equal numbers of colony forming units (CFU) for each strain. The overnight cultures were then adjusted to equal CFU ml^{-1} counts before performing 10^{-2} dilutions in VNSS and incubating in side-arm flasks at room temperature with shaking at 200 rpm. The optical density was measured at 600 nm (NovaSpec® spectrophotometer Pharma Biotech) hourly. An average of three replicates was used to plot the growth curves.

3.2.4.2 Protozoan phagocytotic growth rate

T. pyriformis and *A. castellanii* are capable of growth in nutrient-rich media via pinocytosis and rate of growth would therefore differ to that supported by phagocytosis. The ciliate growth curve was determined by feeding 20 ml of 3 d old cultures with 1 ml of HKB in ventilated tissue culture flasks at room temperature with shaking at 60 rpm. Ciliates were enumerated by counting six 5 µl drops of Lugol's solution (Merck & Co., Inc.) fixed cultures every 2 h over a period of 2 d using a Leica DMLB epi-fluorescence microscope (Leica Microsystems, NSW, Australia) at 10 × magnification. The amoebae growth curve was monitored as follows. The initial 15 ml 3 d old stock was fed with 1 ml of HKB. Subsequently, 1 ml of this feed stock (10^5 amoebae ml^{-1}) was allowed to settle in 24-well tissue culture treated plates (Sarstedt Inc.) for 2 h. An average of six fields of view for three replicates were counted using an inverted light microscope (Olympus INVT200, Olympus Optical Co. Ltd., Tokyo, Japan) at 10 × magnification with 1.5 × intermediate magnification every 2 h over 2 d. The growth rate of protozoa, μ, was determined with the formula, $(\ln N_2 - \ln N_1)/(t_2 - t_1)$, where N_1 and N_2 represent the protozoan abundance at the beginning (t_1) and at the end (t_2), respectively, of exponential growth (183).

3.2.4.3 *Rhynchomonas nasuta* grazing on early stage biofilms

Early single- and mixed-species (four species) biofilms were formed by co-inoculation of 1000 protozoans ml^{-1} with 1 ml of bacterial cultures diluted 10^{-2} (approximately 10^7 CFU ml^{-1}) in VNSS in 24-well tissue culture treated microtitre plates (Sarstedt Inc.) (206). The grazing assays were incubated at room temperature with shaking at 60 rpm over a period of 3 d. *R. nasuta* cultures were quantified at 24, 48 and 72 h using an inverted light microscope (Olympus INVT200, Olympus Optical Co. Ltd., Tokyo, Japan) at 20 × magnification with 1.5 × intermediate magnification and an average of six fields of view for all three replicates. The biofilm biomass for each sampling time point was determined by crystal violet (CV) staining and CFU ml^{-1} counts for both sonicated (Unisonics FXP12MH, Australia) planktonic and biofilm fractions (see below). Blank controls of protozoa with VNSS and HKB and ungrazed control biofilms of single- and mixed-species of the same age were included for comparison.

3.2.4.4 *Tetrahymena pyriformis* grazing on intermediate biofilm and planktonic phases

T. pyriformis was co-inoculated with single- and mixed-species biofilms for 3, 6 and 8 d respectively in 24-well tissue-culture plates (Sarstedt Inc.) as described above (343) with the following amendments. After 3, 6 and 8 d, 100 µl aliquots were removed from the planktonic fraction and fixed with an equal volume of Lugol's solution (Merck & Co., Inc.). Six 5 µl drops of fixed culture were counted for each replicate, using a Leica DMLB epi-fluorescence microscope (Leica Microsystems, NSW, Australia) at 10 × magnification. Blank controls containing protozoa, VNSS and HKB as well as ungrazed biofilms were included. Planktonic fractions were collected for CFU ml^{-1} counts and exposed to two 5 min rounds of 50 Hz sonication in a water bath before plating serial dilutions. Biofilm biomass was determined by CV staining and CFU ml^{-1} determination (see below). Both grazed and ungrazed treatments were conducted in triplicate.

3.2.4.5 *Acanthamoeba castellanii* grazing on well-developed late stage biofilms

The surface feeding amoeba, *A. castellanii*, was co-inoculated with single- and mixed-species biofilms and incubated for 3, 6 and 8 d in 24-well tissue culture treated plates (Sarstedt, Inc.) as described above (136). Spent media was replaced every 2 d with fresh VNSS and care was taken to minimise biofilm disturbance to prevent amoeba detachment. Amoeba growth was monitored by averaging triplicate samples of six fields of views each, using an inverted light microscope (Olympus INVT200, Olympus Optical Co. Ltd., Tokyo, Japan) at 10 × magnification with 1.5 × intermediate magnification. Blank and ungrazed controls were included for comparison of the impact of grazer presence on both plankton and biofilms. Planktonic fractions were sonicated in a water bath sonicator at 50 Hz twice, for 5 min to disrupt any suspended aggregates to obtain single cells for CFU ml^{-1} determination. Biofilm biomass was quantified as described below.

3.2.4.6 Quantification of biofilm biomass

The biofilm biomass of single- and mixed-species grazed and ungrazed biofilms was quantified by CV staining (170) and by CFU ml^{-1} counts of serial dilutions. Planktonic fractions were discarded and the biofilms were washed three times with an initial volume of 1.25 ml of phosphate buffered saline (PBS) pH 7.2, followed by increments of 0.25 ml. Subsequently, they were stained for 20 min with 1 ml of 0.3% (wt vol^{-1}) 0.22 µm filtered CV, and then washed three times with PBS. The CV-stained wells were de-stained with 100% ethanol and the absorbance measured at 490 nm on a spectrophotometer (NovaSpec® spectrophotometer Pharma Biotech). The OD_{490nm} of the actual biofilm biomass was adjusted by subtracting the OD_{490nm} of the blank controls.

Grazed and ungrazed biofilm CFU ml^{-1} counts were obtained by removal of the planktonic fraction for plating, followed by washing of the biofilms three times with sterile PBS. 1 ml of VNSS was added to each well and biofilms detached from the surface with a blunt sterile toothpick. Biofilm aggregates were subjected to three 5 min rounds of 50 Hz sonication in a water bath for disintegration into single cells. Serial dilutions of these sonicated biofilm samples were then plated

on VNSS agar and incubated at room temperature, until growth was observed. The CFU ml^{-1} obtained were plotted as a function of time using Prism ® Version 5 for Windows (GraphPad Software Inc.).

3.2.5 Calculating relative abundance of bacterial species in the biofilm community using species evenness and niche-specific indices

The impact of protozoan grazing on the relative abundance of the bacterial species was measured at each sampling time point using evenness indices (E). Evenness is a measurement of how similar the abundance of each species is within a community and is defined by E equals $[(-\sum P_i \ln P_i)/\ln S]$ where S, the species richness, is defined by the number of species present in the community (251).

The niche-specific index (NI) is a calculation of the niche specific distribution of species in a community for the identification of preferential persistence in either planktonic or biofilm fractions, when exposed to protozoan grazers. The NI is determined by the ratio of the planktonic proportion of a species to the biofilm portion in the community. The NI is an arbitrary value where $NI < 1$ indicates a selective niche preference for the biofilm fraction and an $NI > 5$ indicates preferential persistence as plankton (170).

3.2.6 Statistical analyses

Statistical analyses were performed using Minitab 15 package for Windows (Minitab Inc., State College, PA, USA). The data from the grazing assays were analyzed using one, two and three-factorial analyses of variance (ANOVAs) with time (day 1, 2 and 3 for *R. nasuta* and days 3, 6 and 8 for *T. pyriformis* and *A. castellanii*), community (single or mixed) and treatment (grazed or ungrazed) as independent factors. Tukey's post hoc tests were used for the comparison of every group of means with every other group of means. Values obtained from the niche-specific indices were analyzed with student's t-tests using Prism ® Version 5 for Windows (GraphPad Software Inc.).

3.3 Results

3.3.1 Mixed-species early stage biofilms are more resistant to protozoan grazing than single-species biofilms

Current experimental systems employed for the study of bacterial-protozoan interactions usually involve a settled-suspended system requiring the attachment of protozoa onto a surface submerged in liquid media (206, 264, 343). In contrast, agar-based experimental systems are relatively rare, in spite of the fact that these systems are not affected by overlying media perturbations as is the case in settled-suspended systems. Therefore, such systems are particularly useful for microscopic observations of amoeba movements towards bacteria and for determining the impact of their interactions via 'amoeba trails' created on the agar. The amoebae plate assay described above was used to study the impact of grazing on the resistance of early stage biofilms formed by using one-, two-, three- and four-species combinations, for the model epiphytic community.

In this assay, the resistance to predation by *A. castellanii* of each strain when grown as a single- or mixed-species consortia was assessed by their ability to survive in the presence of the amoeba. Resistance is expressed as the percentage survival, as determined by the number of colonies developed in the presence of amoeba (non-grazed treatments) (Figure 3.1). As mono-species biofilms, *M. phyllosphaerae* and *A. lwoffii* were sensitive to grazing (13.00 % and 23.60 % survival respectively), whereas *S. japonica* and *D. donghaensis* were more resistant (50.00% survival). Dual-species biofilms consisting of different combinations of the four epiphytic isolates were in general more resistant to *A. castellanii* grazing than single-species, except for early biofilm formed by *M. phyllosphaerae* and *D. donghaensis* (24.00 % survival compared to > 50.00 % survival for other dual-species combinations). Two out of the four possible combinations of three-species biofilms also displayed higher levels of resistance, when compared to single-species biofilms (abd = 66.00 % and bcd = 53.00 % survival, where a = *M. phyllosphaerae*, b = *S. japonica*, c = *D. donghaensis* and d = *A. lwoffii*), whereas the other two were sensitive to grazing (abc = 38.00 % and acd = 37.00 % survival). When grown as a four-species biofilm, the grazing resistance was enhanced compared to single-species early biofilms (percentage survival 56.00 %) (Figure 3.1). The grazing resistances varied significantly

depending on the members present in the biofilm consortia (a vs. ad, bc, bd, abd; d vs. ad, bd; ab vs. bc; ac vs. ad, bc, bd; ad vs. abc, acd; and bc vs. abc, $P < 0.05$, analysis of variance). In general, mixed-species consortia were more resistant to *A. castellanii* grazing than single-species consortia, when grown as early stage biofilms.

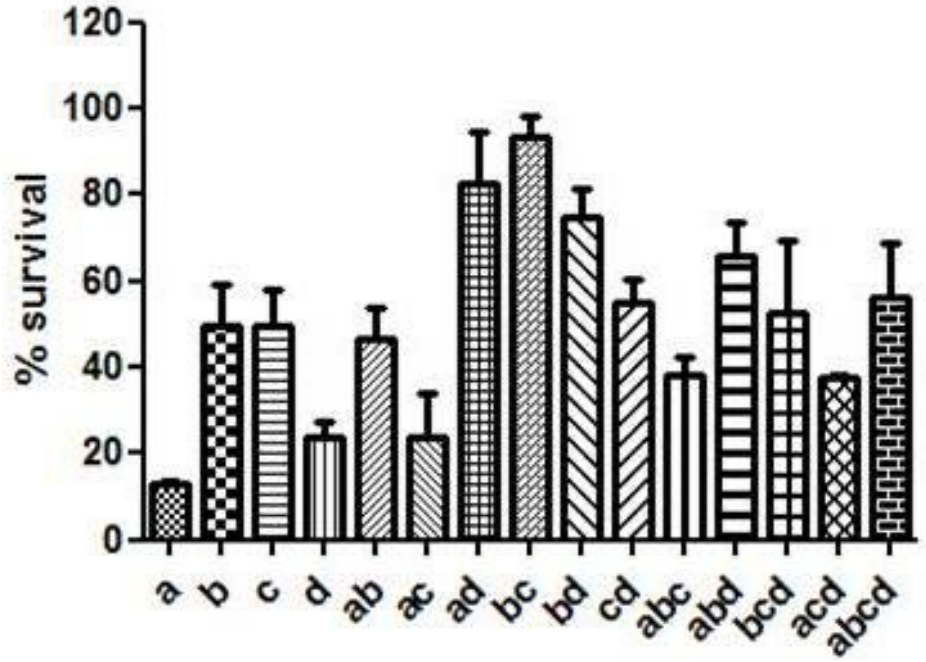


Figure 3.1: Mean grazing resistance of combinations (one-, two-, three- and four-species consortia) of *U. australis* epiphytic isolates; *M. phyllosphaerae* (a), *S. japonica* (b), *D. donghaensis* (c) and *A. lwoffii* (d), against *A. castellanii*. The resistance of each consortium is expressed as the percentage survival under grazing pressure exerted by *A. castellanii.* The grazing resistances displayed by the consortia consisting of different species combinations were significantly different to each other as determined by analysis of variance; $P < 0.05$. Error bars represent standard deviation of four replicates.

3.3.2 Selective feeding preferences of *Acanthamoeba castellanii*

To determine if *A. castellanii* displays feeding selectivity which can potentially affect the community composition of a mixed community, feeding preference was assessed by providing the amoebae with a 'choice' of the four epiphytic isolates. Each bacterial strain was aligned at an equal distance from a central inoculum of

amoeba and allowed to grow before *A. castellanii* was added. *A. lwoffii* was preferentially consumed by *A. castellanii* eight days into the experiment as indicated by the grazing front, which reduced the original bacterial inoculum by 4.2 mm (Figure 3.2A), while *S. japonica* was reduced by 4.25 mm after 14 days. In contrast, *M. phyllosphaerae* and *D. donghaensis* remained ungrazed for the duration of the experiment.

The production of bacterial chemical cues with attractant or repellent properties affecting grazer migration was also examined. The number of amoebae migrating towards each strain was determined microscopically. There was no significant increase or decrease in the number of amoebae approaching each bacterial strain (Figure 3.2B). The amoebae trail radiated outwards from the centre of the drop in all directions, indicating that there was no repellant or attractant effects. Furthermore, amoebae migrated towards the bacterial front of *M. phyllosphaerae* suggesting that protozoan movement was random and that the resistance of *M. phyllosphaerae* to grazing by *A. castellanii* was dependent on contact.

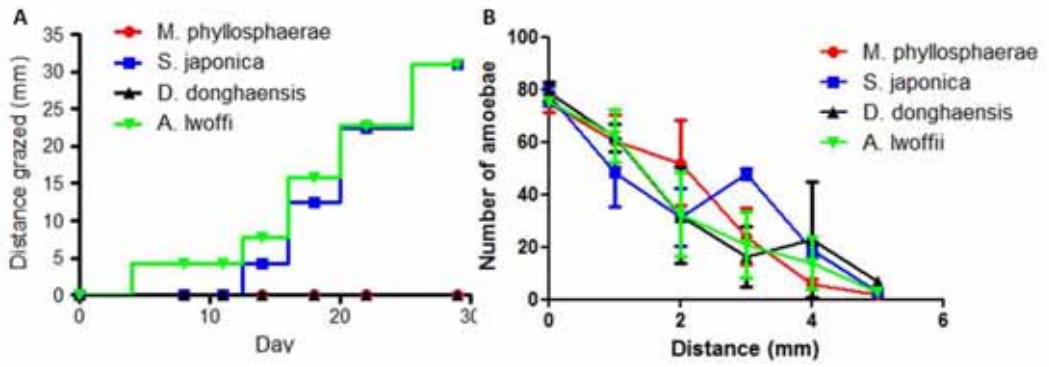


Figure 3.2: Preferential feeding displayed by *A. castellanii* in the presence of the four marine epiphytic isolates. The distance each isolate was grazed was determined by measuring the distance between the grazing front and the starting position of the bacterial streak (A). Migration of amoebae was monitored over time by enumeration of amoebae at various distances away from the protozoan inoculum (B).

3.3.3 Grazing on epiphytic isolates by *Rhynchomonas nasuta*

The flagellated grazer *R. nasuta* grazed differently on single- and mixed-species biofilms of *U. australis* epiphytic isolates, and showed varying growth responses

to the biofilms (Figure 3.3). The growth rates (μ) of protozoa feeding on single-species biofilms demonstrated that growth of *R. nasuta* on *S. japonica* (μ = 2.31 d^{-1}) was significantly faster than on all other strains (P < 0.001, analysis of variance), followed by *D. donghaensis*, *M. phyllosphaerae* and *A. lwoffii* (μ = 2.00 d^{-1}, 1.95 d^{-1} and 1.86 d^{-1} respectively). On mixed-species biofilms, protozoan numbers were comparable to numbers on single-species biofilms for all strains, except *S. japonica* where the numbers of protozoa were 2.8-fold higher (Figure 3.3A). *R. nasuta* numbers did not correlate with biofilm biomass in either single- or mixed-species biofilms (Figure 3.3B to F). Neither *M. phyllosphaerae* nor *S. japonica* single-species biofilms showed any significant differences when grown in the presence, as compared to the absence of *R. nasuta* (P > 0.05, student's *t*-test) (Figure 3.3B to C). Even so, *S. japonica* was able to sustain the highest numbers of *R. nasuta*. In contrast, the increase in biofilm biomass in single-species biofilms of *A. lwoffii* in the presence of the grazer did not result in increases in protozoan numbers (P > 0.05, student's *t*-test) (Figure 3.3E and F).

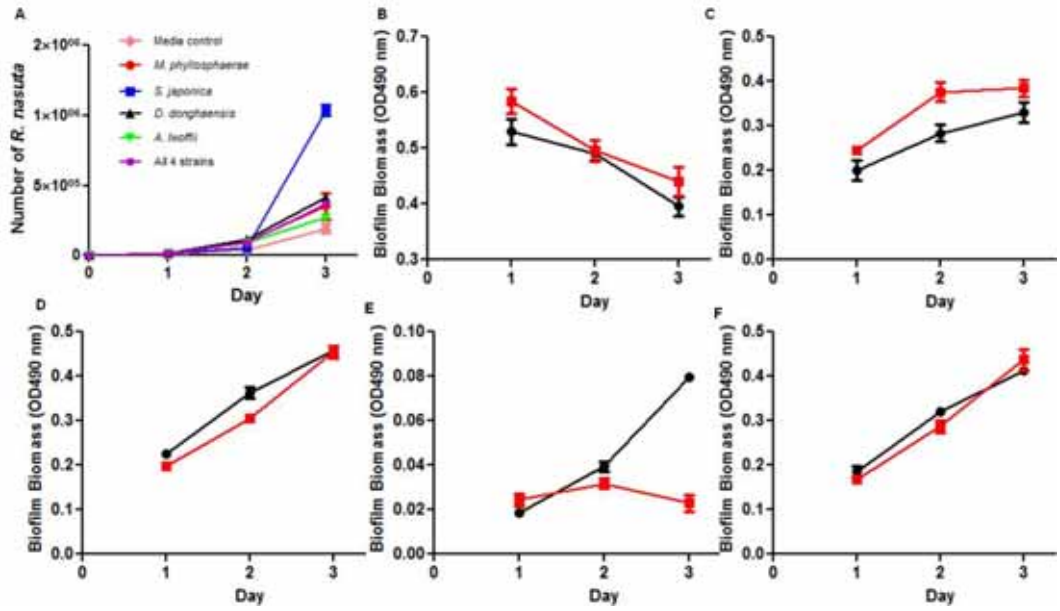


Figure 3.3: Mean persistence of single- and mixed-species early stage biofilms when exposed to grazing by the flagellate, *R. nasuta.* The abundances of *R. nasuta* (A) and the biofilm biomass of single species *M. phyllosphaerae* (B), *S. japonica* (C), *D. donghaensis* (D) and *A. lwoffii* (E), and the four-species biofilms (F) formed in grazed (black) and ungrazed (red) treatments were monitored over 3 d. Error bars represent standard deviations of three replicates.

The effect of grazing on the abundances of biofilm and planktonic bacteria of single- and mixed-species communities was also examined. Further, the impact of *R. nasuta* grazing on the community composition of mixed species biofilms was investigated. Biofilms were established and exposed to *R. nasuta* as described in section 3.2.4.3. Initial inocula of mono-species biofilms for *M. phyllosphaerae, S. japonica, D. donghaensis* and *A. lwoffii* were $2.28 \pm 0.47 \times 10^7$, $8.85 \pm 0.07 \times 10^7$, $2.09 \pm 1.03 \times 10^7$ and $3.78 \pm 0.89 \times 10^7$ CFU ml^{-1}, respectively. The proportions of each species in the mixed-species biofilms were within the range of $2.34 \times 10^6 \pm 1.63 \times 10^6$ to $8.43 \times 10^6 \pm 2.59 \times 10^6$ CFU ml^{-1} giving a final inoculum of $2.00 \times 10^7 \pm 5.2 \times 10^6$ CFU ml^{-1} (similar to single-species inocula).

When grown as mono-species biofilms, the abundance of the biofilm of all four epiphytic isolates was higher than when grown as a mixed-species biofilm in grazed and non-grazed treatments ($P = 0.006$ for *S. japonica* and $P < 0.001$ for the remaining three strains, analysis of variance) (Figure 3.4A to D). The total bacterial abundance of biofilm and planktonic phases of the mixed community was approximately equal to the total bacterial abundance of single species communities, suggesting the decreased biomass of each species in the mixed community was due to limitations in nutrient availability and space rather than to competition between species. The *M. phyllosphaerae* single-species abundance was reduced over 3 d (- 16.70 % on day 1, - 10.60 % on day 2 and – 47.20 % on day 3). The resistance of *M. phyllosphaerae* to *R. nasuta* grazing was significantly different when grown as a mixed-species biofilm, increasing over time as indicated by higher biofilm abundance in grazed treatments (1.17-fold increase on day 1 to 6.86-fold increase on day 3; $P = 0.009$, analysis of variance) (Figure 3.4A). Similarly, in single-species *M. phyllosphaerae* planktonic fractions, reductions in bacterial abundance under grazing pressure were observed over time (Appendix IA, $P = 0.027$, analysis of variance).

S. japonica biofilm bacterial abundance increased in the presence of grazers on days 1 and 2 both in single- and mixed-species biofilms. However, on day 3 a reduction in biofilm abundance occurred under grazing pressure (- 85.70 % in single and - 18.70 % in mixed; $P < 0.001$, analysis of variance) (Figure 3.4B). A comparison of planktonic and biofilm bacterial abundance indicated that *S.*

japonica planktonic counts were ca. 100-fold higher, than biofilm counts in both single- and mixed-species consortia (Figure 3.4B and Appendix IB, $P = 0.001$, analysis of variance). The planktonic bacterial abundance was reduced over time, more so in the single- than in the mixed-species samples (- 96.50 % in single compared to - 74.60 % in mixed on day 3) (Appendix IB).

Decreases in both biofilm and planktonic abundance of *D. donghaensis* occurred in the mixed-species community compared to single-species cultures indicating antagonistic interactions (Figure 3.4C and Appendix IC). Despite this, both planktonic and biofilm fractions of *D. donghaensis* were protected from grazing in the mixed-species consortia, compared to the single-species condition where both biofilm and planktonic bacterial abundance were significantly grazed on day 3 (- 79.50 % in biofilms and - 97.70 % in plankton, $P < 0.001$, analysis of variance) (Figure 3.4C and Appendix IC).

Of all the epiphytic isolates, *A. lwoffii* had the lowest CFU ml^{-1} counts in both single- (ca. 10^5 to 10^6) and mixed-species biofilms (ca. 10^4 to 10^5) in the presence and absence of *R. nasuta* (Figure 3.4D and Appendix ID). Although, the impact of grazing on *A. lwoffii* increased over time for both mixed- (- 17.90 % on day 1, - 70.60 % on day 2 and - 88.20 % on day 3) and single-species biofilms (- 15.40 % on day 1, 1.04-fold increase on day 2 and - 72.30 % on day 3), no significant differences were observed ($P > 0.05$, analysis of variance). Planktonic *A. lwoffii* abundance did not show significant grazing effects over time ($P > 0.05$, analysis of variance).

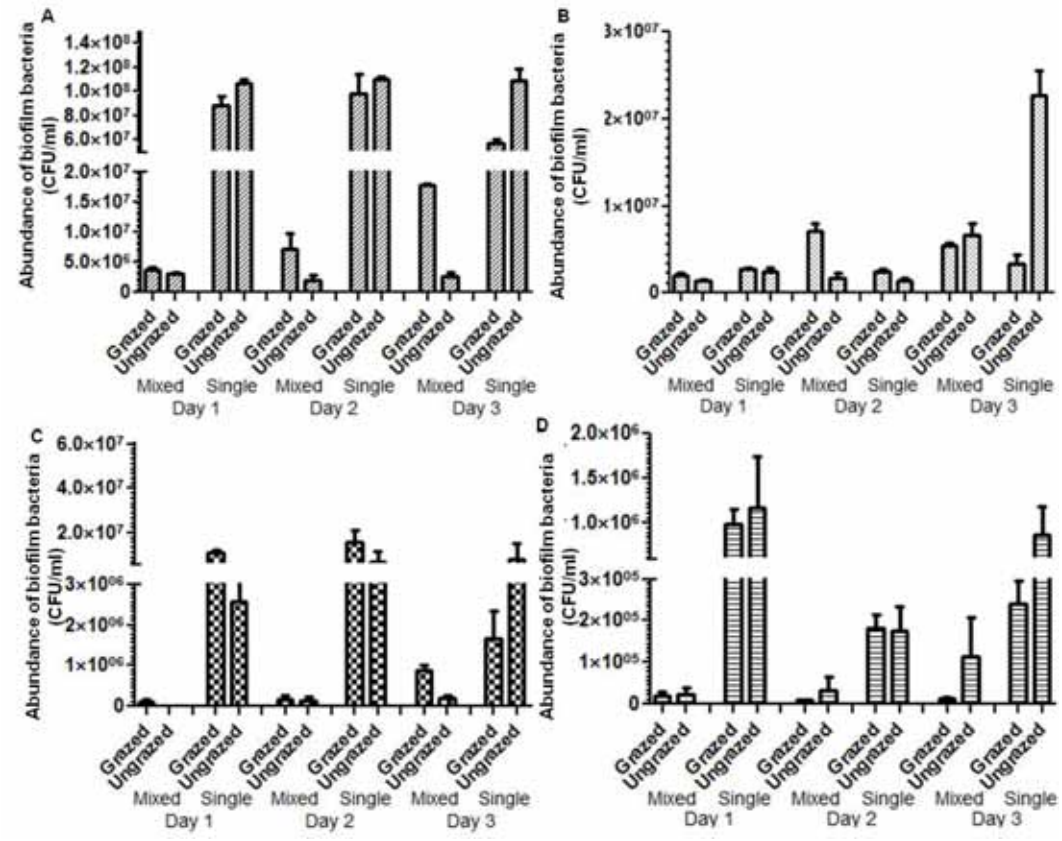


Figure 3.4: Comparison of the mean resistance of single- and mixed-species (four species) biofilms grown in the presence and absence of *R. nasuta* over 3 d. The biofilm fractions of *M. phyllosphaerae* (A), *S. japonica* (B), *D. donghaensis* (C) and *A. lwoffii* (D) in each consortium were harvested. Bacterial counts from grazed and ungrazed treatments were expressed as colony-forming units (CFU ml^{-1}). Days 1, 2 and 3 indicate the number of days both mixed- and single-species biofilms were co-incubated with *R. nasuta*. Error bars represent standard deviation of three replicates.

3.3.4 *Rhynchomonas nasuta* grazing impacts on the evenness of the mixed-species biofilm and planktonic communities

Protozoan mediated grazing activities can result in high levels of prokaryotic mortalities and selective feeding preferences can cause significant impacts on marine prokaryotic communities (e.g. diversity and evenness) (247). Evenness indices were calculated for biofilm and planktonic abundance of each species in the mixed community sampled over 3 d to determine if *R. nasuta* grazing can invoke changes in bacterial community composition and evenness of the mixed community by enriching for or selectively removing particular species. As the defined mixed species community was grown in a semi-continuous closed system,

the species richness remained constant in both biofilms and planktonic fractions (data not shown). Both biofilm and planktonic evenness indices were below 1, indicating very different species abundances in the communities (Figure 3.5A and B). Despite this, evenness indices were not significantly different in the presence compared to absence of the protozoan grazer, *R. nasuta* ($P > 0.05$, student's *t-test*), suggesting the lack of evenness was due to inter-specific interactions rather than grazing pressure.

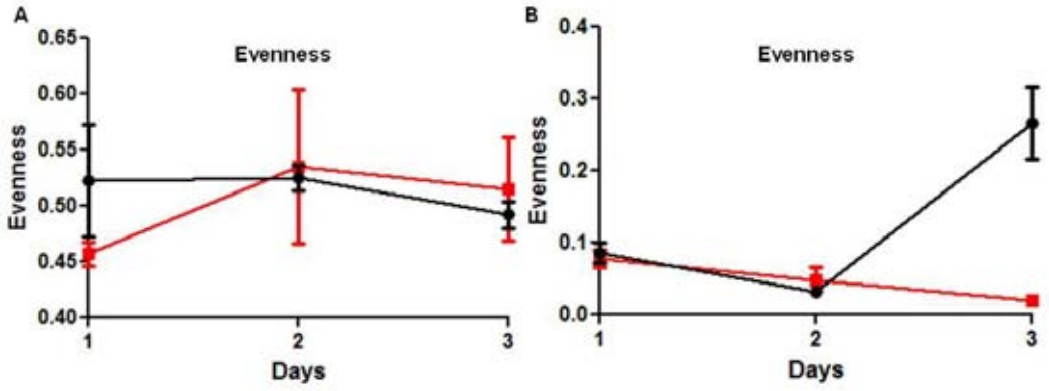


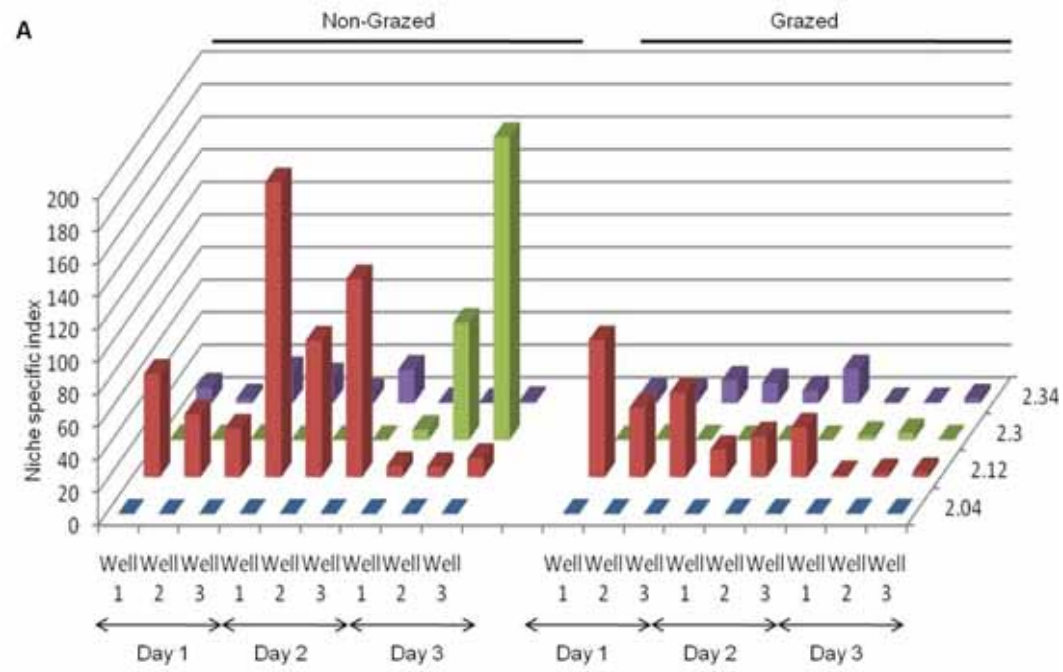
Figure 3.5: Comparison of the mean evenness of mixed species biofilm (A) and planktonic phases (B) when co-inoculated with (black) and without (red) of *R. nasuta* for 3 d. Error bars represent standard deviations of three replicates.

3.3.5 Grazing by *Rhynchomonas nasuta* induces shifts in ecological niche preferences

The niche-specific index (170), is an arbitrary value, where NI < 1 indicates a preference for the biofilm fraction, while a NI > 5 indicates a preference for the planktonic phase. The niche specific preference of members of the mixed-species consortia was investigated in order to determine if grazing by *R. nasuta* influenced species niche preferences. Differences in niche specific distribution were observed for some members of the mixed-species community, despite evenness indices showing no significant differences between grazed and non-grazed treatments. Single-species biofilms were resilient to niche-specific changes as co-inoculation with *R. nasuta* on days 1 and 2 did not result in any clear shifts in the niche specific distributions of the four epiphytic isolates (Figure 3.6A).

However, on day 3, grazing pressure induced a shift by *D. donghaensis* from the planktonic fraction to the biofilm fraction.

M. phyllosphaerae exhibited a niche preference for biofilms in both the presence and absence of *R. nasuta* when in mixed-species consortia (Figure 3.6B). *S. japonica* showed a shift in niche preference towards the planktonic fraction in the presence of *R. nasuta*, whereas in the absence of *R. nasuta*, the biofilm fraction was the preferential niche. *D. donghaensis* did not show any significant niche preference or distinct shifts towards biofilm or planktonic fractions. A comparison between grazed and ungrazed treatments indicated a preferential shift in *A. lwoffii* towards the planktonic fraction in the presence of the surface grazer, *R. nasuta*, when in a mixed-species consortia (Figure 3.6B).



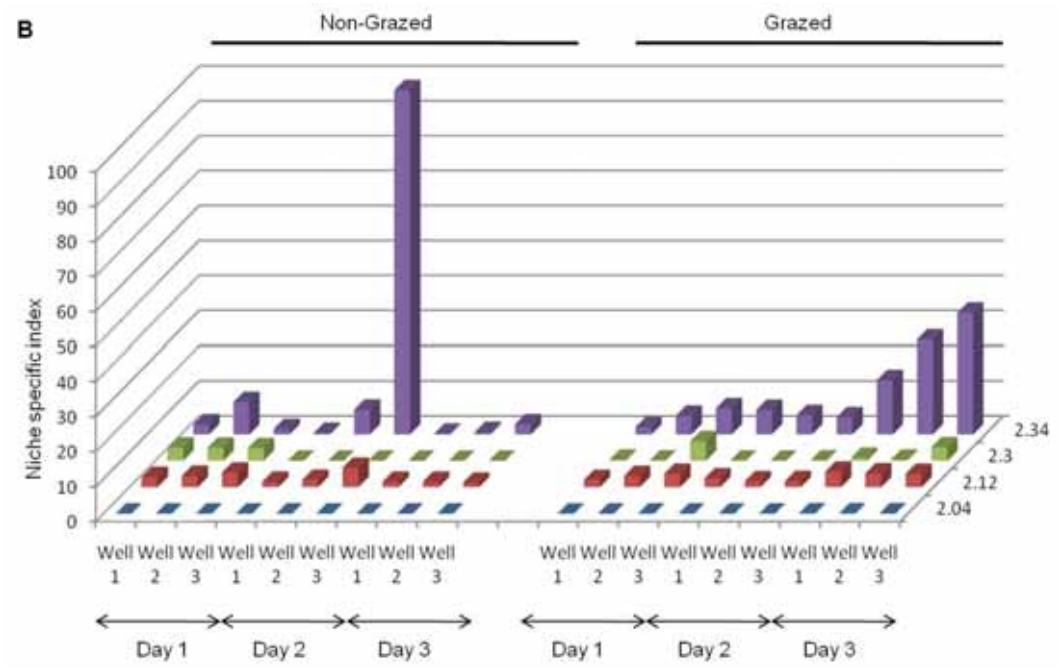


Figure 3.6: Impact of *R. nasuta* grazing on the niche preference of the four epiphytic isolates (2.04 – *M. phyllosphaerae,* 2.12 – *S. japonica,* 2.3 – *D. donghaensis* and 2.34 – *A. lwoffii*) grown as single species (A) and mixed (B) species consortia. The ecological niche index was calculated for non-grazed and grazed treatments over a period of 3 d.

3.3.6 *Tetrahymena pyriformis* displays selective feeding preferences which affect mixed microbial communities

Different groups of protozoa are adept at grazing on different forms of bacterial communities and hence have varying grazing niche preferences. For example, ciliate grazing plays a major role in controlling bacterial abundances in pelagic systems (83). However, a recent study by Weitere *et al.* (343) which demonstrated the ability of *Tetrahymena* sp. to reduce late-stage biofilms, indicated that ciliates are efficient grazers of both suspended and sessile microbial communities. The impact of grazing by *T. pyriformis* on single- and mixed-species biofilms of *U. australis* epiphytic isolates was investigated. The epiphytic isolates exhibited varying degrees of persistence when exposed to grazing which in turn contributed to different growth rates of *T. pyriformis* (Figure 3.7).

In general, single- and mixed-species biofilms of the epiphytic isolates can be categorized into two groups (sustaining or suppressing), based on the numbers of *T. pyriformis* (Figure 3.7A). The growth rate of *T. pyriformis* (μ) was highest on

single-species biofilms of *A. lwoffii* (μ = 0.626 d^{-1}) followed by *D. donghaensis* (μ = 0.607 d^{-1}). It appeared that these species were capable of sustaining *T. pyriformis* growth, as indicated by the increase in *T. pyriformis* numbers when feeding on biofilms of these strains (Figure 3.7A). In contrast, *M. phyllosphaerae* and *S. japonica* biofilms suppressed protozoan growth despite being grazed (μ = – 0.115 d^{-1} and – 0.018 d^{-1} respectively) (Figure 3.7A to C). Mixed-species biofilms also suppressed growth of *T. pyriformis* (Fig. 3.7A, μ = 0.018 d^{-1}). The anti-predatory fitness of each species could be categorized based on the impacts on both protozoan numbers and bacterial biomass: (i) susceptible (e.g. *A. lwoffii* sustains protozoan growth but suffers decreased bacterial biomass) (ii) susceptible but toxic at high numbers (e.g. *S. japonica* supports initial increase in protozoa numbers but results in death of the grazer at higher biofilm biomass), (iii) resilient (e.g. *D. donghaensis* is capable of supporting high levels of protozoa via high bacterial growth rates) and (iv) resistant strains (e.g. *M. phyllosphaerae*, which can resist grazing by killing the grazer through contact-dependent or post-ingestional defense strategies) (Figure 3.7). As for the mixed-species biofilms, the anti-predatory fitness can be classified as resistant due to similarities in bacterial and protozoan growth trends to that of the single species *M. phyllosphaerae* biofilm.

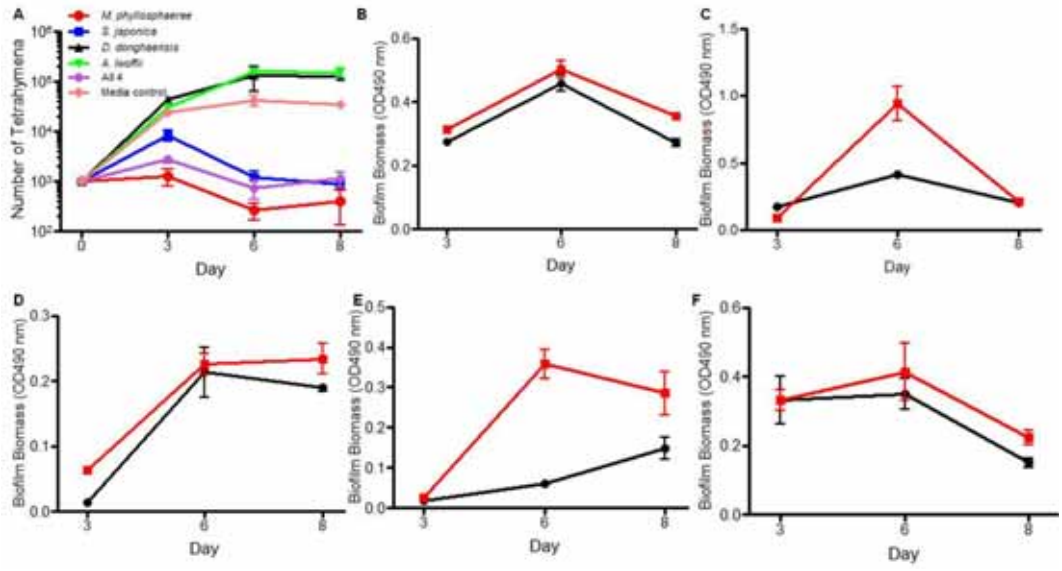


Figure 3.7: Mean impact of grazing on intermediate and late stage single and mixed species biofilms by the browsing ciliate, *T. pyriformis.* The abundances of *T. pyriformis* (used for calculating μ) (A) and the biofilm biomass of the single species *M. phyllosphaerae* (B), *S. japonica* (C), *D. donghaensis* (D) and *A. lwoffii* (E), and the four-

species biofilm (F) formed in grazed (black) and ungrazed (red) treatments were monitored over 3, 6 and 8 d. Error bars represent standard deviations of three replicates.

The effect of *T. pyriformis* grazing on the community composition of intermediate- to late-stage biofilms was also examined. Biofilms were established and exposed to *T. pyriformis* as described above. Initial inocula used for mono-species biofilms for *M. phyllosphaerae, S. japonica, D. donghaensis* and *A. lwoffii* were $5.30 \pm 0.14 \times 10^7$, $1.01 \pm 0.20 \times 10^8$, $2.03 \pm 0.65 \times 10^7$ and $4.30 \pm 0.14 \times 10^7$ CFU ml^{-1} respectively. In mixed-species biofilms, initial inocula ranged between $4.00 \pm 1.41 \times 10^6$ to $4.60 \pm 0.28 \times 10^7$ CFU ml^{-1} of each species to give a final inoculum of $9.95 \pm 1.62 \times 10^7$ CFU ml^{-1}.

Grazing by *T. pyriformis* was significantly different for epiphytic isolates in both biofilms ($P = 0.007$, analysis of variance) and planktonic fractions ($P = 0.005$, analysis of variance) of single- and mixed-species communities (Figure 3.8). As single species cultures, both *M. phyllosphaerae* and *S. japonica* showed an increase in biofilm ($P = 0.027$ and $P < 0.05$ respectively, analysis of variance) and planktonic ($P < 0.05$ and $P < 0.01$ respectively, analysis of variance) bacterial abundance, in response to grazing (Figure 3.8A, B and Appendix IIA, B). In contrast, single species biofilm and planktonic fractions of *D. donghaensis* (biofilm reduction of 98.40 % on day 3, 99.40 % on day 6 and 83.30 % on day 8, $P = 0.02$, analysis of variance; planktonic reduction of > 99.00 % over 3, 6 and 8 d, $P < 0.001$, analysis of variance) (Figure 3.8C and Appendix IIC) and *A. lwoffii* were significantly reduced during grazing (– 99.90 %, – 51.00 % and – 66.80 % on days 3, 6 and 8, respectively; $P = 0.003$, analysis of variance) (Figure 3.8D, Appendix IID).

In mixed-species biofilms, all four epiphytic isolates showed grazing resistance and susceptibility profiles, which were similar to single species biofilms ($P = 0.846$, analysis of variance). However, higher biofilm bacterial abundance was observed for *M. phyllosphaerae* and *A. lwoffii* single-species biofilms in the absence of *T. pyriformis* compared to mixed-species biofilms (Figure 3.8A and D). The lower abundances of *S. japonica* and *D. donghaensis* single-species biofilms, compared to mixed-species biofilms indicated that the mixed-species consortia are synergistic to certain members (Figure 3.8B and C).

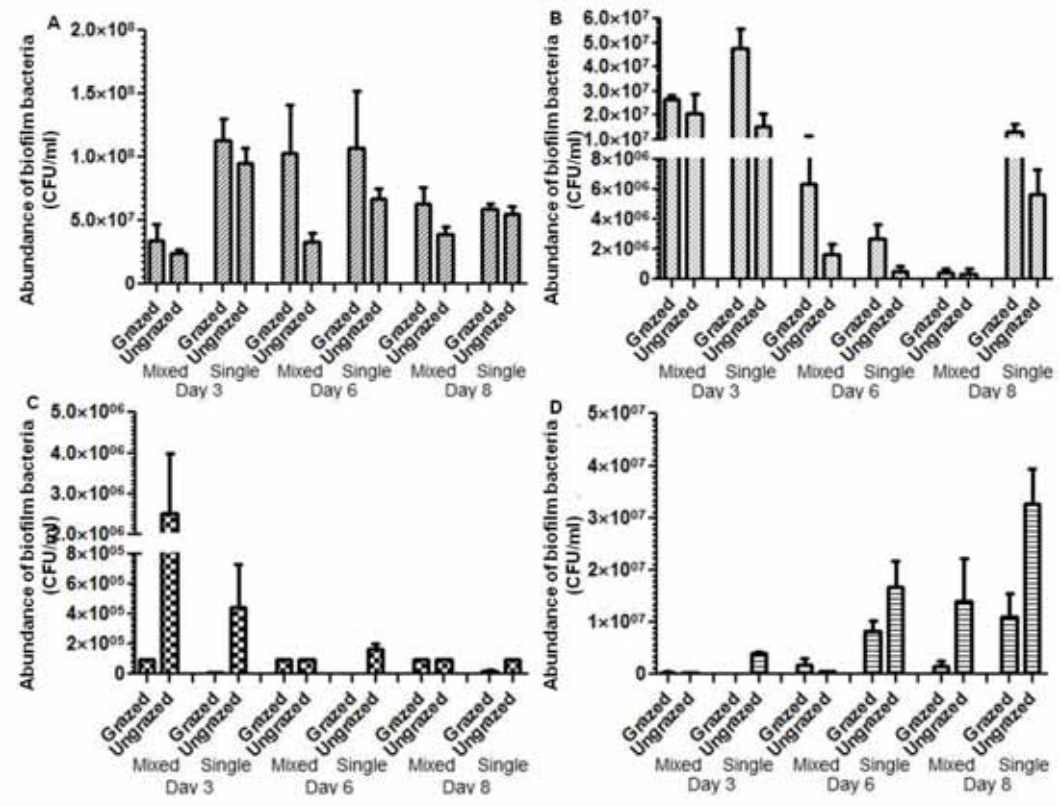


Figure 3.8: Persistence of intermediate and late stage biofilms of single and mixed species (four species) biofilms grown with (grazed) and without (ungrazed) *T. pyriformis*. The biofilm phases of *M. phyllosphaerae* (A), *S. japonica* (B), *D. donghaensis* (C) and *A. lwoffii* (D) were harvested as described in Section 3.2.4.4 and bacterial abundance from grazed and ungrazed treatments expressed as CFU ml^{-1}. Day 3, 6 and 8 indicate the number of days of co-inoculation with *T. pyriformis* prior to harvesting. Error bars represent standard deviation of three replicates.

3.3.7 Species evenness in defined mixed-species consortia was not significantly altered by *Tetrahymena pyriformis* grazing

The ciliate, *T. pyriformis* is not restricted to specific grazing niches and thus feeds effectively on biofilm and suspended cells. The resistance to *T. pyriformis* grazing of the mixed-species community over 3, 6 and 8 d, was investigated to establish the effects of intense grazing pressure on community composition. Species richness remained constant in both biofilm and planktonic fractions (data not shown) due to the closed system. Evenness indices for biofilm and planktonic fractions were < 1, suggesting that the species abundances of the epiphytic

isolates differed (Figure 3.9A and B). Comparisons of evenness between grazed and ungrazed treatments were not significantly different, indicating that differences in species abundance were affected more by inter-species interactions than by grazing pressure ($P > 0.05$, student's *t-test*).

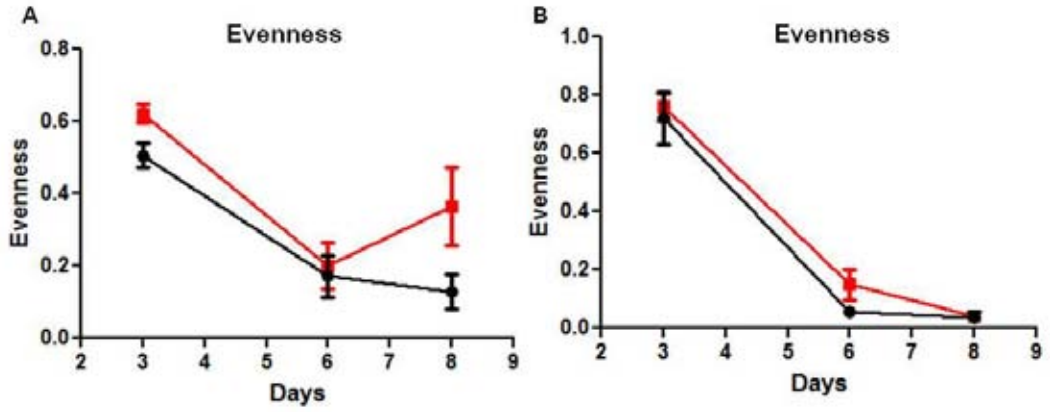


Figure 3.9: Comparison of mean evenness of mixed-species biofilm (A) and planktonic (B) phases grown with (black) and without (red) *T. pyriformis* over 3, 6 and 8 d. Error bars represent standard deviations of three replicates.

3.3.8 *Tetrahymena pyriformis* grazing effects on niche preference of epiphytic isolates are dependent on growth as single-species or mixed-species consortia

The impact of *T. pyriformis* grazing on the niche distribution of members of single-and mixed-species consortia was investigated. Despite the lack of significant differences in evenness indices between grazed and non-grazed treatments, differences in niche specific distribution were observed for members of a mixed-species community exposed to grazing pressure. Single-species cultures of *M. phyllosphaerae*, *S. japonica* and *D. donghaensis* were resilient to niche-specific changes when co-inoculated with *T. pyriformis* (Figure 3.10A). All three strains maintained an ecological niche preference for the planktonic fraction. As for *A. lwoffii*, grazing pressure exerted by *T. pyriformis* resulted in an early shift to the planktonic phase on day 3, after which the preference was for the biofilm phase in grazed and ungrazed samples.

In contrast, mixed-species biofilms demonstrated a niche preference contrary to that observed in single-species biofilms (Figure 3.10B). Both *M. phyllosphaerae* and *S. japonica* exhibited a niche preference for the biofilm fraction in the presence and absence of *T. pyriformis. D. donghaensis* and *A. lwoffii* showed an initial preference for the planktonic fraction on day 3 but for the biofilm at later sampling times. The introduction of the ciliated grazer stimulated the shift from planktonic to biofilm fractions from the period of day 8 to day 6 (Figure 3.10B).

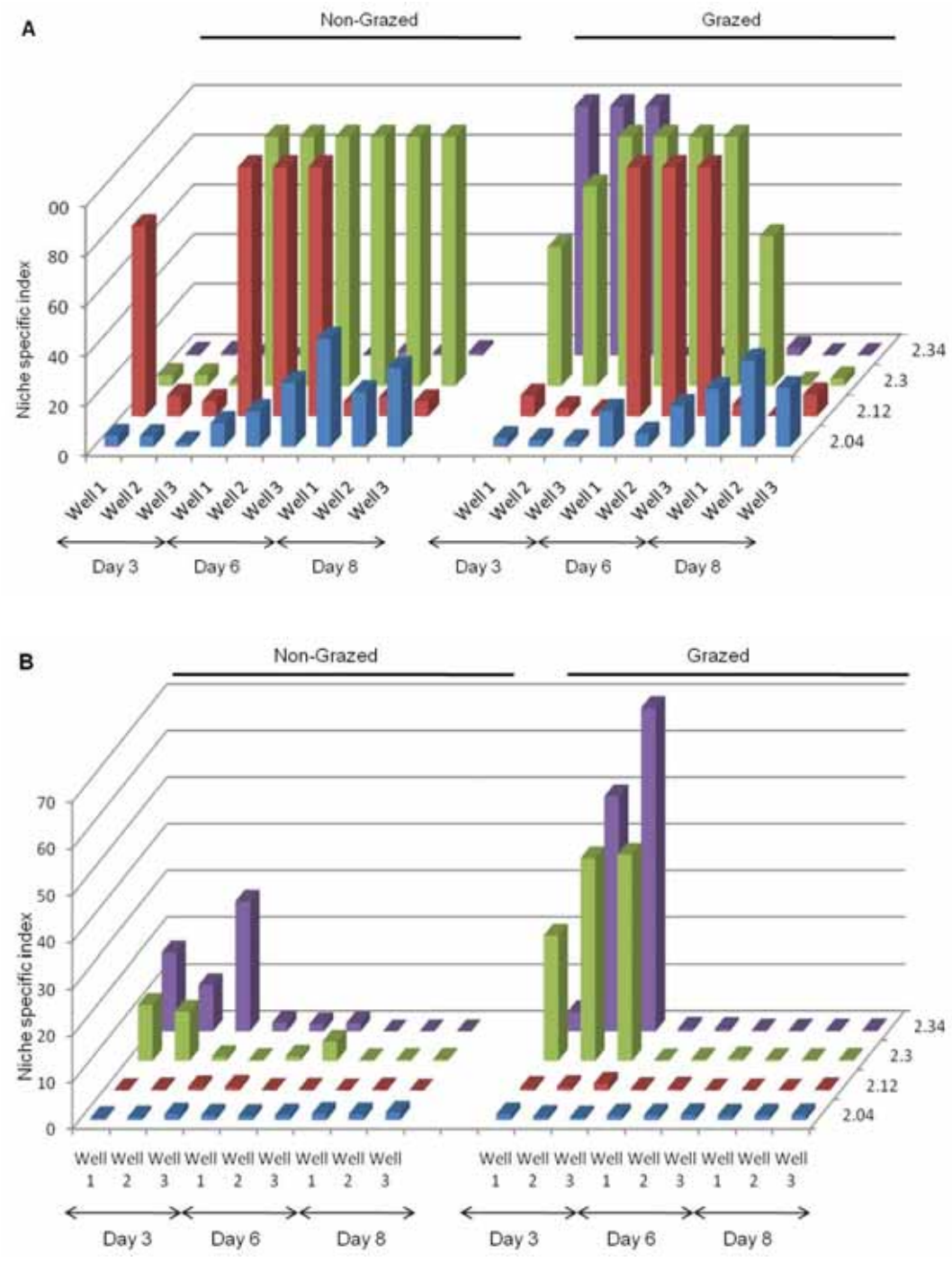


Figure 3.10: Niche specific distribution of the four epiphytic isolates (2.04 – *M. phyllosphaerae,* 2.12 – *S. japonica,* 2.3 – *D. donghaensis* and 2.34 – *A. lwoffii*) in single (A) and mixed (B) species biofilms when exposed to grazing by *T. pyriformis*. The ecological niche index was calculated for comparison of non-grazed and grazed treatments over 3, 6 and 8 d.

3.3.9 Susceptibility of epiphytic isolates to amoeba grazing is dependent on growth as single- or mixed-species consortia

The surface associated grazer, *A. castellanii* can alter the species composition of environmental microbial biofilms (136), in both soil and fresh water bacterial communities (279, 298). In response, bacterial communities have evolved a variety of resistance mechanisms for protection against amoeba grazing (264). Therefore, the impact of grazing by *A. castellanii* on intermediate and late stage biofilms formed by single and a mixed species consortium of *U. australis* epiphytic isolates was examined.

Single species biofilms were not affected by the presence of the amoeba grazer with the exception of *A. lwoffii* (Figure 3.11A to D) where there was an increase in biofilm biomass under grazing pressure. The presence of *A. castellanii* trophozoites suggested that active feeding occurred in the *A. lwoffii* biofilm. The increase in biofilm biomass indicated a 'reverse grazer effect', where biofilm growth was stimulated through nutrient re-mineralization (Figure 3.11D). *M. phyllosphaerae* biofilm biomass was similar for both grazed and ungrazed cultures but decreased over time (Figure 3.11A). The biofilm biomass of *S. japonica* and *D. donghaensis* cultures increased over time but there was no difference between grazed and ungrazed biofilms. The appearance of inactive cysts in the *S. japonica* and *D. donghaensis* biofilms suggested that the biofilms were inhibitory to *A. castellanii* (Figure 3.11B and C). The four species mixed community biofilm was thicker than the single species biofilms and resulted in encystment of amoeba. There was no significant difference between grazed and ungrazed treatments on biofilm biomass ($P > 0.05$, student's *t*-test) (Figure 3.11E).

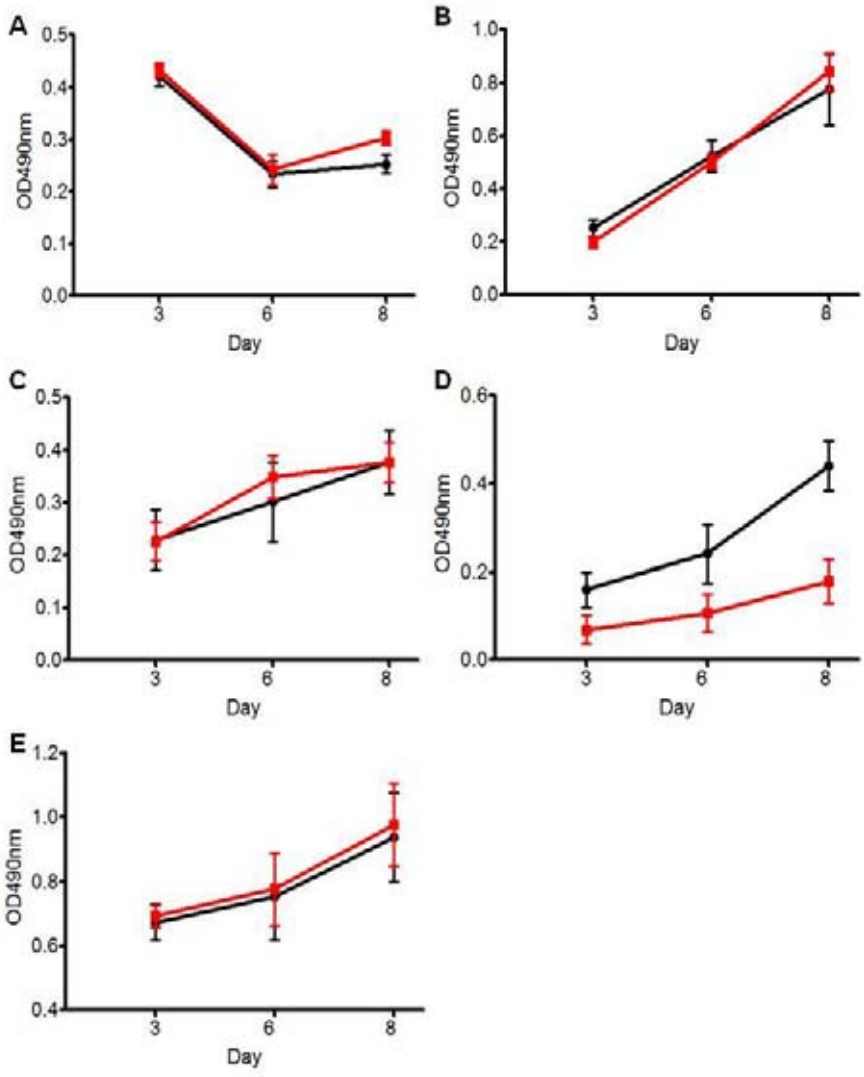


Figure 3.11: Mean resistance of intermediate and late stage biofilms exposed to grazing by the amoeba, *A. castellanii* over 3, 6 and 8 d. The biofilm biomass of single-species *M. phyllosphaerae* (A), *S. japonica* (B), *D. donghaensis* (C) and *A. lwoffii* (D), and four-species biofilms (E) in grazed (black) and ungrazed (red) treatments. Error bars represent standard deviations of 3 replicates.

A. castellanii was co-inoculated with intermediate to late stages mixed- and single-species biofilms to investigate the impact of a well-adapted surface feeder on a microbial community. The initial inocula for *M. phyllosphaerae, S. japonica, D. donghaensis* and *A. lwoffii* single-species biofilms were $3.15 \pm 0.21 \times 10^7$, $1.67 \pm 0.57 \times 10^7$, $2.39 \pm 0.48 \times 10^7$ and $2.1 \pm 0.43 \times 10^7$ CFU ml^{-1} respectively. For mixed-species biofilms, initial inocula of all four species were adjusted to

between 2.93 ± 2.02 × 10^6 and 4.43 ± 1.31 × 10^6 CFU ml^{-1} to give a final inoculum of 1.42 ± 0.67 × 10^7 CFU ml^{-1}.

A. castellanii grazing on the intermediate- and late-stage single- and mixed-species biofilms had no significant effect ($P > 0.05$, analysis of variance). Both *M. phyllosphaerae* and *S. japonica* in single, and mixed-species biofilms, were resistant to *A. castellanii* grazing (Figure 3.12A and B). However, higher abundance of *S. japonica* was observed, when grown as a single species, suggesting the presence of negative interactions when it was grown in the mixed-species community (Figure 3.12B). In addition, both *M. phyllosphaerae* and *S. japonica* reached a higher biofilm bacterial abundance when grazed, suggesting a 'reverse grazer effect'. Significant differences in mixed- and single-species consortia were observed for both *D. donghaensis* and *A. lwoffii* ($P = 0.040$ and $P < 0.001$ respectively, analysis of variance) with higher bacterial abundance in mono-cultured biofilms compared to mixed-species consortia in the absence of grazers (Figure 3.12C and D). *D. donghaensis* displayed resistance against *A. castellanii* grazing in the mixed-species consortia but not in single-species biofilms. It appeared that synergistic interactions were present in the mixed biofilm and that associational resistance is experienced by *D. donghaensis* (Figure 3.12C). The response of *A. lwoffii* to grazing was more variable in both mixed- and single-species consortia; it was protected from grazing on both day 6 and 8.

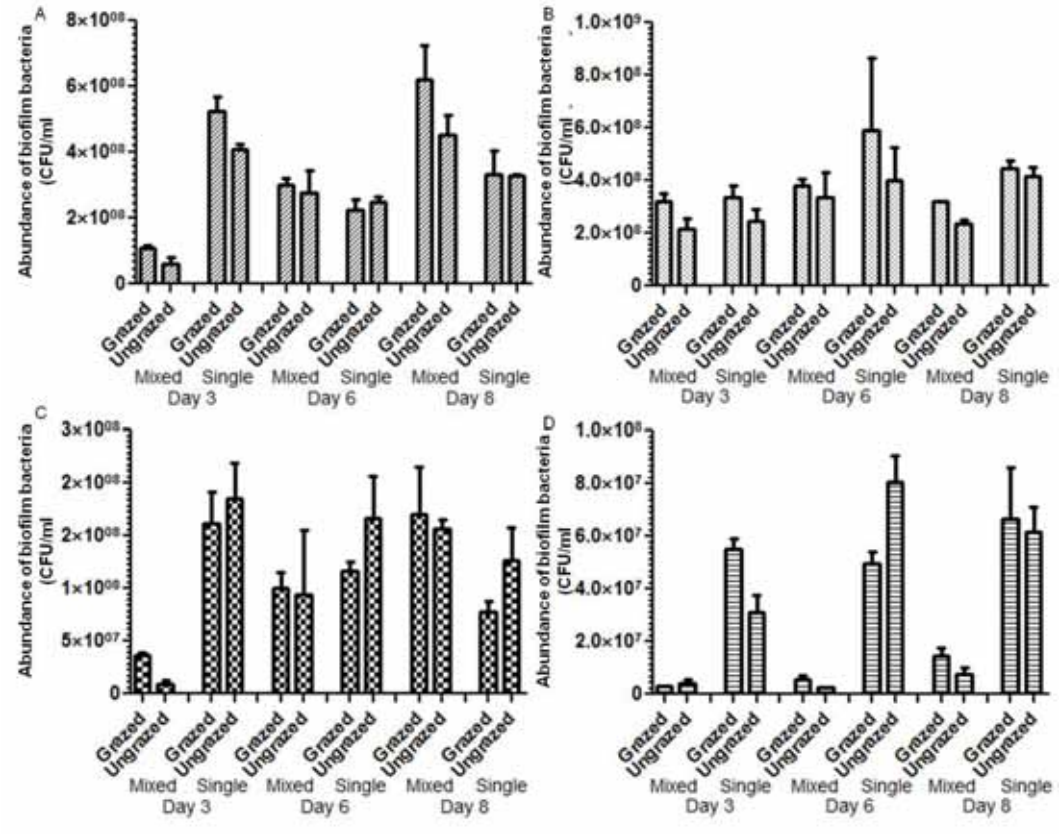


Figure 3.12: Mean grazing resistance of intermediate and late stages biofilms of single- and mixed-species (four species) grown in the presence (grazed) and absence (ungrazed) of *A. castellanii* over 3, 6 and 8 d. The biofilm fractions of *M. phyllosphaerae* (A), *S. japonica* (B), *D. donghaensis* (C) and *A. lwoffii* (D) were harvested, plated and expressed as colony-forming units (CFU ml^{-1}). Error bars represent standard deviation of three replicates.

3.3.10 *Acanthamoeba castellanii* affects species evenness in planktonic and biofilm phases

Amoebae are restricted to feeding on sessile bacterial communities due to their requirement for surface attachment. As a result, they are commonly associated with late stage biofilms. The effects of *A. castellanii* grazing on the evenness of mixed-species communities were explored. Species richness was a constant (data not shown) in the semi-continuous closed system. Evenness indices for biofilm fractions indicated that abundances of the four species were becoming more similar over time, as the index approaches 1. In contrast, the abundance of the four species in planktonic fractions shifted from a relatively well-distributed community to a less evenly distributed one (Figure 3.13A and B). Comparison

between species evenness of grazed and ungrazed planktonic and biofilm treatments of the mixed-species consortia, were not significantly different (P > 0.05, student's *t-test*).

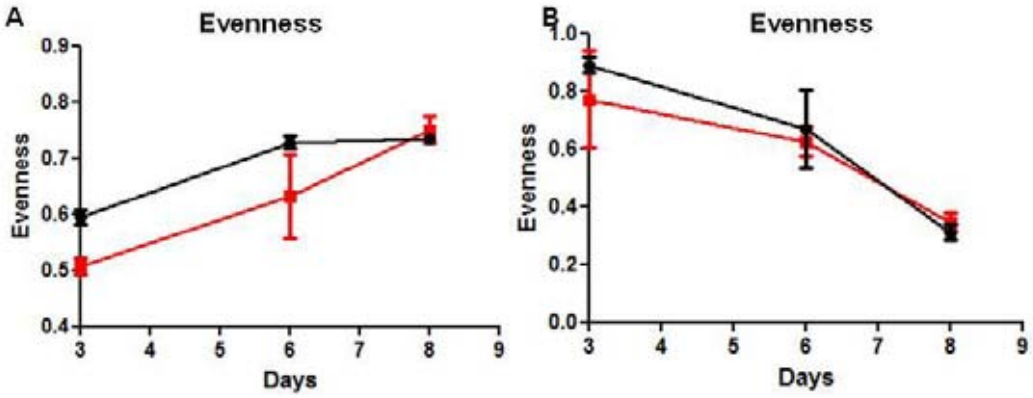


Figure 3.13: Mean evenness of intermediate- to late-stage mixed-species biofilm (A) and planktonic phases (B) in the presence (black) and absence (red) of grazing pressure exerted by *A. castellanii* over periods of 3, 6 and 8 d, respectively. Error bars represent standard deviations of three replicates.

3.3.11 Shifts in ecological niche preferences caused by *Acanthamoeba castellanii* grazing

A. castellanii was introduced to single- and mixed-species biofilms to determine if changes in ecological niche distributions of members occurred. Distinct shifts in niche preferences depended on whether the isolates were grown as mixed- or single-species biofilms. *M. phyllosphaerae, S. japonica* and *D. donghaensis* were resilient to niche specific changes, when grown as single-species co-inoculated with *A. castellanii* (Figure 3.14A), as all maintained a preference for the planktonic fraction. The addition of *A. castellanii* to *A. lwoffii* single-species consortia accelerated the shift towards the biofilm fraction, which occurred on day 3 in the grazed samples compared to day 6 in ungrazed samples.

For mixed-species biofilms the ecological niche distribution of each isolate was opposite to that observed in single-species biofilms (Figure 3.14B). *M. phyllosphaerae, S. japonica* and *D. donghaensis* exhibited a niche preference for the biofilm fraction in both grazed and ungrazed treatments of the mixed-species

consortia. The introduction of *A. castellanii* resulted in *A. lwoffii* shifting its ecological niche preference towards the planktonic fraction on day 8 (Figure 3.14B).

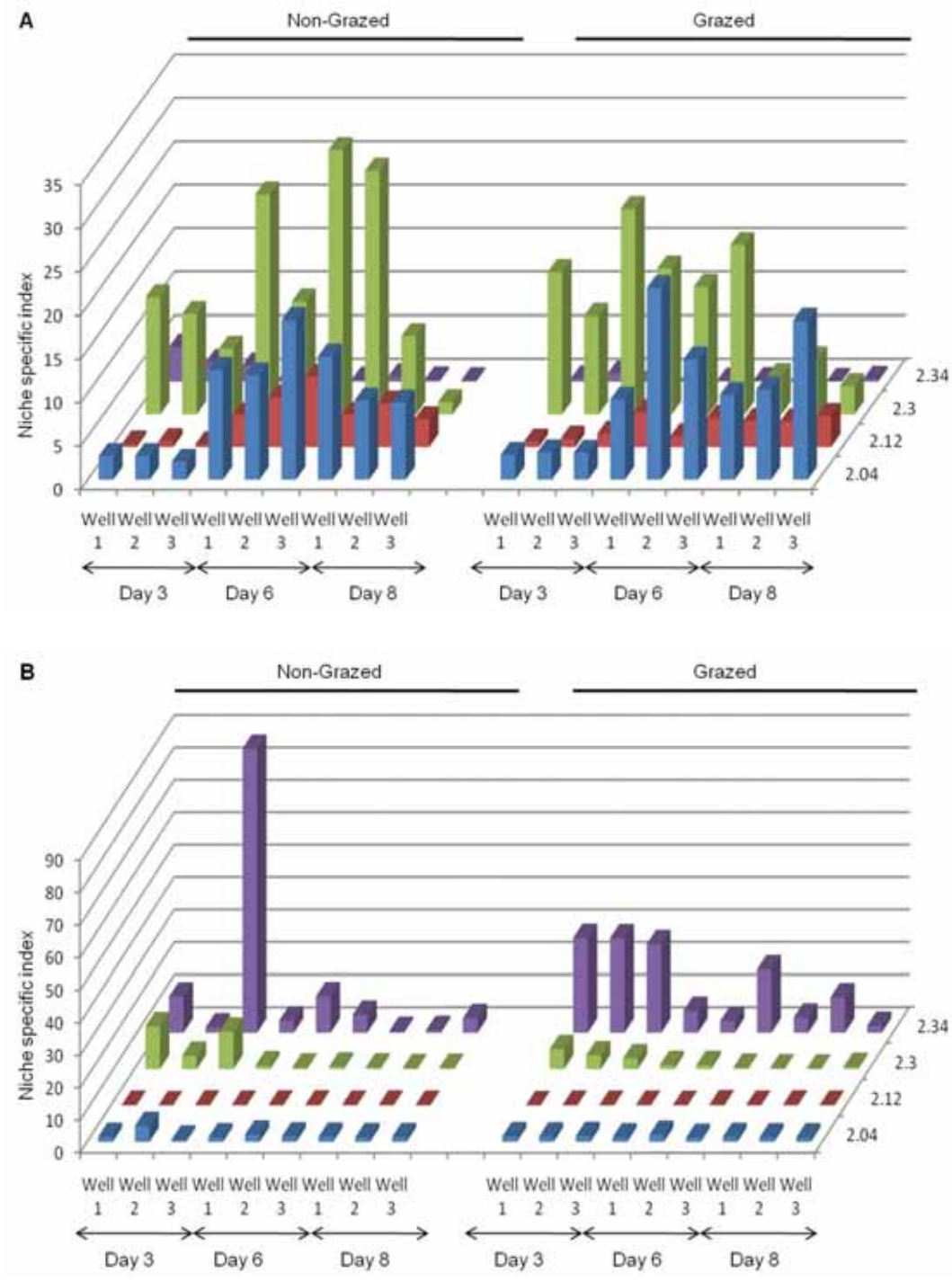


Figure 3.14: Comparison of the niche specific distribution of four epiphytic isolates (2.04 – *M. phyllosphaerae*, 2.12 – *S. japonica*, 2.3 – *D. donghaensis* and 2.34 – *A. lwoffii*) in single- (A) and mixed- (B) species biofilms in the absence (non-grazed) and presence (grazed) of *A. castellanii* over 3, 6 and 8 d.

3.3.12 Removal of *Microbacterium phyllosphaerae* altered the dynamics of a mixed-species biofilm

The defined four species epiphytic community became dominated by *M. phyllosphaerae* over time, in both the presence (22.93 % to 62.40 %) and absence (21.29 % to 61.62 %) of *A. castellanii* grazing (data not shown). *S. japonica*, which was dominant in the early stages of the four species biofilm, gradually became less dominant in both grazed (68.70 % to 21.70 %, Figure 3.15D to F) and ungrazed (74.20 % to 25.20 %, Figure 3.15A to C) treatments. *D. donghaensis* and *A. lwoffii* were less dominant in the ungrazed four species community compared to *M. phyllosphaerae* and *S. japonica*. In the four species grazed treatments, *D. donghaensis* was resilient to changes over time (7.73 %, 15.64 % and 15.40 % from day 3 to 8 in grazed compared to 3.14 %, 22.19 % and 10.27 % from day 3 to 8 in ungrazed). *A. lwoffii* had the lowest numbers in the four species community and was capable of resisting grazing (0.64 % on day 3 and day 6 and 0.47 % on day 8) (Figure 3.15D to F).

The effects of the removal of *M. phyllosphaerae* from the four species community on the relative abundance of the remaining species were examined. In both grazed and ungrazed treatments, removal of *M. phyllosphaerae* resulted in the domination of the mixed three-species consortia by *S. japonica* (Figure 3.15A to F, $P < 0.001$, student's *t-test*). No significant grazing impact on *S. japonica* was observed (Appendix IIIA and IVA, $P > 0.05$, analysis of variance). The three-species community was also detrimental to *D. donghaensis*, as its proportion in the community was lower compared to other strains and to its numbers in the four-species mixed biofilm (Figure 3.15A to F). The three-species community was more beneficial to *A. lwoffii* than the four-species community, as proportions were higher than that in the latter (Figure 3.15D to E, $P < 0.05$, student's *t-test*). However, *A. lwoffii* was also outcompeted over time in the three-species consortia (Figure 3.15A to C and Appendix IVC). The grazing impact on *A. lwoffii* in the three-species community was not as drastic as that observed in single-species biofilms (Figure IIIC and Appendix IVC, $P = 0.005$, analysis of variance).

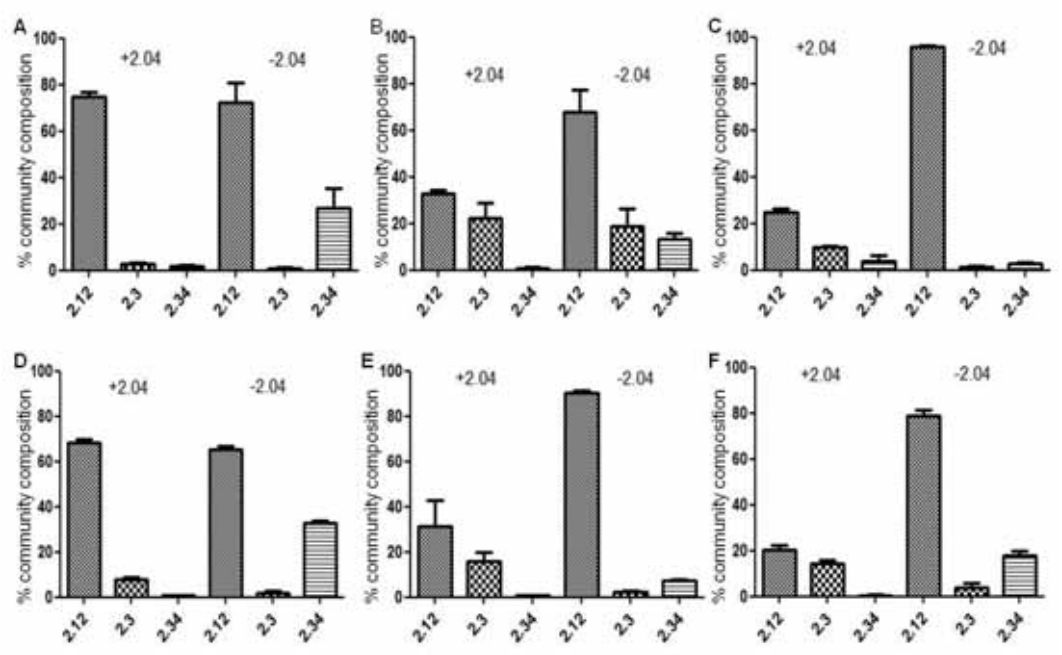


Figure 3.15: Comparison of mean proportions of members in the mixed-species community; 2.12 – *S. japonica*, 2.3 – *D. donghaensis* and 2.34 – *A. lwoffii*, after removal of *M. phyllosphaerae* (2.04). Biofilms were established in the absence (A to C) and in the presence (D to F) of *A. castellanii* over 3 (A and D), 6 (B and E) and 8 (C and F) d. Error bars indicate standard deviations of three replicates.

3.3.13 Removal of *Microbacterium phyllosphaerae* from the mixed community did not affect the ecological niche preference of other members

The impact of *M. phyllosphaerae* removal on the ecological niche preference of the remaining community was investigated, in the absence and presence of grazing by *A. castellanii*. A comparison of the niche specific preferences of each strain when grown in four and three species communities revealed some similarities. For example, *S. japonica* displayed a niche preference for the biofilm fraction in both communities (Figure 3.14B and 3.16) and the addition of *A. castellanii* to either community did not result in a shift in niche preference (Figure 3.14B and 3.16). Similarly, *A. lwoffii* also showed no variation in niche preference, when grown in either four or three member communities, maintaining its ecological niche preference for the planktonic fraction in both the presence and absence of *A. castellanii* (Figure 3.14B and 3.16). *D. donghaensis* was the only strain to show differences in niche preferences. Whilst a preference for the biofilm

fraction was observed on day 6 and 8 in the four species biofilm in both grazed and ungrazed treatments, no preference was observed in the ungrazed three species community (Figure 3.14B). However, when *A. castellanii* was added to the three species community, a late shift by *D. donghaensis* towards biofilm niche preference was observed on day 8 (Figure 3.16).

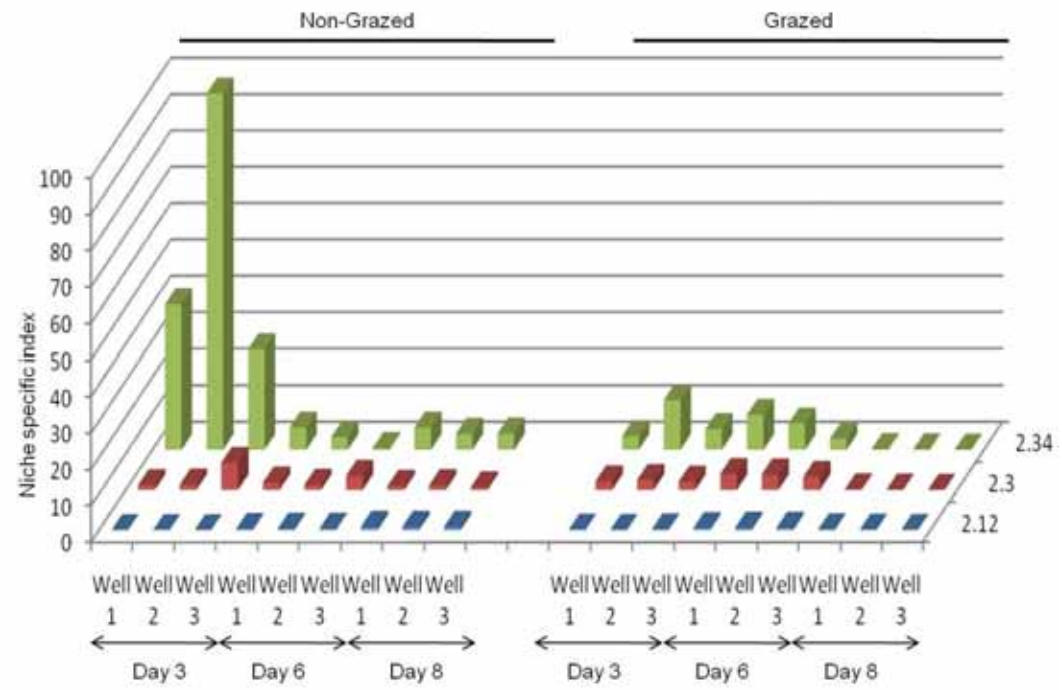


Figure 3.16: Niche specific distribution of the epiphytic isolates (2.12 – *S. japonica,* 2.3 – *D. donghaensis* and 2.34 – *A. lwoffii*) in the three species mixed community after removal of *M. phyllosphaerae*. Biofilms were established in the absence (non-grazed) and presence (grazed) of *A. castellanii* and were monitored over 3, 6 and 8 d.

The removal of *A. lwoffii* from the four-species mixed community to give a three-species mixed biofilm did not alter the dynamics of the community nor affect the ecological niche preferences of the remaining strains. *M. phyllosphaerae* remained the dominant strain in the three-species mixed community followed by *S. japonica* and *D. donghaensis*. The niche preferences of the each strain in the three-species community were identical to that for the four-species community (data not shown).

3.4 Discussion

This chapter describes the impact of predation by grazers with different feeding strategies on the responses and interactions of members in a defined mixed community. The composition of environmental microbial communities is a consequence of coexistence between members experiencing synergistic and/or antagonistic interactions which are affected by levels of disturbances and the occurrence of population bottlenecks (40). Population bottlenecks are evolutionary events that result in the purging of a significant portion of a population, affecting the genetic diversity of the community (45, 48). Environmental disturbances are common and are responsible for increasing biological and functional diversity in the ecosystem, thereby contributing to its stability (46, 211). For example, protozoan grazing can destabilize microbial communities through size selective grazing or stabilize them by prey switching (335). Biofilm formation by bacterial communities is a form of collective behaviour that protects members from protozoan grazing, which accounts for major mortalities in bacterial communities (177). For example, grazing pressure from bacterioplankton grazers induced phenotypic diversification in *V. cholerae* biofilms, preferentially selecting for the rugose variant which overproduces EPS and preferentially exists in the biofilm phase (206). Even though the formation of biofilms is a grazing resistance strategy, they may also be susceptible to predation by surface-associated grazers such as *A. castellanii* (343). The use of different community compositions in this current study allowed evaluation of the importance of the roles played by particular members in the consortia.

3.4.1 Different protozoan grazers are adapted to feed on different biofilm stages and different bacterial strains

Environmental biofilms, particularly those found in aquatic systems, need to be highly dynamic to survive the constant exposure to a variety of stresses, including different nutrient regimes and a variety of predators (275). For example, *Serratia marcescens* biofilms can either be in the form of microcolonies that allow protection from grazing by early biofilm colonizing flagellate grazers, or filamentous biofilms which are resistant to *Acanthamoeba polyphaga* grazing (264). Similarly, microcolony formation by *P. aeruginosa* provides sufficient

protection against early biofilm colonizing protozoa, but not to the intermediate and late colonizing protozoa (343).

The results presented here demonstrated that protozoan predators of different feeding types are adapted to grazing on different biofilm stages and exhibit selective feeding preferences (Figure 3.2A). Single- and mixed-species associations of members of the bacterial community also differentially contribute to the various fitness responses of the epiphytic isolates against different grazer types (Figure 3.3, 3.7 and 3.13). Early stage biofilms were tested in the agar-based APA and mixed-species consortia were observed to be generally more resistant than single-species, suggesting that members in the biofilm may have contributed to associational resistance, although negative interactions (i.e. competition) could account for the susceptibility of some species (321). Single-species biofilms of *M. phyllosphaerae* and *A. lwoffii* were susceptible to the surface-associated grazer *A. castellanii* while *S. japonica* and *D. donghaensis* were resistant. Bacterial strains with short generation times resist grazing effects by growth compensation and the differential resistance observed here is no exception (177). For example, the ability of *S. japonica* and *D. donghaensis* to develop colonies faster than *M. phyllosphaerae* and *A. lwoffii* provided them with a slight advantage.

The single species biofilms were established in a semi-continuous flow system in order to mimic environmental flow conditions. The biofilms were exposed to different-stage grazers in order to evaluate their fitness. Surprisingly, even though the early colonizer, *R. nasuta*, displayed a selective preference for grazing on biofilms and flocs and was disadvantaged when grazing on planktonic cells (47), all biofilms and planktonic fractions of the four-epiphytic isolates were observed to be vulnerable to grazing (Figure 3.4 and Appendix I). Thus, no shifts in ecological niches were observed (Figure 3.6A). The only early stage single-species biofilm (3 d old) grazed by *A. castellanii* was that of *D. donghaensis* while *T. pyriformis* grazed on *D. donghaensis* and *A. lwoffii* (Figures 3.4, 3.8C and D and 3.12C). The inability of *A. castellanii* to graze on early-stage biofilms reflects grazer specificity for late biofilm stages (343).

Varying degrees of resistance were observed for the mixed-species communities. The four single-species intermediate-stage biofilms were subjected to grazing by *T. pyriformis* which is well adapted to grazing on both planktonic and biofilm

fractions. Both *M. phyllosphaerae* and *S. japonica* were resistant to *T. pyriformis* grazing with higher biofilm bacterial abundance observed in the presence of grazing (Figure 3.8A and B). Such an observation could be attributed to the 'reverse grazer effect', where nutrient remineralization encourages biofilm growth as observed in *Vibrio cholerae* A1552 (84). Ciliated protozoans are well known to cause biofilm disruption through their feeding activities, causing sloughing of smaller chunks of biofilms (10), increasing the surface area exposed and hence contributing to nutrient delivery. Grazing resistance could also be due to inedible cells as well as production of antiprotozoal compounds (318). The negative growth rate of *T. pyriformis* and the absence of a shift in the ecological niche could account for *M. phyllosphaerae* and *S. japonica* grazing resistance (Figure 3.7A). Ciliates have the ability to graze on biofilms (343) despite exhibiting a preference for grazing on planktonic communities (152). This could explain the significant grazing impacts observed in both biofilms and planktonic fractions of *D. donghaensis* and *A. lwoffii* exposed to ciliate grazing (Figure 3.8C and D).

The epiphytic isolates were pre-established as late-stage biofilms prior to exposure to *A. castellanii,* a late-stage grazer, in the agar-based selective grazing assay. Both *M. phyllosphaerae* and *D. donghaensis* exhibited resistance against *A. castellanii* grazing while *A. lwoffii* and *S. japonica* were preferentially grazed. The higher rates of consumption of *A. lwoffii* compared to *S. japonica* suggested that prey quality could contribute to differential prey consumption by *A. castellanii*. Chemo-attractants or repellants released in the form of volatiles have the potential to deter/attract herbivorous grazers from/to a focal plant (75). Similarly, in microbial systems, the production of such allelochemicals contributes to chemotaxis, chemokinesis and even phagocytosis in protozoan grazers (76, 284, 349). The preferential grazing and resistance observed was not attributed to allelochemicals secreted by bacteria as similar levels of amoebae migration was observed towards bacterial growth fronts (Figure 3.2C). Hence, the rationale for the susceptibility of *M. phyllosphaerae* to grazing in the APA and its resistance to grazing in the selective grazing assay could be attributed to density-dependent cell to cell contact, between *M. phyllosphaerae* and *A. castellanii*. Similarly, when the four single-species biofilms were exposed to the late-stage biofilm feeder, *A. castellanii* in the semi-continuous systems, amoebae exhibited only slight grazing effects on *D. donghaensis* and *A. lwoffii* (Figure 3.12C and D) and the inability of

A. castellanii to graze effectively on biofilms of other species was amplified. The grazing pattern displayed by amoeba showed a selective preference for strains with a slow growth rate. Therefore, strains like *M. phyllosphaerae* and *S. japonica* with short generation times were effectively protected due to growth compensated resistance, a common grazing resistant strategy adopted by bacteria (177, 205).

The anti-predator fitness displayed by the epiphytic isolates against grazing by *A. castellanii* is likely to be multifactorial. For example, toxins produced by many bacteria are membrane bound and thus can be released upon lysis of the cell or may be secreted as in the case of the pore-forming cytolysins (301). The type-III secretion system of *Pseudomonas aeruginosa* which secretes virulence factors into host cells (214, 280), also has effective anti-protozoan activity (207). Selective feeding by the protozoa could also contribute to differential resistance of the strains. Gram-negative bacteria are generally preferentially grazed compared to Gram-positives, due to the differences in cell wall structure which affects handling time by the grazer (4, 278). Moreover, the cell surface charge of bacterial prey also affects palatability and influences the degree of grazing (202). The resistance displayed by *D. donghaensis* in both agar-based assays suggested that phenotypic properties such as the ability to form elongated rods of up to 25 µm could play a role in grazing resistance (356). As protozoans demonstrate size-selective feeding, *D. donghaensis*, a distinct lineage within the *Cytophaga-Flavobacterium-Bacteriodes* (CFB) group, becomes protected due to size exclusion (298, 356). While the presence of grazers might have accelerated the rate of filament formation by increasing growth rates through nutrient remineralization, it could also result in other ecological consequences such as size shifts in the community composition from one consisting of smaller edible rods to one with inedible filaments (111). Surprisingly, even though *D. donghaensis* exhibited grazing resistance against amoebal grazers in the agar-based systems, it was susceptible to grazing in the semi-continuous systems, suggesting extrinsic factors could contribute to the biofilm structure and hence grazing resistance.

3.4.2 Grazing on mixed-species biofilms by different grazers has different ecological consequences

Environmental bacterial communities are faced with a variety of stresses including but not limited to changing environmental conditions, predation, competition and invasion (44, 162). As a result, the inherent functional diversity of mixed-species consortia can act to protect bacterial members from mortality and hence facilitate persistence in the environment. Many studies have attempted to link diversity (both structural and functional) with community stability (141, 211). It is therefore possible that this positive diversity-stability relationship is widespread in natural biofilms. This diversity-stability link has also been demonstrated in single-species systems, where the production of phenotypic variants increases the stability of the biofilm. For example, in single-species biofilms of *S. marcescens*, phenotypic diversification has been suggested to be responsible for resilience against stress and protozoan grazers (170). This 'insurance hypothesis' or 'bet-hedging' has also been demonstrated in *P. aeruginosa* where the occurrence of phenotypic variants increases the functional diversity of a biofilm, facilitating survival under adverse conditions (33). Therefore, a mixed-species community should ideally be more resilient to grazing disturbances compared to their single-species counterparts, due to higher structural and functional diversity.

When grown as a defined mixed-species consortium, both synergistic and antagonistic interactions were observed (Figures 3.4, 3.8 and 3.12). All four epiphytic members in the biofilm experienced inter-species competition, which could reflect nutrient, oxygen and space limitation. Positive commensal interactions were observed during predation by *R. nasuta* (Figure 3.4). In the mixed-species biofilm, *A. lwoffii* was the only member grazed by *R. nasuta*, while other members exhibited increased biofilm bacterial abundances, suggesting that the contribution of remineralized nutrients due to grazing activity resulted in a 'reverse grazer' effect for these strains. Grazing by *R. nasuta* also induces a shift of *S. japonica* from the biofilm to the planktonic phase (Figure 3.6B). The selective grazing of *A. lwoffii* in the mixed community, displayed by *R. nasuta* suggests that it could be due to the fact that it is a specialist grazer. However, *R. nasuta* is also capable of grazing flexibility. When co-inoculated with single-

species biofilms or when alternative prey is not available, maintaining a selective grazing preference could be costly (29).

Members in the intermediate- and late-stage mixed-species biofilms also experienced competition. A significant grazing impact was observed on *D. donghaensis* in the mixed community, indicating a selective grazing preference for this particular strain. Thus, *T. pyriformis* could also have a tendency to be a specialist grazer. Grazing selection by ciliated protists is density-dependent when the prey that is contacted the most is subjected to the highest levels of predation (139). Surprisingly, the low densities of *D. donghaensis* in the mixed community did not impede the grazing activities of *T. pyriformis* on this species, suggesting other factors such as chemical (surface expressed amino acids or organic nutrients) or mechanical (cirri, membranelles made by the fusion of a bundle of cilia, used for screening prey sizes) cues could contribute to prey recognition and capture (218). *T. pyriformis* grazing caused an ecological shift in niche preference to the biofilm phase by members in the mixed-species biofilm (Figure 3.10).

Mixed-species biofilm exposure to *A. castellanii* grazing was not significantly different to that of *R. nasuta* and *T. pyriformis*. The mixed-species community was found to have commensal properties by serving as a spatial refuge for slow growing poor biofilm forming species, preventing them from being completely eliminated (Figure 3.12) (129). *D. donghaensis* and *A. lwoffii* gained structural support and associational resistance against *A. castellanii* grazing, by associating with the two grazing resistant strains in the mixed community (Figure 3.12). The resistance of *M. phyllosphaerae* appeared to be a consequence of *A. castellanii's* long generation time in the semi-continuous systems (7-8 h on defined media and 17 h on bacterial prey), which failed to match the growth rate of *M. phyllosphaerae* (Figure 3.11A) (68, 281). Hence, overwhelming biofilm formation resulted in no significant grazing impacts.

Grazing by *R. nasuta* and *A. castellanii* did not result in any specific ecological niche shifts by the members of the mixed community; the lack of discriminative feeding by both grazers suggested that they are generalists (129). The selective grazing by *T. pyriformis* did not lead to prey niche restriction of either single- or mixed-species biofilms. The mixed-species biofilm composition was similar in both grazed and non-grazed treatments. The mixed-species consortia appeared

stable in the presence of the three different types of grazers ($P > 0.05$, student's *t-test*). The closed system used for carrying out the grazing assays would have contributed to biofilm stability in the presence of grazers, as there was a limited number of species present. Nevertheless, it is possible for mixed-species community phenotypic diversity to change as a result of exposure to grazing, due to the increase in spontaneous mutants or phenotypic variants, which would increase the functional diversity and possibly the community stability (33). Indeed, disturbances can act to prevent a single taxonomic group from dominating a community and thereby controlling ecological processes (46). Obviously, predation can have drastic effects on community stability. For example, selective feeding without prey switching would be destabilizing to a community as constant grazing pressure exerted on preferred prey could lead to local extinction (311). Depending on the role played by the extinct member, subsequent cascades of local extinctions could occur, thereby indirectly influencing the biodiversity of a community (55).

The first three-species community was generated by removal of *M. phyllosphaerae* from the four-species consortia. *S. japonica* gradually dominated the three-species consortia (Figure 3.15). In the absence of *A. castellanii* both the slow growers, *D. donghaensis* and *A. lwoffii*, appeared to be inferior competitors and were almost competitively excluded from the mixed-species biofilm by *S. japonica* (Figure 3.15C). Under such conditions *M. phyllosphaerae* could dominate the mixed consortia and become a superior competitor, capable of suppressing growth of *S. japonica*. The introduction of amoebae would be expected to alleviate the competitive interactions occurring in the mixed-species biofilm, resulting in growth of *D. donghaensis* and *A. lwoffii*. The similarity in species composition of grazed and non-grazed biofilms infers that the community was stable and unaffected by protozoan grazing. Overall, the stability of both types of mixed-species communities against protozoan grazing reflects the ability of all members to coexist and to resist all types of perturbations (e.g. competition or predation), possibly through resource partitioning or niche differentiation or by associational resistance (52).

Using phylogeny as a basis for understanding the function of an ecosystem is clearly not sufficient. Instead, the structural and functional diversity of a microbial community in the environment depends on modifications brought about by

environmental perturbations (46, 185). It is therefore crucial to understand the inter- and intra-species interactions occurring within the community and its surroundings, fundamental contributions to ecological processes. Eukaryotic defense theories can be utilized and incorporated into microbial systems to further comprehend the complexity of such relationships. The impacts of environmental conditions such as nutrient limitation on the interactions between members in a mixed community, the defense responses adopted for protection against environmental disturbances and the trade-offs required is explored in Chapter 4.

Chapter 4 : The effect of nutrient availability on grazing resistance of microbial biofilms – the growth or defense trade-off

4.1 Introduction

Predators are a major source of mortality for microorganisms in the environment. It is generally assumed that predator-prey interactions depend on prey availability (289). However, in marine ecosystems, bacterioplankton productivity is controlled by 'bottom-up' factors (availability of inorganic nutrients) in addition to 'top-down' (predation) factors (24, 235), suggesting that the quality of prey also contributes to bacteria-protozoan interactions (289).

Survival strategies employed by organisms depend on environmental conditions where nutrient availability plays a major role in the defense responses expressed by organisms in order to maximize fitness and minimize losses (120, 190, 348). Natural environments, especially aquatic ecosystems, are typically oligotrophic and have limited availability of inorganic nutrients. Hence, inorganic nitrogen (N) and phosphorus (P) function as major factors limiting the growth of bacteria and photosynthetic eukaryotes in the marine environment (247, 294). For example, phosphorus is required by bacteria for basic cell assembly of lipids, nucleic acids, proteins, sugars and as a co-factor for many biochemical reactions (176). Such environmental nutrient limitation necessitates an organism's need for efficient resource allocation for either growth or defense in order to survive (348). In higher plants it has been demonstrated that the availability of nutrients determines whether resources are allocated to growth or defense from herbivory (75, 120). Bacterial biofilms are similar to plant communities as both express morphological, structural and chemical defenses to deter grazers. Therefore, ecological theories developed to describe eukaryote plant ecology provide a conceptual framework in which to investigate microbial predator-prey interactions. Plant-herbivore defense theories can be applied to biofilm-grazer interactions to better predict bacterial defense strategies exhibited in response to environmental conditions.

The trade-off between different fitness traits such as growth and defense is a central concept in eukaryote ecology. For example, the growth-differentiation balance hypothesis (GDB, elaborated in section 1.5.3) developed to describe resource allocation in plants, addresses trade-offs between defense (structural and functional palatability) and growth strategies, both of which are dependent on nutrient availability (190, 289). This type of trade-off between growth and defense has also been demonstrated in microbial systems, resulting in the classification of prey based on the defense strategies adopted. The competition specialist has the capacity to exploit resources and maximize growth, while the defense specialist invests substantially in grazing resistance strategies as it has a competitive disadvantage due to slow growth rate (59, 225, 290, 348). Trade-offs between fitness traits are common in community ecology and sacrificing certain phenotypic traits for other benefits is crucial for species coexistence under intense competition (167).

Other plant ecological theories, such as the carbon:nutrient balance hypothesis (CNB), predict that the availability of nutrients in the environment (e.g. C and N), affects resource allocation and therefore the type of defenses expressed (i.e. C or N-based defenses) (elaborated in section 1.5.3) (114). In microbial systems it has been demonstrated that genotypic and phenotypic plasticity maximizes the exploitation of nutrients for enhanced survival (304). For example, changes in nutrient levels in planktonic systems affect bacterial cell morphology as starved cells become smaller, thereby increasing the surface to volume ratio of the cells and improving nutrient acquisition (100, 178). Biofilm cells are often nutrient limited due to the sessile mode of life. As only the biofilm/liquid interface is exposed to nutrients, transport of nutrients within biofilms is limited (304). Biofilm cells respond to nutrient limitation by altering morphologies. For example, under C and/or N limitation, *Pseudomonas aeruginosa* biofilm cells elongate while maintaining their width thereby increasing the surface area and nutrient accumulation efficiency (304). A link between grazing resistance and nutrient limitation exists as phosphorus (P) limitation results in the formation of elongated and encapsulated bacterial cells whereas C limitation leads to increased cell motility which effectively confers resistance against flagellated grazers (204). As protozoan predators display size-selective grazing, these morphological changes,

which are starvation adaptive responses to nutrient limitation, can also function as a grazing resistance strategy.

The ratio of C and N as well as the total nutrient availability can affect the nutritional content of the bacterial cell and thereby affect protozoan grazing efficiency. For example, in environments with high C:N ratios (C-replete and N-deplete conditions), bacterial cells will channel excess carbon into extracellular polysaccharide (EPS), which can potentially serve as a grazer deterrent (133). As protozoan grazers have strict C:N stoichiometric requirements, they display a preference for prey with balanced C:N ratios rather than prey with high C:N ratios to avoid the metabolic cost of removing excess C (151). Marine flagellates demonstrate selective feeding and have higher clearance rates and faster digestion rates on growing prey when compared to non-growing cells (105). This is attributed to differences in cell wall, protein and nucleic acid composition between actively growing and nutrient limited cells (105).

In order to further explore the complex inter- and intra-species interactions occurring in mixed species biofilm communities, as described in Chapter 3, the effects of nutrient limitation on a defined mixed species community consisting of *Microbacterium phyllosphaerae*, *Shewanella japonica, Dokdonia donghaensis* and *Acinetobacter lwoffii*, and its response to *A. castellanii* grazing was assessed. As *A. castellanii* is inefficient at grazing on early stage biofilms (Chapter 3), emphasis is placed on the comparison of six-day and eight-day old biofilms. The three-species community employed in Chapter 3 (i.e. after the removal of *M. phyllosphaerae*) was also included for comparative purposes to determine if nutrient limitation results in community composition shifts or competitive exclusion. Eukaryotic defense theories (described in section 1.5.3) were used as a framework for investigating the defense responses adopted by each species in the mixed community. The CNB hypothesis was applied to predict the effect of nutrient on the response of the mixed community to *A. castellanii* grazing under different C:N and C:P nutrient regimes for biofilm growth media.

4.2 Materials and Methods

4.2.1 Strains and culturing conditions

The four marine epiphytic bacterial isolates, *Microbacterium phyllosphaerae, Shewanella japonica, Dokdonia donghaensis* and *Acinetobacter lwoffii*, were harvested from the intertidal green macro-alga, *Ulva australis* (44). Bacterial overnight cultures were grown with shaking (200 rpm) in VNSS broth (made with 1 × NSS) or maintained on VNSS agar plates (made by addition of 15 g l^{-1} of agar to the broth) at room temperature (ca. 22°C) for 3 d (195). For defined nutrient limitation studies, *M. phyllosphaerae* and *S. japonica* were grown overnight in complete marine minimal medium, 2M (238). The components of complete 2M consisted of 0.5 × NSS (8.8 g l^{-1} NaCl, 0.735 g l^{-1} Na_2SO_4, 0.04 g l^{-1} $NaHCO_3$, 0.125 g l^{-1} KCl, 0.02 g l^{-1} KBr, 0.935 g l^{-1} $MgCl_2.6H_2O$, 0.205 g l^{-1} $CaCl_2.2H_2O$, 0.004 g l^{-1} $SrCl_2.6H_2O$ and 0.004 g l^{-1} H_3BO_3) 40 mM of tricine, 0.1 mM of $FeSO_4.7H_2O$ and 40 mM MOPS as a buffer. The medium was supplemented with N, P and C (0.132 mM K_2HPO_4, 0.952 mM NH_4Cl and 0.2% glucose (w/v), respectively) (238).

The protozoan, *A. castellanii* ATCC 30234, obtained from the American Type Culture Collection (Manassas, V.A.) was maintained axenically in 15 ml of protease-yeast-glucose (PYG) growth medium, supplemented with 0.1 × M9 and 0.1 M glucose in ventilated capped tissue culture flasks (Sarstedt Inc.) at room temperature (ca. 22°C). Prior to biofilm grazing experiments, 500 µl of *A. castellanii* were acclimatized to phagocytosis and increased salt concentrations used in grazing assays, by passaging in 15 ml of 0.5 × NSS (nutrient-free, marine minimal media) (324) containing 1 ml of heat-killed *P. aeruginosa* (HKB). The amoeba cultures were incubated at 30°C for 3 d before harvesting and the concentration of amoebae was determined by visual enumeration of six 5 µl drops under 10 × magnification on a Leica DMLB epi-fluorescence microscope (Leica Microsystems, NSW, Australia).

4.2.2 Effect of low nutrient conditions on susceptibility of single and mixed species biofilms to *Acanthamoeba castellanii* grazing

Grazing experiments were performed in 24-well tissue culture treated microtitre plates (Sarstedt Inc.) over a period of 8 d. Mono-species biofilms were established by diluting overnight cultures of all four epiphytic isolates 1:100 for initial inocula (approximately 10^7 CFU ml^{-1}) in 10 % VNSS (diluted 1:10 with 1 × NSS) and co-inoculating with 10^3 protozoa ml^{-1} of *A. castellanii*. Seed cultures for mixed species biofilms were prepared by mixing equal proportions of the four isolates. Mixed species biofilms were established by co-inoculating 10^3 *A. castellanii* with 1 ml of 1:100 10 % VNSS diluted seed cultures. All grazing assays were carried out at room temperature with shaking at 60 rpm. Media was refreshed every 2 d by replacing 0.75 ml of spent media with fresh 10 % VNSS while minimizing disturbances to the base of the well. *A. castellanii* was enumerated after 3, 6 and 8 d using an inverted light microscope (Olympus INVT200, Olympus Optical Co. Ltd., Tokyo, Japan) at 10 × magnification and 1.5 × intermediate magnification. Three replicates of grazed and ungrazed treatments were compared for the responses of both biofilm and planktonic phases to the presence of protozoan grazers. Biofilm biomass was quantified as described previously (in section 3.2.4.6) and planktonic biomass was subjected to two 5 min intervals of sonication in a water bath at 50 Hz (Unisonics FXP12MH, Australia) to obtain single cells for plate counts.

4.2.3 The impact of removal of *Microbacterium phyllosphaerae* from the mixed community on resistance to amoeba grazing

Three species mixed biofilms used for comparison with four species biofilms were established by inoculating 1 ml of 1:100 diluted *S. japonica, D. donghaensis* and *A. lwoffii* in 10 % VNSS in 24-well tissue culture treated plates (Sarstedt Inc.). Grazed and ungrazed treatments were co-inoculated in the presence and absence of *A. castellanii* over 3, 6 and 8 d at room temperature with shaking at 60 rpm. Spent media was replaced with fresh 10 % VNSS every second day. Amoebae were enumerated using an inverted light microscope (Olympus INVT200, Olympus Optical Co. Ltd., Tokyo, Japan) at 10 × magnification with 1.5 × intermediate magnification. Planktonic fractions removed prior to biofilm biomass

quantification were sonicated and serially diluted for enumeration of CFUs. Biofilms were quantified as previously described.

4.2.4 Effects of varying carbon, nitrogen and phosphorus concentrations on biofilm responses to *Acanthamoeba castellanii* grazing

The individual resistance of *M. phyllosphaerae* and *S. japonica* to grazing by *A. castellanii* was investigated under nutrient limitation. Single species biofilms were established in 2M media with different carbon (C), nitrogen (N) and phosphorus (P) concentrations in 24-well tissue culture treated microtitre plates (Sarstedt Inc.) over 3, 6 and 8 d at room temperature with shaking at 60 rpm. Grazed biofilms were co-inoculated with 10^3 *A. castellanii*. To examine the effects of varying C and N concentrations, the nutrient conditions tested were as follows with P concentrations unchanged: low C and low N (LC:LN), low C and high N (LC:HN), high C and low N (HC:LN) and high C and high N (HC:HN or 2M media). Similarly, C and P concentrations were also varied (LC:LP, LC:HP, HC: LP and HC:HP) and N kept constant to investigate the effects on biofilm grazing resistance. Low concentrations of C, N and P used were 10 % of the original concentration (0.0132 mM K_2HPO_4, 0.0952 mM NH_4Cl and 0.02% glucose (w/v)) in 2M media and high C, N or P indicates similar concentrations to that used in 2M (0.132 mM K_2HPO_4, 0.952 mM NH_4Cl and 0.2% glucose (w/v)) (238). Planktonic fractions were removed, sonicated for 5 min intervals twice and serially diluted for determination of CFU ml^{-1} whereas biofilm fractions were harvested according to section 3.2.4.6.

4.2.5 Determination of ecological niche preferences

The niche-specific index (NI) was used to determine niche specific distributions of species in the mixed community and was determined by the ratio of the proportion of a species in the plankton phase to the proportion of that species in the biofilm phase. The NI value allows one to identify a preference for either planktonic or biofilm fractions displayed by a species when subjected to grazing (170). NI is an arbitrary value and NI < 1 represents preference for the biofilm

niche whereas a NI > 5 indicates a selective preference for persistence as planktonic cells (170).

4.2.6 Statistical analyses

Graphs of the grazing assays were plotted using Prism ® version 5 for Windows (GraphPad Software Inc.). Statistical analyses were performed using Minitab 15 package for Windows (Minitab Inc., State College, PA, USA). One, two and three-factorial analyses of variance (ANOVAs) taking time (days 3, 6 and 8), community (single or mixed) and treatments (grazed and ungrazed) as independent factors, were used to analyze data from the grazing assays. Tukey's posthoc tests were used for comparison of data between groups.

4.3 Results

4.3.1 Single and mixed species biofilms grown under low nutrient conditions were susceptible to *Acanthamoeba castellanii* grazing

Biofilms were established and exposed to *A. castellanii* (initial inocula of mono-species biofilms for *M. phyllosphaerae, S. japonica, D. donghaensis* and *A. lwoffii* were $1.77 \pm 0.44 \times 10^7$, $2.06 \pm 0.89 \times 10^7$, $2.75 \pm 0.50 \times 10^7$ and $1.40 \pm 0.25 \times 10^7$ CFU ml^{-1}, respectively). The proportion of each species in the multi-species biofilms was within the range of $1.37 \pm 0.64 \times 10^6$ to $5.05 \pm 2.84 \times 10^6$ CFU ml^{-1} for a final inoculum of $1.15 \pm 0.70 \times 10^7$ CFU ml^{-1}.

While there were no significant grazing effects on single or mixed species biofilm consortia under high nutrient conditions (section 3.3.9), significant grazing differences were observed under low nutrient conditions ($P < 0.005$, analysis of variance). In ungrazed treatments of low nutrient mono-species biofilms, all the epiphytic isolates, except *S. japonica*, showed higher biofilm CFU ml^{-1} than when grown in a mixed-species consortia ($P < 0.05$, analysis of variance) (Figure 4.1A to D, Appendix VA to D). The ratios of *M. phyllosphaerae* : *S. japonica* : *D. donghaensis* : *A. lwoffii* in mixed and single species biofilms were highly variable with a ratio of 271 : 105 : 69 : 1 (day 6 mixed) and a ratio of 16 : 2 : 12 : 1 (day 6 single), respectively. This result suggests that *S. japonica* experiences commensal interactions in the mixed-species community while both *M. phyllosphaerae* and *D. donghaensis* play a neutral role within the community. In contrast, *A. lwoffii* appears to be outcompeted in the mixed-species community. Both single- and mixed-species biofilms on day 3 exhibited increased grazing resistance compared to days 6 and 8, suggesting that the media change at day 2 could have contributed to increased resistance due to increases in nutrient levels. As a single-species biofilm, *M. phyllosphaerae* experienced significant reductions in bacterial biofilm abundance on days 6 and 8 (– 81.8 % and – 87.9 % respectively) ($P < 0.05$, analysis of variance) (*A. castellanii* growth rate, $\mu = 0.455$ d^{-1}). However, when grown as a mixed-species biofilm, *M. phyllosphaerae* was shown to be a superior competitor capable of maintaining dominance over other species in the community. The reduction in biofilm abundance for *M. phyllosphaerae* was significantly lower than that seen for other species (– 62.9 % and – 76.4 % on day 6 and 8 respectively; $P < 0.05$, analysis of variance) (Figure 4.1A).

The impact of grazing by *A. castellanii* did not result in any significant grazing differences between single- and mixed-species biofilms for *S. japonica* and grazing trends on day 6 were similar to those displayed on day 8 ($P > 0.05$, analysis of variance). However, on each day, significant reductions in single- and mixed-species biofilm bacterial abundance were observed (– 97.6 % and – 95.7 % for single and – 95.9 % and – 94.9 % for mixed on day 6 and 8 respectively) ($P < 0.005$, analysis of variance) ($\mu = 0.492$ d^{-1} on single species *S. japonica*) (Figure 4.1B). A comparison of planktonic and biofilm abundance of *S. japonica* in single- and mixed-species biofilms revealed higher planktonic CFU ml^{-1} counts for single-species treatments (Figure 4.1B and Appendix VB) and higher biofilm CFU ml^{-1} when grown as a mixed species consortia. *S. japonica* appeared to experience synergistic interactions in the mixed-species consortia, which include possible scaffolding effects or commensal interactions occurring in the mixed community biofilm.

Both single- and mixed-species biofilms of *D. donghaensis* showed significant susceptibility to *A. castellanii* grazing ($P < 0.05$, analysis of variance). However, the impact of grazing on *D. donghaensis* in the mixed-species biofilm was less that in the single-species biofilm (– 90.6 % and – 94.1 % for single and – 84.2 % and – 89.8 % for mixed on day 6 and 8 respectively), suggesting there was selective grazing for other species present in the mixed community ($P = 0.014$, analysis of variance) ($\mu = 0.430$ d^{-1} on single species *D. donghaensis*) (Figure 4.1C).

Of all the epiphytic isolates, *A. lwoffii* had the lowest CFU ml^{-1} in single- and mixed-species biofilms both in the presence and absence of *A. castellanii*, suggesting that it is a poor biofilm former (Figure 4.1D and Appendix VD). Although reductions in biofilm abundance of *A. lwoffii* were observed in both mixed- (– 45.9 % and – 24.8 % on day 6 and 8 respectively) and single-species biofilms (– 5.0 % and – 77.2 % on day 6 and 8 respectively), the grazing impact was not significantly different for either single vs. mixed or grazed vs. ungrazed ($P > 0.05$, analysis of variance) ($\mu = 0.370$ d^{-1} on single vs. $\mu = 0.476$ d^{-1} on mixed species).

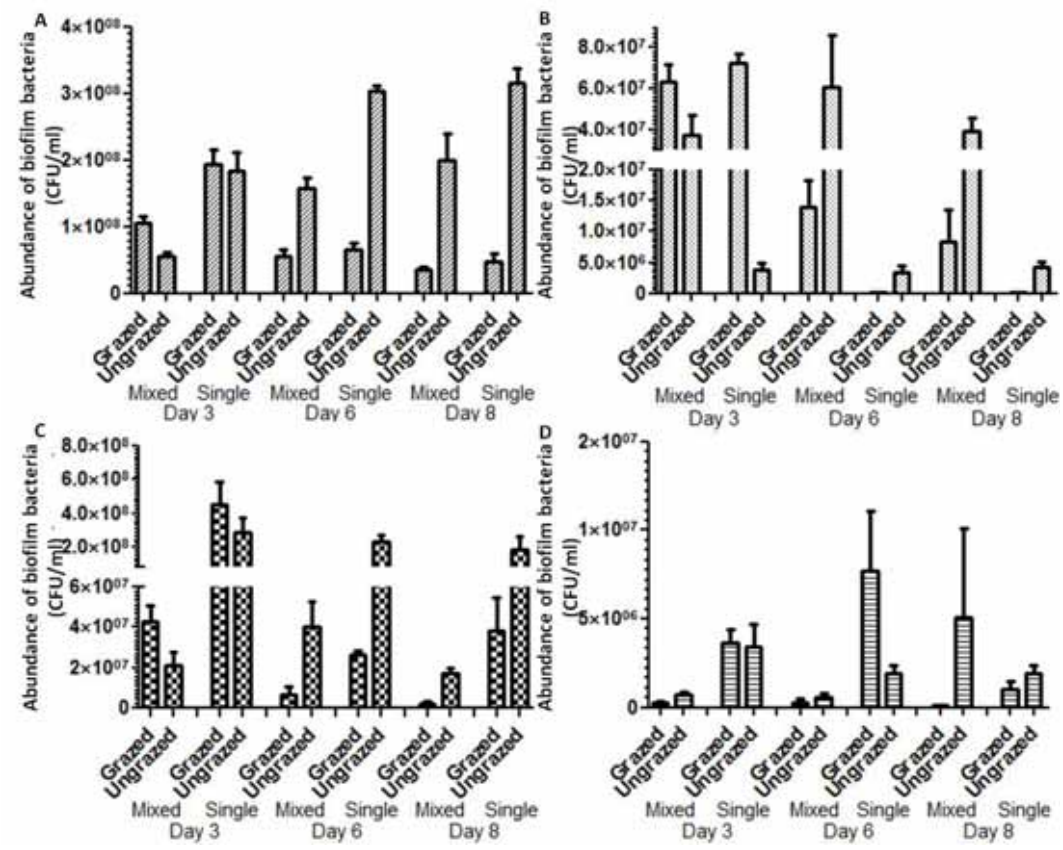


Figure 4.1: Mean differential resistance of each epiphytic isolate grown in single- and mixed-species biofilms under low nutrient conditions in the presence (grazed) and absence (ungrazed) of the surface feeding protozoan, *A. castellanii*. The biofilm bacterial abundance of *M. phyllosphaerae* (A), *S. japonica* (B), *D. donghaensis* (C) and *A. lwoffii* (D) was expressed as CFU ml^{-1}. Day 3, 6 and 8 represent the length of exposure to *A. castellanii* grazing. Error bars indicate standard deviation of three replicates.

4.3.2 Nutrient conditions and presence of grazers led to shifts in ecological niche preferences

The impact of *A. castellanii* grazing and low nutrient conditions on single- and mixed-species biofilms was investigated to determine if changes in ecological niche distributions of members occurred. Differences in niche preferences were observed for strains in the mixed community when grown under different nutrient conditions. Under high nutrient conditions, *M. phyllosphaerae* did not show any distinct niche preference in the mixed community (Chapter 3; Figure 3.14B). However, under low nutrient conditions, a niche preference for the biofilm phase was observed in both grazed and ungrazed treatments of *M. phyllosphaerae*

(Figure 4.2). Similarly, *S. japonica* in both grazed and ungrazed high nutrient mixed species biofilms displayed a preference for the biofilm phase (Figure 3.14B). In contrast, under low nutrient conditions there was a preference for the planktonic phase which resulted in a lack of distinct preferences on day 3, followed by a shift to biofilm phase on day 6 and no preference on day 8, after the introduction of *A. castellanii* (Figure 4.2). A similar niche preference pattern was observed for *D. donghaensis* where the biofilm phase was preferred under high nutrient conditions (Figure 3.14B) but the presence of *A. castellanii* under low nutrient conditions led to a switch away from the biofilm phase. *A. lwoffii* was the only strain in the mixed community that maintained a niche preference for the planktonic phase under different nutrient conditions and in both the presence and absence of *A. castellanii* (Figure 3.14B and 4.3). This suggests that *A. lwoffii* is a poor biofilm former, predominantly found in the planktonic phase, and the mixed community does not result in maintenance of *A. lwoffii* in the biofilm.

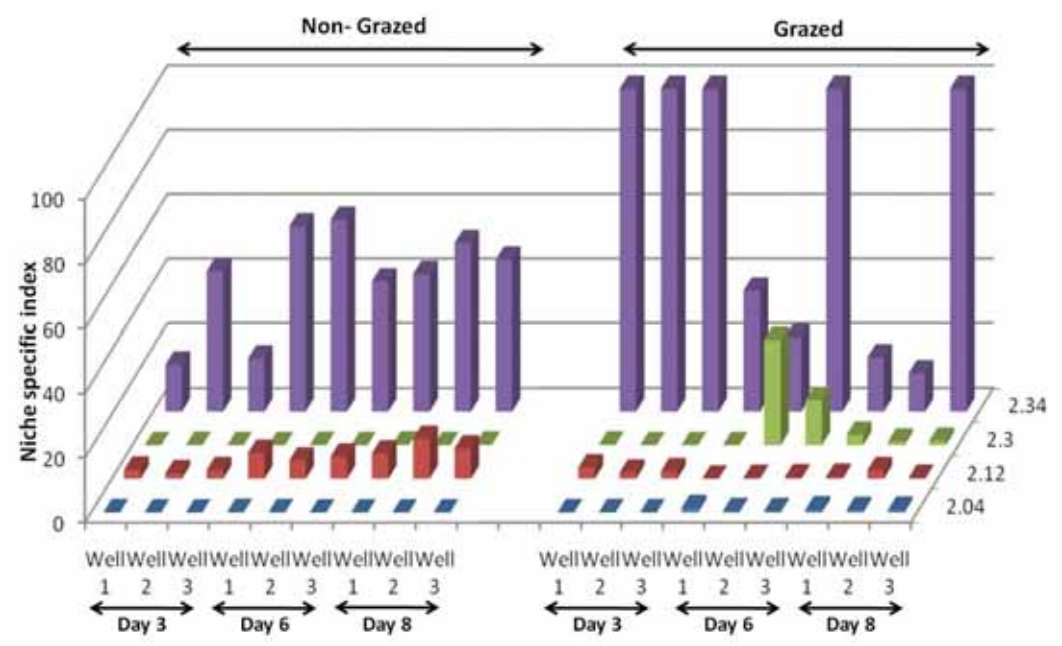


Figure 4.2: Impact of *A. castellanii* grazing on the ecological niche preferences of *M. phyllosphaerae* (2.04), *S. japonica* (2.12), *D. donghaensis* (2.3) and *A. lwoffii* (2.34) when grown under low nutrient conditions in a mixed species biofilm consortia. The niche specific index was calculated for both grazed and ungrazed treatments over days 3, 6 and 8.

4.3.3 Nutrient limitation and *Microbacterium phyllosphaerae* removal altered the mixed-species community composition

Under low nutrient conditions, the defined four-species community was dominated by *M. phyllosphaerae* over time (57.40 % to 69.00 %) and the addition of *A. castellanii* to the biofilm stimulated further dominance of *M. phyllosphaerae* (51.20 % on day 3 to 89.20 % on day 8) (data not shown). *S. japonica*, which was the next most dominant strain in the low nutrient four species mixed biofilm maintained its proportion in the absence of *A. castellanii* (Figure 4.3A to C). The presence of *A. castellanii* in the four-species biofilm decreased the proportion of *S. japonica* in the mixed-species consortia (27.58 % to 6.62 %, Figure 4.3D to F) but *S. japonica* remained the second most dominant member of the consortia after *M. phyllosphaerae*. The percentage of *D. donghaensis* decreased over time in the four-species biofilm in both the absence (13.70 % to 6.90 % from day 3 to 8) and presence of the grazer (21.07 % on day 3 to 3.88 % on day 8). *A. lwoffii*, the least dominant member in the four-species community, exhibited a decrease in abundance in the absence of grazers (1.10 % on day 3 to 0.07 % on day 8) (Figure 4.3A to C). The presence of *A. castellanii* resulted in fluctuations in numbers of the biofilm members but did not lead to consistent change over time (Figure 4.3D to F).

To investigate whether the removal of *M. phyllosphaerae* from the four-species low nutrient biofilm altered the proportion of strains in the remaining three-species community or affected the stability of the biofilms under *A. castellanii* grazing pressure, the experiments were repeated with a three-species mixed community. The proportion of *S. japonica, D. donghaensis* and *A. lwoffii* in low nutrient biofilms with four-species was significantly different to that of the three-species mixed biofilms, where higher species proportions of each member were observed in the three-species biofilms, a possible consequence of increased nutrient availability to members in the three-species community ($P < 0.05$, students *t*-test). In four-species biofilm communities, members were more resilient to changes in species proportions in both the presence and absence of *A. castellanii*. In comparison, three-species biofilms were less stable as species compositions of the biofilms did not follow a fixed trend of increase or decrease. However, exceptions were observed for *D. donghaensis*, where a decrease in percentage proportion was shown in the ungrazed (from 80.60 % to 45.30 %)

treatment, and *A. lwoffii*, which exhibited a decrease in grazed (from 25.80 % to 4.60 %) treatments over time (Figure 4.3). In the ungrazed treatments, *D. donghaensis* dominated the three-species biofilm community on days 3 and 6 (Figure 4.3A and B); the abundance of *S. japonica* was 1 % more than that of *D. donghaensis* (Figure 4.3C). In the grazed treatment, the mixed community was dominated by *D. donghaensis* on day 3 and day 8 (Figure 4.3D and F) and by *S. japonica* on day 6 (Figure 4.3E). *A. lwoffii* was regularly the least dominant in both grazed and ungrazed treatments (Figure 4.3A, C to F) with the exception of ungrazed day 6 treatments (Figure 4.3B).

Despite no significant grazing impacts on *S. japonica* being observed for the single- and three-species communities ($P > 0.05$, analysis of variance), the three-species community was commensal to *S. japonica* as growth was stimulated ($P < 0.005$, analysis of variance) (Appendix VIA). *D. donghaensis* also experienced commensal effects in the mixed community resulting in growth stimulation on days 3, 6 and 8 and grazing resistance on day 8 (Appendix VIB, $P = 0.024$, analysis of variance). In ungrazed treatments, higher biofilm (Appendix VIC) and planktonic (data not shown) bacterial abundance of *A. lwoffii* occurred in the mixed- compared to single-species community, indicating that the three-species community was commensal for *A. lwoffii*. However, the high grazing rates observed suggested that the three-species community could also be detrimental to *A. lwoffii* when in association with other species, even though the grazing differences were not significant (Appendix VIC, $P > 0.05$, students *t*-test).

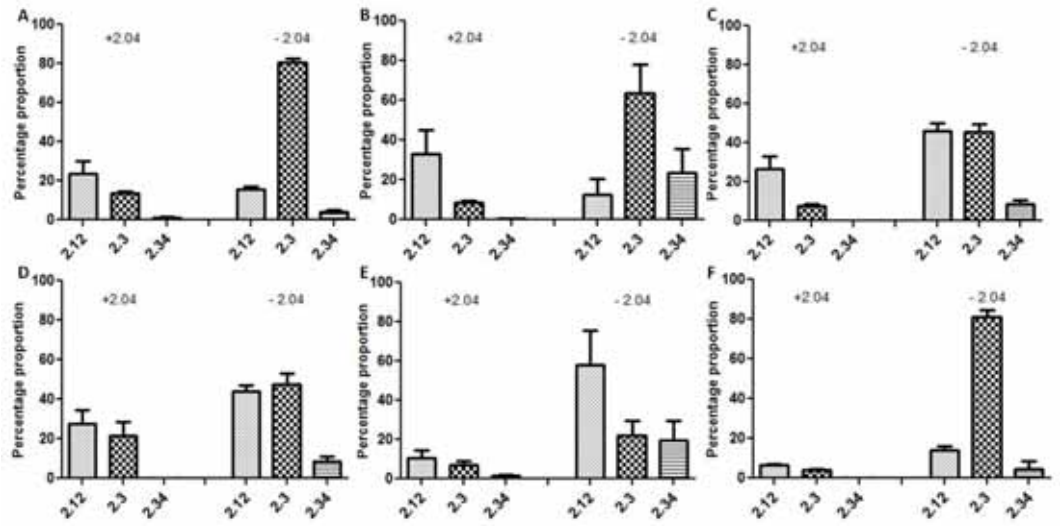


Figure 4.3: Comparison of mean changes in proportions of *S. japonica* (2.12), *D. donghaensis* (2.3) and *A. lwoffii* (2.34) before (+2.04) and after (-2.04) the removal of *M. phyllosphaerae* (2.04) from the mixed-species community. The biofilms were established in the absence (A to C) and presence (D to F) of *A. castellanii* over 3 (A and D), 6 (B and E) and 8 (C and F) days. Error bars indicate standard deviations of three replicates.

4.3.4 *Microbacterium phyllosphaerae* is grazing resistant regardless of C, N or P concentration

M. phyllosphaerae late stage biofilms exhibit grazing resistance against *A. castellanii* (Chapter 3). The impact of *A. castellanii* grazing on intermediate to late stage single-species biofilms of *M. phyllosphaerae* when grown under different C, N and P concentrations was investigated. The initial inocula for *M. phyllosphaerae* for C and N limitation were $2.21 \pm 0.38 \times 10^7$, $1.77 \pm 0.28 \times 10^7$, $3.00 \pm 0.57 \times 10^7$ and $1.76 \pm 0.19 \times 10^7$ CFU ml^{-1} (LC:LN, LC:HN, HC:LN and HC:HN, respectively) and $4.35 \pm 0.86 \times 10^7$, $4.78 \pm 0.07 \times 10^7$, $5.44 \pm 0.71 \times 10^7$, 4.92×10^7 CFU ml^{-1} (LC:LP, LC:HP, HC:LP and HC:HP, respectively) for C and P limitation. The significantly higher CFU ml^{-1} counts on days 6 and 8 in both biofilms and planktonic phases of grazed treatments compared to ungrazed treatments suggested that *M. phyllosphaerae* was resistant to *A. castellanii* grazing under C, N and P nutrient limitation ($P = 0.013$, analysis of variance) (Figure 4.4A to D). On day 6, under C and N limitation, all grazed treatments exhibited more than a 10-fold increase in biofilm biomass and more than 300-fold increase in planktonic biomass compared to ungrazed treatments (Figure 4.4A and C). In contrast, grazed treatments grown under C and P limitation showed up to a five-fold increase in biofilm biomass and 50- to 200-fold increase in planktonic biomass ($P < 0.005$, analysis of variance) (Figure 4.4A and C). On day 8, the increase in biofilm bacterial abundance of grazed *M. phyllosphaerae* for C and N limitation and C and P limitation was slightly lower than on day 6 (Figure 4.4B). Similarly, the planktonic bacterial abundance of both treatments also exhibited a significantly greater increase on day 6 compared to that on day 8 ($P < 0.005$, analysis of variance) (Figure 4.4D).

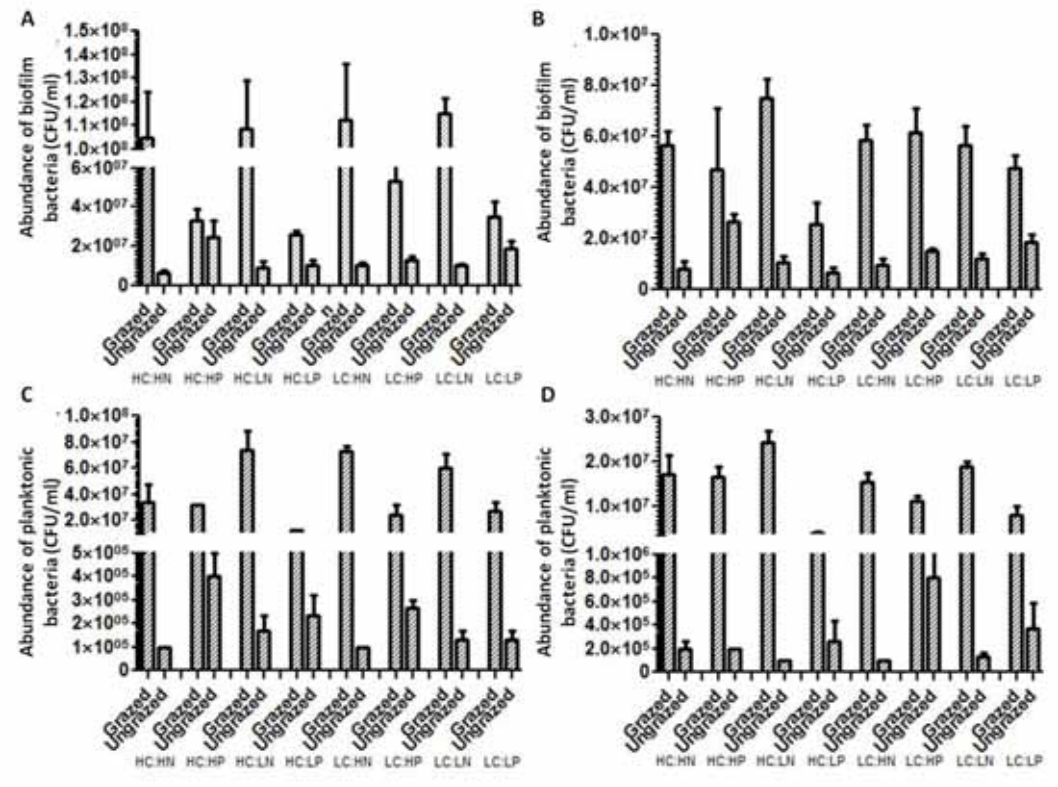


Figure 4.4: Comparison of the mean persistence of *M. phyllosphaerae* biofilms (A and B) and planktonic phase (C and D) grown under different combinations of C, N and P limitation; HC:HN, HC:HP, HC:LN, HC:LP, LC:HN, LC:HP, LC:LN and LC:LP. Biofilm and planktonic bacterial abundance exposed in the presence (Grazed) and absence (Ungrazed) of *A. castellanii* were monitored over 6 (A and C) and 8 (B and D) d and expressed as CFU ml^{-1}. Error bars represent standard deviations of three replicates.

4.3.5 *Shewanella japonica* exhibits different levels of grazing resistance under C, N and P limitation

The effects of altering C:N and C:P ratios in 2M media on the growth of *S. japonica* single species biofilms and the ability of this strain to resist *A. castellanii* grazing were tested. The initial inocula for *S. japonica* under C and N limitation were $3.00 \pm 0.28 \times 10^7$, 3.70×10^7, 3.70×10^7 and $3.55 \pm 0.35 \times 10^7$ CFU ml^{-1} (LC:LN, LC:HN, HC:LN and HC:HN, respectively) and $4.30 \pm 0.42 \times 10^7$, $4.25 \pm 0.50 \times 10^7$, $4.10 \pm 0.30 \times 10^7$, $4.00 \pm 0.85 \times 10^7$ CFU ml^{-1} (LC:LP, LC:HP, HC:LP and HC:HP, respectively) for C and P limitation. The impact of *A. castellanii* grazing on the biofilm and planktonic bacterial abundance of *S. japonica* grown under C, N and P limitation on days 6 and 8 was significantly different ($P < 0.005$, analysis of variance) (Figure 4.5). Under C and N limiting

conditions, only HC:HN treatments exhibited higher biofilm CFU ml^{-1} in the presence as compared to the absence of *A. castellanii* on days 6 and 8 (1.75- and 1.34-fold increase respectively) (Figure 4.5A and B) ($P < 0.005$, analysis of variance). *S. japonica* biofilms grown under all other treatments (HC:LN, LC:HN and LC:LN) were susceptible to grazing on days 6 (-58.00 %, -64.00 % and -70.00 %, respectively) and 8 (2.26-fold increase, -87.00 % and -90.00 %, respectively), except under HC:LN where grazing resistance was observed on day 8 (Figure 4.5A and B) ($P < 0.05$, analysis of variance).

Grazing resistance of *S. japonica* biofilms grown under C and P limitation differed significantly on days 6 and 8 ($P = 0.006$, analysis of variance) (Figure 4.10A and B). Higher biofilm bacterial abundance observed for grazed treatments on day 6 suggested that *S. japonica* was resistant to grazing under all nutrient conditions on that day (with 1-, 6.54-, 4.80- and 2.60-fold increases for HC:HP, HC:LP, LC:HP and LC:LP, respectively) (Figure 4.5A) ($P < 0.005$, analysis of variance). The opposite was observed on day 8 with *S. japonica* biofilms grown under all C and P conditions where susceptibility to *A. castellanii* grazing was observed (-33.00 %, -28.00 %, -73.00 % and -56.00 % for HC:HP, HC:LP, LC:HP and LC:LP, respectively) ($P < 0.005$, analysis of variance) (Figure 4.5B). Resistance to *A. castellanii* grazing was displayed under high C conditions (HC:HP and HC:LP) on day 6 (with 1.42- and 1.86-fold increases, respectively), while predation susceptibility was observed under low C conditions (LC:HN and LC:LN) (-77.00 % and –41.00 %, respectively) ($P < 0.005$, analysis of variance) (Figure 4.10C). On day 8, the planktonic bacterial abundance increased 1.6- and 1.2-fold for HC:HP and HC:LP, respectively, while it decreased by 84.00 % and 94.00 % under low C conditions (LC:HN and LC:LN) ($P < 0.005$, analysis of variance) (Figure 4.10D).

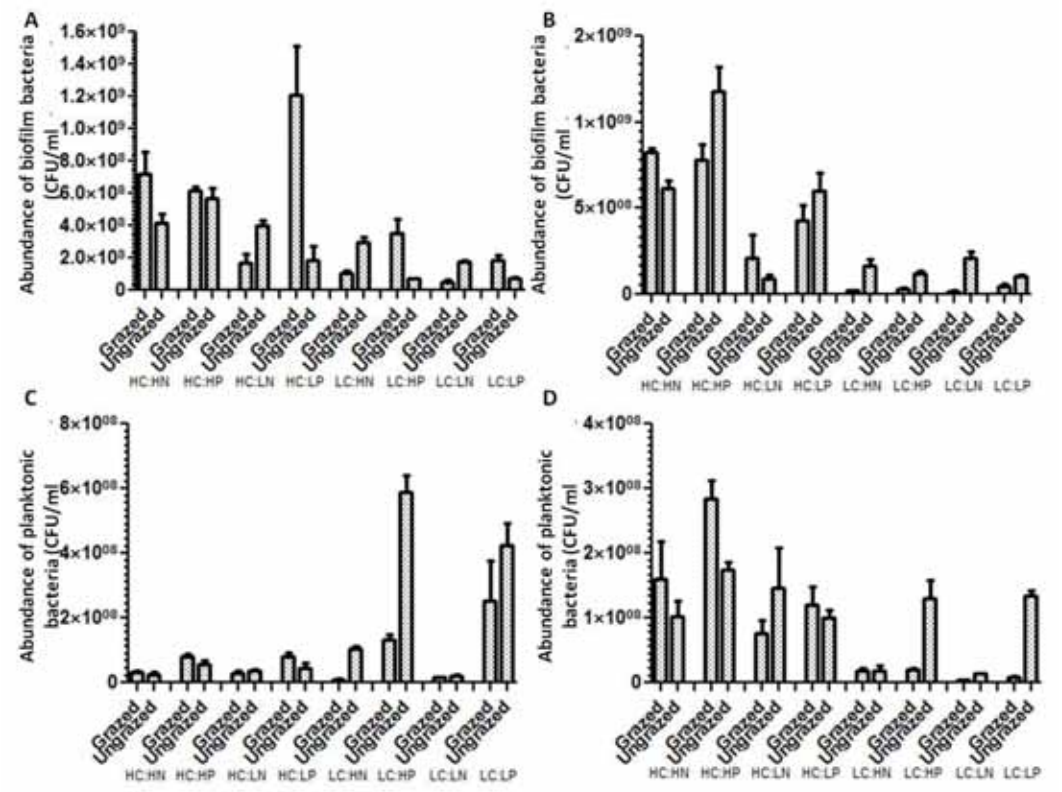


Figure 4.5: Comparison of the mean resistance of *S. japonica* biofilms (A and B) and planktonic phase (C and D) to *A. castellanii* grazing when grown under different concentrations of C, N and P; HC:HN, HC:HP, HC:LN, HC:LP, LC:HN, LC:HP, LC:LN and LC:LP. Grazed and ungrazed treatments of biofilms and planktonic bacterial abundance were monitored over 6 (A and C) and 8 (B and D) days and expressed as colony-forming units (CFU ml^{-1}). Error bars represent standard deviations of three replicates.

4.4 Discussion

Single- and mixed-species biofilms, grown under high nutrient conditions display varying degrees of resistance against protozoan grazers (Chapter 3). Protozoan grazers have selective preferences for certain members of mixed-species communities and display preferences for different biofilm stages depending on the feeding habits of the protozoan (343). *R. nasuta* grazes selectively on *A. lwoffii* in mixed-species communities, leading to nutrient remineralization and thus encouraging growth of other members of the community. Similarly, in Chapter 3, *R. nasuta*, an initial biofilm surface grazer demonstrated the ability to graze on all early stage single species biofilms, displaying a selective preference for biofilms

rather than planktonic cells. In contrast, *A. castellanii,* a predominantly surface-associated late biofilm grazer, is unable to graze on early stage biofilms. *T. pyriformis* is adapted to grazing on both biofilm and planktonic cells. As mixed-species biofilms demonstrated resistance to *A. castellanii* grazing under high nutrient conditions, it was deemed appropriate, in this chapter, to investigate the response of the community to *A. castellanii* grazing under low nutrient conditions and test eukaryotic defense theories for their application to microbial predatory-prey systems.

Oligotrophic conditions are common to aquatic systems and limitations in C, N or P affect an organism's chemical stoichiometry, indirectly affecting predator-prey interactions (216). Bacterial communities have evolved survival strategies, which allow them to adapt and exploit available nutrients and to maximize fitness against predators. Such microbial survival strategies may require trade-offs between structural and functional defense and growth (167), and these responses can be accurately modeled using eukaryotic defense theories like the GDB and CNB hypotheses, both derived from terrestrial systems (reviewed in Chapter 1) (190, 289). In this chapter, the impact of low nutrient conditions on the response of a defined mixed-species community to grazing was investigated. As both plants and bacterial biofilms are known to regulate the expression of structural or chemical defenses in response to grazing, it was deemed relevant to apply well-developed eukaryotic defense theories to microbial systems. The CNB hypothesis, a eukaryotic defense theory, was chosen to be tested as a predictive model for defenses regulated by mixed microbial species communities in response to protozoan grazing.

4.4.1 Low nutrient conditions increased the susceptibility of a mixed species community to *Acanthamoeba castellanii* grazing

The majority of studies investigating the response of bacteria to predation have been performed on planktonic systems (25-27, 30). In this study, a mixed species biofilm system was used to determine the consequences of nutrient limitation on the defense response of the community. Low nutrient conditions had a significant impact on the grazing resistance of a mixed-species biofilm community. In contrast to high nutrient conditions where the mixed-species community was

resistant to *A. castellanii* grazing, under low nutrient conditions, the mixed community became vulnerable to predation (Figure 4.1). Eukaryote defense theories like the GDB (reviewed in Chapter 1) encompass the CNB and the growth rate hypotheses, both of which can be used to predict defense responses and survival strategies adopted under nutrient limitation and in the presence of grazers (59, 120). The *A. castellanii* grazing resistance displayed by the mixed-species biofilm under high nutrient conditions indicated that the growth rate hypothesis might be applicable as competitive growth is prioritized over chemical defense, leading to growth compensated resistance. However, under space limitation in a closed system, resource partitioning might occur and resources provided in excess to growth requirements can be allocated to defense responses (128). In contrast, under low nutrient conditions, growth demands may not be satisfied and thus resources are allocated to growth instead of allelopathy whenever available. The findings reported in this chapter demonstrated resource partitioning in the mixed community and also the relevance of the growth rate hypothesis, one of the eukaryote defense theories discussed.

The survival strategy adopted by a species is dependent on the trade-off made between growth and differentiation (167), and many species can be categorized as competition or defense specialists. In a recent study, Simek *et al.* (294) have proposed that bacteria may be able to adopt three types of survival strategies when confronted with protozoan grazing. The first is the ability to exploit environmental nutrients for high growth potential, while sacrificing grazing resistance as a consequence (167, 294). The second strategy is the simultaneous optimization of nutrient uptake for growth and grazing defense through the ability to acquire alternative nutrients or to more efficiently utilize available nutrients, for example by reducing the cell size (319). The third survival strategy against protozoan grazers pertains to defense specialists, which are typically poor competitors generally characterized by having small or inedible morphotypes (298). Defense specialists have a competitive advantage when grazing pressure is intense and density-dependent, as superior competitors would experience the highest mortality rate (149). *S. japonica* and *D. donghaensis* have high growth rates and are fed upon by *A. castellanii* and thus are competition specialists under low nutrient conditions, whereas *M. phyllosphaerae* can be classified as both a competition and defense specialist, reflecting its ability to exploit nutrients to

dominate the community while also being grazing resistant. *A. lwoffii*, an inferior competitor, can be classified as a defense specialist given its ability to evade *A. castellanii* and reduce grazing losses by remaining in the planktonic phase rather than being associated with the mixed-species biofilm.

Eukaryotic communities such as those observed in terrestrial systems, exhibit niche differentiation and resource partitioning which allow coexistence of species in the community (130, 226). Members of the community occupy specific niches in order to exploit nutrients and metabolic products that are needed and at the same time, limit competition (316). Such spatial stratifications allow members to minimize competitive interactions, facilitating coexistence of members within the community (266). Generally, members with the same nutrient requirements cannot remain stable in equilibrium in the same niche. According to Hardin (118), competition often results in the competitive exclusion of the inferior competitor, unless species diversify and occupy different niches (ecological differentiation). Therefore, the stability of the four-species community investigated in this chapter, suggests that members have the ability to utilize different nutrient sources (resource partitioning) in their specific niches. Low nutrient conditions also induced shifts in the niche preference for three of the four epiphytic isolates, while *A. lwoffii* maintained its niche preference for the planktonic phase (Figure 4.2). *M. phyllosphaerae* dominated the mixed-species community, followed by *S. japonica, D. donghaensis* and *A. lwoffii*.

Protozoan grazing is important for nutrient remineralization in the environment, in particular for recycling of N and P (247). The presence of alternative prey can stimulate grazing activity in a community and hence nutrient regeneration and subsequent growth (193). The increase in biofilm bacterial abundance of both mixed- and single-species communities seen on day 3 in the studies reported in this thesis (Figure 4.1), may reflect the introduction of nutrients on day 2 during media change which led to increased prey concentrations, encouraging density-dependent grazing and further nutrient regeneration and regrowth. Density-dependent grazing by protozoa only occurs above a certain bacterial prey threshold (i.e. the active feeding threshold) (139, 193). Protozoa graze in a way that is not overly exploitative, but rather satisfies current demands, thereby allowing prey to persist as a future food source (2). Therefore, in single-species communities, slow growers that are unable to achieve growth beyond this active

feeding threshold become effectively protected, whereas fast growers that are capable of exceeding this threshold rapidly will experience higher grazing pressure. As a consequence, association within a mixed community can be detrimental to slow growers such as *A. lwoffii*, as the total prey threshold is shifted above the protozoan active feeding threshold, resulting in increased grazing pressure.

M. phyllosphaerae was the most abundant member of the biofilm community formed by the four epiphytic isolates, followed by *S. japonica*, then *D. donghaensis* and finally *A. lwoffii*. Even though *M. phyllosphaerae* was the most abundant species in the mixed community, the effect of grazing exerted by *A. castellanii* was less pronounced than for *S. japonica* and *A. lwoffii*. This indicated that *M. phyllosphaerae* growth was able to compensate for any negative grazing effects, or that other factors such as prey preference for Gram-negatives, could have contributed to the selective feeding (4, 279). However, *A. lwoffii*, which is also a Gram-negative bacterium, was the least grazed. The low total CFU ml^{-1} of *A. lwoffii*, together with its preference for the planktonic phase (Figure 4.2), suggests that it is an inferior biofilm competitor. In single-species biofilms, its slow growth rate (i.e. takes longer to achieve the active feeding threshold) and its preference for the planktonic phase, provides protection from surface grazers. In mix heterogeneous systems, the probability of predator capture of low-density prey decreases due to reduced predator-prey contact. In addition, the preference for *A. lwoffii* to exist in the planktonic phase, provides an evasive mechanism through spatial refuges against the predominantly surface feeding, *A. castellanii* (260). Thus, low nutrient conditions may result in resource partitioning and as a result, niche restriction for some species in the mixed community (130).

The removal of *M. phyllosphaerae* from the four-species community destabilized the subsequent three-species community, resulting in no distinct trend in species abundance (Figure 4.3). This indicated that the presence of *M. phyllosphaerae* was crucial in suppressing the growth of other strains resulting in a reproducible hierarchy of dominance in the four-species community (*M. phyllosphaerae*, *S. japonica*, *D. donghaensis* and *A. lwoffii*) (Figure 4.3). High numbers of *S. japonica* in the single species plankton and mixed species biofilm, suggest that the four-species community also resulted in synergistic interactions by improving nutrient acquisition, cooperative metabolism or in providing scaffolding support

for *S. japonica*, allowing it to remain in the biofilm and benefit when in the mixed community (Figure 4.1B and Appendix VB). In comparison, ratios and numbers of *A. lwoffii* in the mixed community compared to single-species biofilms indicated that the four-species community might be detrimental to its fitness, as it is an inferior competitor in terms of nutrient and space acquisition, which could have resulted in it being outcompeted (Figure 4.1D).

Predation intensity can have positive, negative or neutral effects between prey species (52). The habitat shift of *D. donghaensis* away from the biofilm phase (Figure 4.2) is an example of predator induced prey behaviour affecting population density and interactions between species (52, 129). Predators can also affect community composition by altering the diversity of prey resources. For example, in density-dependent grazing, predation on competitive dominants might promote species coexistence by preventing competitive exclusion (2, 130).

4.4.2 Varying resistance of single species communities to grazing observed under C, N and P limitation

Copiotrophs represent the culturable fraction of marine bacterioplankton community and are usually found in association with higher organisms (169). They adopt a feast and famine lifestyle, and are capable of undergoing cellular developmental changes under multiple nutrient limitation. During nutrient upshift, copiotrophs exploit the available nutrients to maximize growth, leading to rapid proliferation (169). During nutrient limitation, starvation adaptive responses allow the copiotrophic cell to shift from active growth to starvation stasis, which is characterized by a series of specific phenotypes such as increased stress resistance (302). In response to P limitation, Gram-positive species undergo a pronounced starvation-induced reorganization event, which includes substitution of cell wall components where phosphate-based teichoic acids are replaced by carboxyl-based teichuronic acids (106). In this way low P conditions can result in increased grazing resistance as changes in the cell wall composition might contribute to discriminatory feeding by *A. castellanii*. The Gram-positive *M. phyllosphaerae,* was grazing resistant under both high and low P concentrations, indicating that there might not have been a change in palatability for this strain (Figure 4.3) (204).

Nutrient remineralization from grazing activities could also have contributed to higher biomass in grazed as compared to non-grazed biofilms.

Polyphosphates accumulate in some Gram-negative bacteria during nutrient imbalances and occur as inclusion bodies within cells, often resulting in an increase in cell length (142). The larger cell size could function as a grazing deterrent for *S. japonica* on day 6 due to prey size discrimination by protozoa (Figure 4.5A). Alternatively, excess C resulting in the production of EPS could also have contributed to grazing resistance in *S. japonica* on day 6 (Figure 4.5A). However, other aspects of the starvation response such as substitutions of outer membrane proteins, lipids and lipopolysaccharides (LPS) might also occur (176). Such outer membrane structures may also provide grazing resistance against different types of protozoa (339). Thus, the susceptibility of *S. japonica* biofilms to *A. castellanii* grazing on day 8 shown in all C and P conditions suggests that *S. japonica* might have undergone cell reorganization on day 8 due to depletion of polyphosphate stores.

At high C and low N concentrations, bacteria often channel the excess C towards production of EPS (51, 133). EPS can function as a grazing defense (133, 204). Other structural defenses such as filament and microcolony formation also contribute to grazing defense (205). The higher planktonic biomass in grazed compared to ungrazed treatments, indicated that detachment of *M. phyllosphaerae* from the biofilm and residence in the planktonic phase served as refuge against the surface grazer, *A. castellanii* (Figure 4.4A to D). C limitation has been demonstrated to affect the hydrophobicity of cells, which can affect ‘palatability’ and prey recognition by predators, thereby altering grazing losses (74, 199). Biofilm detachment can also be triggered under C and N limitation, leading to a shift in cells from biofilm to planktonic phases, effectively providing grazing resistance. Thus, resistance against protozoan predators are dependent on both the ecological niche preference of prey (planktonic or biofilm phase) and the type of grazer (suspension or surface-associated) (74).

A comparison of all the C:N (fixed P) and C:P (fixed N) treatments of *M. phyllosphaerae* revealed that the grazing resistance displayed were significantly higher than that observed in C:P treatments (Figure 4.4A and B). The higher biofilm biomass observed in grazed treatments of all ratios of C:N and C:P

suggested that grazing by *A. castellanii* resulted in nutrient remineralization (Figure 4.4A and B). Grazing-induced mortality can increase the rate DNA release, which can serve as potential C, N and P sources. For example, cleavage of nitrogenous bases from the sugar-phosphate backbone of DNA by nucleoside phosphorylases can increase N availability (229, 283). In contrast, the rates of P regeneration from the sugar-phosphate backbone require a further cleavage and thus are slower compared to N regeneration. This could have accounted for the higher biofilm biomass observed in grazed C:N treatments compared to C:P treatments. The higher grazing resistance to *A. castellanii* could be attributed to C:P defenses being related to structural changes (e.g. cell elongation) whereas C:N defenses are often reported to be linked to chemical or bioactive responses (e.g. production of toxins and virulence factors), which require N as the 'basic building block'. For example, N starvation in *Salmonella typhi*, a Gram-negative bacterium, increases the expression of the *rfaH* gene, which is required for proper expression of LPS (180, 277). Strategies of these kinds could be adopted by either *M. phyllosphaerae* or *S. japonica* during N limitations in the presence of a grazer. The results obtained also showed that under low C concentrations, *S. japonica* was susceptible to *A. castellanii* grazing whereas it was resistant at high C (Figure 4.5A and B). The grazing susceptibility observed in *S. japonica* suggests a trade-off between growth and defense, indicating that resistance against amoeba predation might be a C-based defense for this bacterium.

Overall, the results presented here have demonstrated that bacterial biofilm communities are controlled by both 'top-down' and 'bottom-up' factors. However, under nutrient limitations, 'top-down' factors play a more significant role in determining community composition compared to 'bottom-up' factors (296, 311). Nutrient conditions are crucial in determining the defense strategies adopted and, as a consequence, alter the functional responses of the bacterial community as well as its composition. The experimental approach taken in this chapter and the results obtained have demonstrated that eukaryotic defense theories are useful tools that successfully provide a framework for predicting microbial defense strategies.

Chapter 5 : General Discussion

Bacterial communities are highly diverse assemblages that exhibit complex interactions between members and serve as important drivers of biogeochemical cycles (7, 94). The structural and functional diversity of a community is often determined by both antagonistic and synergistic interactions, which develop as a result of nutrient and space limitation (185). Antagonistic interactions, as derived from allelochemical production by members in a microbial community, can arise from intense competition for nutrients and space, in addition to the need for predatory defense. These interactions allow cells or populations to exploit and/or interfere with other potential competitors or predators and, in this thesis, served as the basis for the bioactive screening in Chapter 2 (125, 200). Synergistic interactions, such as mutualism and commensalism, whereby members benefit from cooperative activities, for example as a result of metabolic cooperation, also occur within mixed species consortia and affect community diversity (54). Protozoan grazing, which exerts major control over bacterial abundances in the environment, can also select for predation resistant strains and result in further changes in community composition (111). In this thesis, a defined mixed community was chosen for exploring interspecies interactions and the impacts of nutrient limitation on grazing resistance and community structure (Chapters 3 and 4).

Understanding the types of interactions occurring in environmental communities is essential for determining ecosystem structure and function. A major limitation in addressing this challenge is the lack of incorporating ecological theories as foundations for generating predictive models for microbial community behaviour (185). Our knowledge of microbial community interactions lags behind that of larger eukaryote systems, largely due to historical methodological constraints. These, however, are rapidly being overcome with the constant advent of new technologies. Consequently, the conceptual framework founded in eukaryote ecology provides a wealth of ecological theories that could equally apply to microbial communities. Indeed, microbial ecologists have begun applying such theories to microbial systems. The insurance hypothesis, which postulates that increased community diversity enhances resistance to disturbances to ensure

community survival, is one example of a eukaryote-derived theory tested in microbial systems (211, 352). Applying this model, Matz *et al.* (206) showed that predation on planktonic cells of *Vibrio cholerae* led to phenotypic diversification resulting in selection for biofilm-enhanced variants.

The results presented in this thesis demonstrate the utility of ecological theory beyond the scope for which it was originally devised by investigating a microbial community in a broader ecological conceptual context. Firstly, the system to be investigated was characterized by using allelochemicals derived from bacterial biofilms to identify novel bioactives against parasitic protozoa and nematodes (Chapter 2). This provided the framework to further investigate the microbial community at the focus of this study. The response of mixed-species biofilm communities to predation at different stages of biofilm development provided insights into the community ecology of the microbial system being studied (Chapter 3). In addition, investigating microbial interactions under different nutrient conditions provided information on how biofilms respond to varying nutrient balance and availability, and on the effect that nutrients have on growth and grazing resistance of these communities (Chapter 4). Moreover, this thesis has provided examples on how eukaryotic defense theories like the growth-differentiation balance (GDB) and carbon:nutrient balance (CNB) hypotheses can be successfully applied to microbial systems for prediction of defense responses. More broadly, results presented here suggest that eukaryotic ecological theories may offer an appropriate context and predictive power in microbial ecology based studies.

5.1 Extension of bioactive screening to complex communities?

Marine microbial ecosystems remain relatively underexplored despite impressive technological advances and the generation of much new data in recent years (58, 147-148, 287). Bacteria in the marine environment are usually found in mixed species consortia and in marine coastal ecosystems associated with higher organisms such as algae or invertebrates in a range of relationships from transient to obligate (11, 147). These associations select for bacterial adaptations which can include inhibitory metabolite production. For example, marine sponges form symbiotic relationships with bacteria; *Pseudoceratina purpurea* harbours surface-

associated bacteria, which are responsible for inhibitory activities against secondary microbial fouling organisms (158). The species-specific associations of bacteria and invertebrates and the diversity of invertebrate species indicate that there is potentially a great diversity in bacterial metabolic flexibility, suggesting that inhibitory metabolites produced for chemical defense would also be diverse (147). Therefore, these communities would be ideal targets for the exploration and identification of novel bioactives.

In Chapter 2, the amoebae plate assay (APA), the selective grazing assay and the *Tetrahymena* sp. motility assay (TMA) were validated using two groups of pigmented and non-pigmented *Pseudoalteromonas* strains, representing a genus of marine bacteria well known for its diverse secondary metabolite production (125). Results presented in this thesis were in agreement with previous findings that pigmented strains exhibit higher levels of biological activity (81). Cell-free media surrounding the bacterial biofilms that were inhibitory against amoeba were tested using the TMA. However, no cytotoxic activity against *Tetrahymena* sp. was observed in these samples, demonstrating that higher levels of toxicity were associated with biofilm cells. Bacterial strains can also produce multiple inhibitory compounds with different biological activity. Perhaps the most well known example of such a bacterium is *Pseudoalteromonas tunicata*, which produces an antifungal and an antimicrobial compound that are additionally cytotoxic to protozoa (208, 320). Such broad spectrum activities were also observed in strains from the environmental library, with inhibition recorded against both nematodes and protozoa (Table 2.4). However, these strains appear relatively infrequently in the screened environmental libraries as compared to the more common occurrence of strains with narrow spectrum activity. Screening for antagonistic activity from different environmental sources also showed that epiphytic communities isolated from algal surfaces displayed higher levels of inhibition compared to activated sludge communities. Different types of interspecies interactions could have accounted for differences in the presence of bioactivity. For example, ammensalism is a common strategy adopted in aquatic systems for achieving a competitive advantage in nutrient and space acquisition (144). In contrast, activated sludge systems are functionally bioactive only when members coexist (144). Thus, one would expect that activated sludge

communities are highly cooperative and coordinated in order to perform the required bioprocesses.

A major constraint in bioactive discovery is the culturing of unique isolates and successful growth on appropriate media for bioactive production (148). The screening methods developed in Chapter 2 are only appropriate for readily culturable bacteria, which represent in the order of 0.1 % of pelagic systems and 3.4 – 11 % of surface-associated bacteria (148). Thus, culture-independent methods may also be considered in bioactive screening. For example, in functional metagenomics, DNA extracted from a community can be cloned into an easily culturable host before screening for expressed bioactives (185). While there are several limitations currently inherent in such an approach, these can in part be addressed through continuous rounds of optimizations. These include improving: DNA extraction protocols, which are currently biased by differential resistance to cell lysis; incompatible host expression systems, which may impair expression of activity; and screening systems, which often display poor resolution, hence leading to failure to capture the wider spectrum of bioactivity (65, 95, 115, 168). Alternatively, a targeted approach of searching for gene homologues of interest (i.e. genes encoding secondary metabolites), followed by the expression in a host, can be achieved using functional genomics (185), although this is restricted to known classes of metabolites. This approach involves designing gene-specific primers to probe a mixed community for the metabolic gene of interest. The major constraints to this gene-targeted approach include failure to obtain full length gene fragments and the bias in primer design, which usually skews toward known gene sequences. Hence, newly evolved sequences with similar functions may not be identified (66). Proteomics can also be used to discover novel bioactives when genomic data from a community such as a defined mixed species consortium is available. This can be achieved from information provided by the sequenced and annotated genomes which can potentially provide a massive platform of diversity of hypothetical and conserved proteins that could exhibit biological activity (95, 115). While proteomics has facilitated the discovery of novel functional genes and metabolic pathways (102, 265), linking protein expression and function to gene sequences still poses a challenge. Pure bacterial cultures are generally still required in order for the biological and molecular characterization of novel proteins (269).

While there are several limitations to using culturable organisms, there are also several advantages to using mixed-species biofilms as a whole entity during screening. For example, interspecies interactions can result in metabolic cooperation and thus the isolation of individual species from the complex mixed community would impair secondary metabolite production. In such cases, metabolites secreted into the planktonic phase from complex community biofilms can be harvested and tested in liquid-based screening assays for secreted metabolites. However, this will only allow for the detection of secreted metabolites. The different screening systems optimized in Chapter 2, provide the flexibility of detecting both secreted and non-secreted metabolites, thus ensuring a wider coverage of biological activity. In addition, the direct screening of the culturable fraction is less time consuming and avoids some of the limitations posed by expression systems used in functional metagenomics as discussed above.

5.2 Linking the understanding of microbial interactions from laboratory to environmental systems

Microbial systems can be easily manipulated and are therefore ideal for the study of positive and negative interactions in mixed-species communities in laboratory-based systems. However, the role of such synergistic and antagonistic interactions has not been fully appreciated for natural microbial communities. As microbial environmental communities are highly diverse, the interactions occurring between and within different trophic levels are complex and likely to be vital for ecosystem function (94). According to the diversity-stability theory or the insurance hypothesis, when environmental conditions are highly variable an increase in the number of species in a community increases its stability against perturbations such as predation and invasion (49, 211). Indeed, in this study mixed communities of two, three and four species were more resistant to *A. castellanii* grazing compared to their single species counterparts (Chapter 3), which could be due to pronounced interactions between community members (49). Protozoan prey preferences could have also contributed to the differential grazing resistance observed in Chapter 3. *Acinetobacter lwoffii* was grazed preferentially by *A. castellanii*, followed by *Shewanella japonica*, whereas *Microbacterium phyllosphaerae* and *Dokdonia donghaensis* were resistant. In the four-species

mixed community, *T. pyriformis* preferentially grazed on *D. donghaensis*, despite its low densities. Surface structures such as chemical (e.g. receptor-epitope) and mechanical (e.g. cirri) cues can be responsible for prey recognition, discrimination and grazing in amoebae and *T. pyriformis*, respectively (3, 218). Other factors contributing to selectivity of this kind, such as those reported by Schuster *et al.* (284), include a chemotactic response by *A. castellanii* to bacteria and their secreted metabolites. However, the lack of chemotaxis towards all four marine epiphytic isolates indicated that the resistance or susceptibility dependents on cell-to-cell contact with the protozoan.

Predation and competition are fundamental interactions between species (52). The presence of predators and the intensity of their grazing activity can alter the competitive outcomes by either increasing or decreasing interspecies interactions between members in a community (52, 272). For example, species coexistence and microbial diversity are promoted when a predator grazes on the competitive dominants, thereby balancing the competitive abilities of members in the community. Such occasional disturbances are healthy for the community as the inferior competitors are given a chance to increase in abundance while superior competitors are kept under control, thus preventing competitive exclusion (272, 348). These interactions are well documented in eukaryote ecology, especially for marine ecology where manipulative field experimentation led to the formulation of the keystone species concept (236). The increased stability to environmental perturbations, associated with increased species diversity, supports coexistence amongst competitors as members can occupy different niches depending on habitat and resource partitioning (130, 348). Predation by specialists can also select for habitat or behavioural shifts in prey communities (129). As such grazers show selective adaptations for certain niches; this provides spatial refuges for species occupying other niches that experience low grazing intensities (130). For example, *A. lwoffii* exhibits a preference for the planktonic phase, thus effectively being protected from *A. castellanii*, a predominantly surface-associated grazer that is less able to prey on plankton (Chapter 4). The lack of floc formation by *A. lwoffii* reduced attachment sites in the plankton, which also decreased *A. castellanii's* grazing effect. In the environment, habitat partitioning in prey can lead to more drastic ecological effects when predation involves different non-migratory predator species. Predator populations that display specific prey

preferences can become spatially segregated depending on prey location (130). In contrast, migratory movements of predators and their random foraging habits will promote coexistence, as prey will be allowed to replenish when predators migrate to patches with higher yields of alternative prey (130). The preferential growth of *A. lwoffii* in the planktonic phase serves as a refuge against surface grazers, indicating that in the environment, the presence of such spatial refuges, are thus likely to prevent species local extinction and promote regrowth of prey away from predators when threshold numbers become too low (129).

Many studies have shown that protozoan grazing induces taxonomical, size and shape shifts in mixed-species microbial communities (reviewed in Chapter 1), causing significant changes in community evenness. For example, in Chapter 3, the impact of *Rhynchomonas nasuta* grazing on a mixed community induced a shift in ecological niche preference of *S. japonica* and *A. lwoffii* towards the planktonic phase. This is in agreement with observations reported by Koh (170), demonstrating that grazing by surface-adapted protozoans such as *A. castellanii* and *R. nasuta*, has the potential to induce shifts in niche preferences. Size-selective grazing can lead to microdiversification of a single species into strains characterized by different sizes that are grazing resistant (31). For example, the amoeba, *Naegleria* sp., grazes readily on filamentous cyanobacteria but not on aggregates (186). In contrast, the amoebal grazer, *Acanthamoebae polyphaga* is capable of grazing on microcolonies but not filamentous biofilms (264). The effectiveness of planktonic phase spatial refuges and anti-grazing biofilm structures in the environment remains unclear. The huge diversity of protozoans in the environment suggests that their associated feeding habits are equally as diverse, thus such inedible morphologies do not provide absolute resistance. This study demonstrated that early-stage biofilms are effectively grazed by *R. nasuta*, intermediate-stage biofilms by *T. pyriformis* and late-stage single species biofilms by *A. castellanii* (Chapter 3). In contrast, mixed-species communities were more complex as the synergistic interactions such as associational resistance between members increased stability and resistance to disturbance, such as presented by *A. castellanii* grazing and dominant strain removal. Selective grazing observed on different prey in the mixed community by *R. nasuta* and *T. pyriformis* (Chapter 3) could result from prey preference or by associational resistance. Nevertheless, the different prey preferences by different protozoans preferentially reduced different

bacterial strains, thus preventing the complete dominance of one single species in a mixed-species community (247). The grazing resistance displayed by any microorganism is thus differentially effective depending on the target predator (247). The results presented in Chapter 3 provide valuable insights into the complexities of bacteria-protozoan interactions occurring in the environment.

Clearly, protozoan grazing can control bacterial abundance across different species, with grazing observed also on less abundant bacteria (Chapter 3). Certain protozoan groups, such as flagellates, may display more distinct preferences for certain prey morphotypes, thus impacting community diversity, as presented in this thesis, a phenomenon that may occur frequently in the environment (14, 199). Many studies have attempted to understand the specific impacts of grazing in the environment while also taking into account a range of potential contributing and competing factors. Overall, protozoan grazing accounts for substantial bacterial mortality, which coupled with considerable viral-induced mortality greatly contribute to nutrient recycling and sustaining diversity (247, 298, 358). The predator-prey interactions become more complex when predators compete directly with bacteria for nutrients, thus exerting control over bacteria via 'top-down' and 'bottom-up' factors, which indirectly affects community diversity (247). It is suggested that when studying microbial communities, all types of organisms such as viruses, protozoans and metazoans, should be included, so that important factors controlling the different types of interactions are encompassed (94). Therefore, in future studies, the impact of metazoans or viruses could also be introduced into defined bacterial consortia (such as those used in Chapter 3) in order to construct a more complete picture resembling mixed species community diversity and function.

5.3 Predation resistance under nutrient limitation

Nutrient limitation affects the level of resistance of a mixed-species microbial community (Chapter 4). For example, biofilms grown on high nutrient media were resistant to *A. castellanii* grazing (Chapter 3), whereas low nutrient grown biofilms remained susceptible (Chapter 4). According to the growth rate hypothesis, competitive growth is prioritized under high nutrient conditions, thus, metabolic resources would be preferentially allocated to growth (59, 120). The

high biofilm biomass of the mixed-species biofilms under high nutrient conditions is an example of the growth rate hypothesis as the grazing resistance displayed by the community against *A. castellanii* was a result of growth-compensated resistance. Alternatively, resource partitioning could also have occurred in the semi-continuous biofilm system, leading to nutrients in excess of growth requirements being diverted to grazing defense (43).

The eukaryotic carbon-nutrient balance (CNB) hypothesis predicts that defenses adopted by a biofilm community are determined by the available levels of carbon, nitrogen and phosphorus. In aquatic ecosystems, nutrient limitation can lead to habitat partitioning of microbial species into either core or occasional species on a temporal and spatial scales. Core species are characterized as being the most abundant due to well developed adaptations for the particular ecosystem (243). Thus, they are postulated to play a crucial role in carbon and energy flow in the ecosystem which is fuelled by predatory activities (243). *M. phyllosphaerae* and *S. japonica*, the two dominant strains in the mixed community, can be classified as core species in the 'laboratory' generated ecosystem. However, when both strains are in their native community on the surface of *Ulva australis*, other forms of synergistic and antagonistic interactions between members of the community, especially those producing allelochemicals, may alter the dominance of both strains. In contrast, occasional species are characterized by sporadic growth due to growth limitation and therefore play less significant roles in ecosystem functions (243). Thus, the results obtained from mixed community studies reported in Chapters 3 and 4, reflect on the contribution of other factors, such as predation, nutrient limitations, synergism, antagonism and habitat partitioning (ecological niche preference), all which could affect the classification of core and occasional species.

Predation can also indirectly exert a selective force on prey nutrient preference and as a result, alter community structure (52). This is achieved when the uptake of certain nutrients increases grazing vulnerability, causing prey to avoid that particular nutrient to increase grazing resistances (52). For example, Bohannan *et al.* (32) reported a trade-off between predatory resistance and competitive ability. The cost of such trade-offs affects the probability of species extinction and thus can lead to a string of environmental consequences which include the changes in community stability, coexistence between species and also invasion resistance

(32). A trade-off between growth and defense was made by *S. japonica* when exposed to *A. castellanii* grazing under nutrient limitation (Chapter 4). Grazing resistance was increased at a cost to growth. The growth differentiation balance (GDB) hypothesis is thus applicable to this microbial system, suggesting that other eukaryote-based defense theories might also have potential for use in microbial ecology.

Bacteria in the environment can adopt a starvation response under oligotrophic conditions, a survival strategy, which includes the stringent response, a subsequent phase of pronounced morphological and physiological reorganization and finally a long-term dormancy phase (165). *S. japonica* and *M. phyllosphaerae* might have adopted this survival strategy under nutrient limitation (Chapter 4). Cellular reorganization such as cell wall substitutions in Gram positive bacteria and polyphosphate accumulation in Gram negative bacteria, could potentially lead to grazing discrimination or handling inefficiency, respectively (165, 204). Grazing activities can be monitored microscopically with an imaging program to determine if such grazing selectivity and/or difficulty occur. In addition, the grazing resistance of *S. japonica* under high carbon availability conditions can also be scrutinized to determine if excess carbon contributed to EPS production by using calcoflour white (333) or if carbon-based defenses (as determined by ethanol/methanol extraction from biofilms or fractionation of cell-free supernatants) were responsible (22). The effect of nutrient limitation on biofilm grazing resistance demonstrated in this study (Chapter 4) provides an additional fundamental platform for understanding biofilm-predator interactions, as most grazing studies on nutrient limitations use planktonic systems to resemble pelagic environments. It also serves as a reference for future mixed species biofilm studies of specific nutrient limitations on biofilm resistance for an improved understanding of how natural biofilms respond to such environmental conditions.

5.4 Suggestions for future work

Much remains to be learned about antagonistic and synergistic interactions in mixed-species microbial communities. The work presented in this thesis serves as a potential starting point for pursuing our exploration and understanding of such community interactions in the environment. The influences of biotic factors such

as competition, parasitism and mutualism, which are all agents of selection, can lead to the formation of phenotypic variants to increase prey survival chances. Thus, a logical follow up experiment would be to investigate mixed community biofilms for the occurrence of phenotypic variants or microdiversification in single species biofilms, and characterize them under intense grazing pressure (31). It has been postulated that chemical cues released by predators might also stimulate grazing resistance (111). Recently, Erken *et al.* (84) suggested that the stimulation of *Vibrio cholerae* biofilms in the presence of *T. pyriformis*, despite segregation of predator from prey with the aid of a filter, could have been a result of chemical cues. Thus, another possible area of work would be to test for the release of chemical stimuli from the predator, and the impact of these on the grazing resistance of mixed species biofilms.

Understanding microbial ecology is one of the intellectual hurdles most environmental microbiologists face, and, there is clearly a lack of application of ecological theories as predictive models into microbial systems. The body of ecological theory is well established for eukaryote ecology, which in fact makes adaptation of such theories into microbial systems a feasible and attractive option (262). The application of the GDB and CNB hypotheses, which address the different trade-offs in exchange for defense, in this thesis, reflects the potential use of eukaryotic ecology to be tested on microbial systems. In the area of community ecology, the impact of biodiversity on ecosystem function can be tested on an artificial microbial community of known species richness and functional contribution. As environmental conditions can differ significantly from those of tightly controlled laboratory systems, shifts in community composition in natural systems can occur in favour of any strain capable of adapting and exploiting current conditions (167). However, defined communities established in laboratory-based systems can also be tested in environmental systems and field trials to investigate the trade-offs made by the community under temporal variations. Several studies have attempted to perform such *in situ* field experiments using environmental chambers with filter membranes of appropriate pore sizes, held together by an autoclavable chamber with a surface in place for bacterial attachment and biofilm formation (213, 328). Bacterial communities and protozoan grazers are injected through inlets in the chamber using syringes. Filters allow media exchange but prevent bacteria and protists within the chamber

from escaping and also environmental organisms from invading. This apparatus enables community response and trade-offs from predation or temporal variation to be compared under either laboratory or environmental conditions.

Phenotypic variation coincides with dispersal events from microcolony type biofilms during biofilm expansion (170, 201). Work done on such morphological variants, isolated from single-species biofilm communities, has demonstrated significant differences in terms of colonization and virulence (170). Moreover, the occurrence of such phenotypic variants also contributes to grazing resistance (206). Koh (170) reported that phenotypic variants of *Serratia marcescens* work in concert to increase biofilm stability against environmental perturbations as well as its competitive ability. These studies serve as foundations for understanding mixed-species communities. Therefore, the presence of phenotypic variants in mixed species communities should also be examined to determine if grazing increases the number of morphological variants and if such variants contribute to increased grazing resistance, virulence or biofilm formation, which could cause shifts in community composition and biological diversity.

In recent years, there is increasing popularity for the application of metagenomics as an approach for community studies (66). However, the information obtained from metagenomics is limited to defining the metabolic potential of a community but not its activity *in situ*. Hence, metatranscriptomics, which provides gene expression profiles of a community, allows one to link and correlate a particular function (e.g. resistance or pathogenicity) to its unique gene expression and it can be used to elaborate on community studies (336). This meta-omics approach is suitable, especially for the study of the response of mixed-species communities to various environmental perturbations. The genomes of members in the defined mixed species community can first be sequenced before metatranscriptomics is performed on single- and mixed-species biofilms to look for differential gene expressions to identify any interactions unique to the mixed community (102, 336). Alternatively, grazers can be added to both single and mixed species community to investigate adaptive responses and expression or regulation of defense responses against protozoan grazers. Different levels of control such as manipulating nutrient limitation, can also be included to increase the level of complexity in the responses regulated by the community. Thus, the meta-omics approach, which provides many different possible ways of cross comparison, will

likely expand our current understanding of interactions occurring in mixed species communities, providing a more complete and elaborate picture of how mixed communities function.

Appendix I

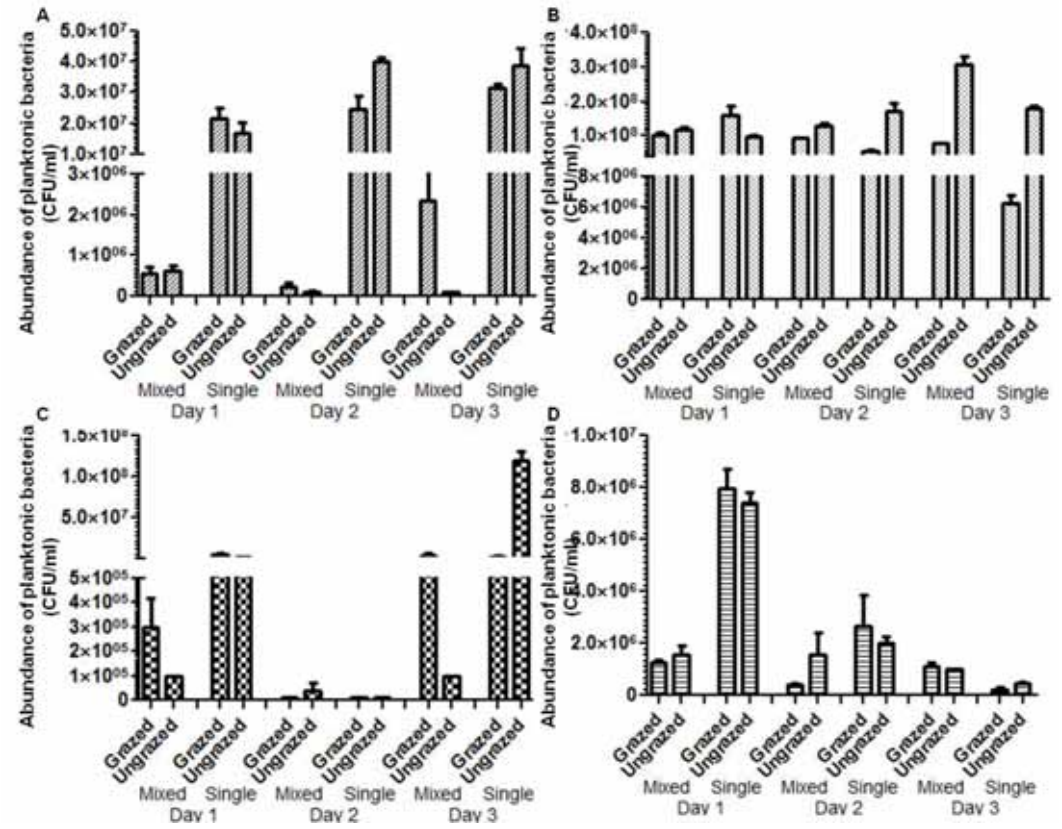


Figure I: Comparison of the resistance of single- and mixed-species (4 species) planktonic fractions harvested from early biofilms grown in the presence and absence of *R. nasuta* over 3 d. The planktonic fractions of *M. phyllosphaerae* (A), *S. japonica* (B), *D. donghaensis* (C) and *A. lwoffii* (D) in each consortium were harvest as described in section 3.2.4.3. Planktonic counts from grazed and ungrazed treatments were expressed as colony-forming units (CFU/ml). Day 1, 2 and 3 indicates the number of days both mixed and single species biofilms were co-inoculated with *R. nasuta*. Error bars represent standard deviation of three replicates.

Appendix II

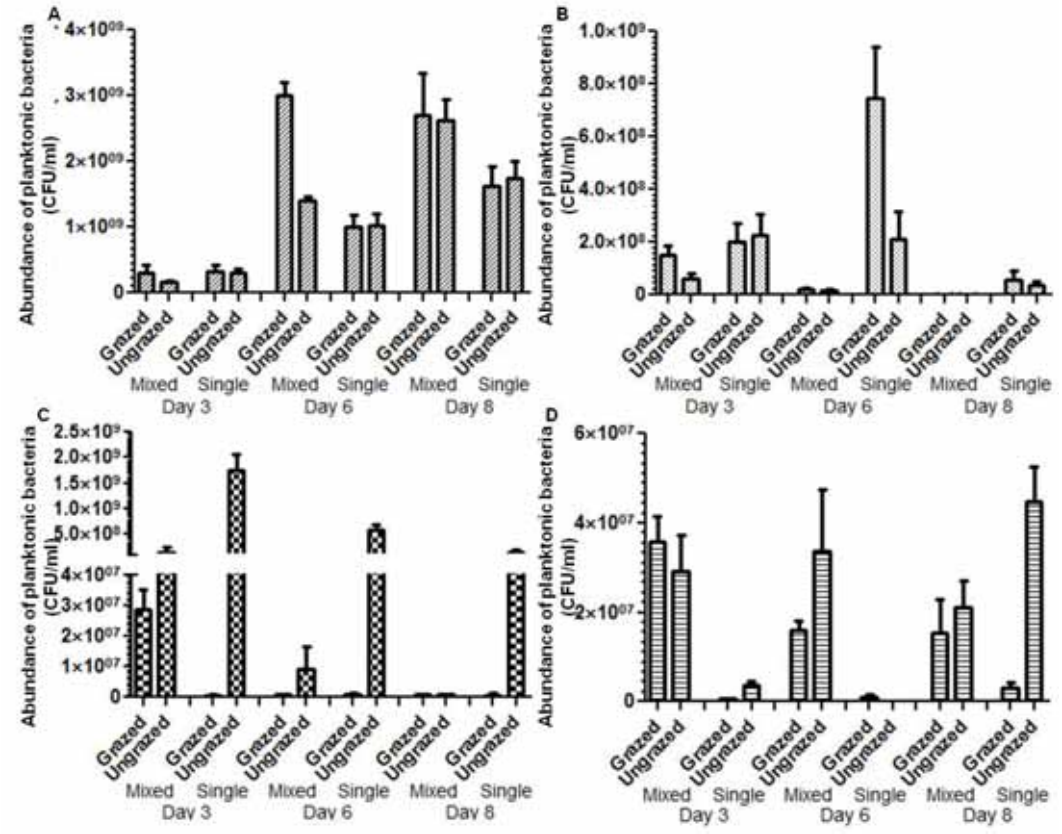


Figure II: Comparison of the resistance of planktonic fractions, expressed as colony-forming units (CFU/ml), harvested from grazed and ungrazed intermediate- and late-stages single- and mixed-species (4 species) biofilm consortia. The planktonic fractions of *M. phyllosphaerae* (A), *S. japonica* (B), *D. donghaensis* (C) and *A. lwoffii* (D) in each consortium were harvested as described in section 3.2.4.4. Day 3, 6 and 8 reflects the numbers of days of co-inoculation with *T. pyriformis* before planktonic fractions were harvested. Error bars represent standard deviation of three replicates.

Appendix III

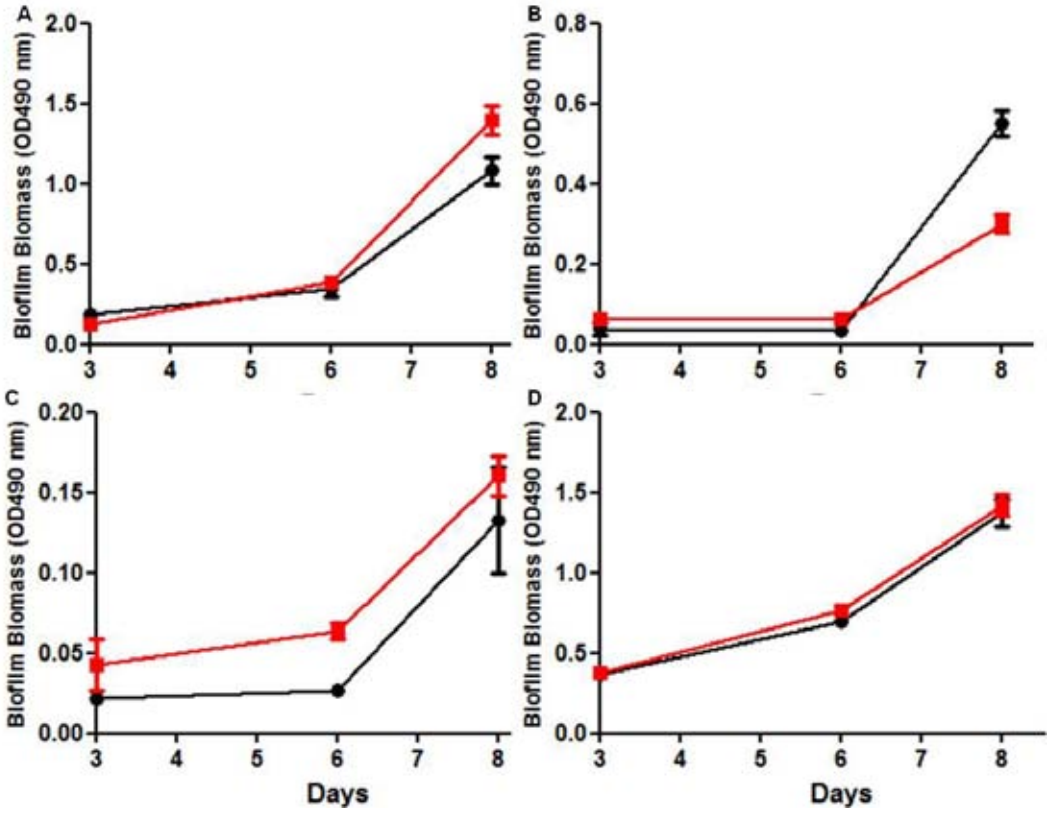


Figure III: Resistance of single- and mixed-species biofilms to grazing by the amoebae over 3, 6 and 8 d. The biofilm biomasses shown are of the single-species *S. japonica* (A), *D. donghaensis* (B), *A. lwoffii* (C) and all three species (D) formed in grazed (black) and ungrazed (red) treatments. Error bars indicate standard deviation of three replicates.

Appendix IV

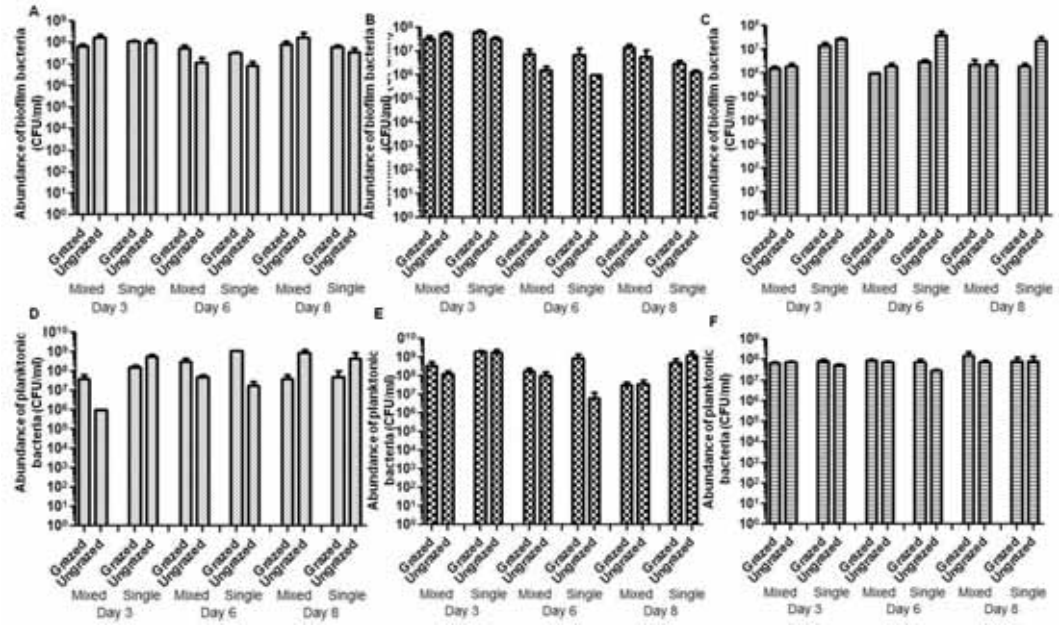


Figure IV: Comparison of the resistance of biofilm (A to C) and planktonic fractions (D to E), expressed as colony-forming units (CFU/ml), harvested from single- and mixed-species (3 species consisting of *S. japonica* (A and C), *D. donghaensis* (B and D) and *A. lwoffii* (E and F)) biofilm consortia. Day 3, 6 and 8 reflects the numbers of days of co-inoculated with (grazed) and without (ungrazed) *A. castellanii*. Error bars represent standard deviation of three replicates.

Appendix V

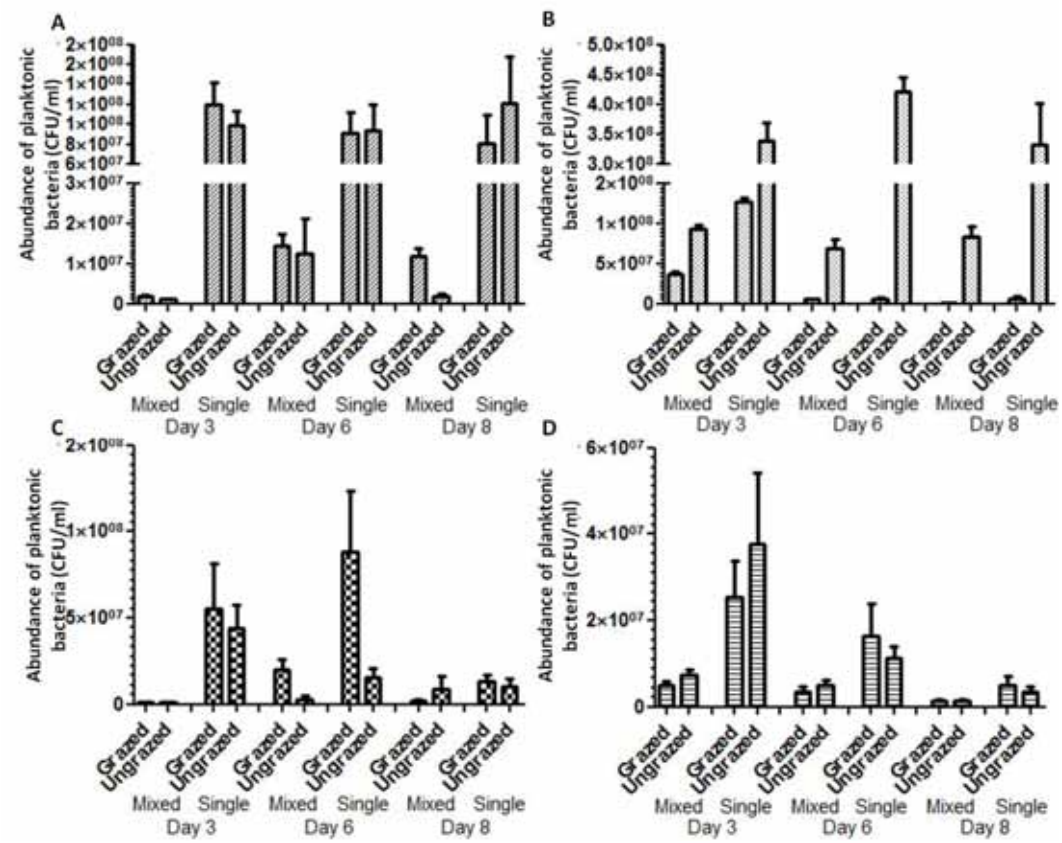


Figure V: Differential resistance of the planktonic phases of each epiphytic isolate grown in single- and mixed-species consortia under low nutrient conditions in the presence (Grazed) and absence (Ungrazed) of the surface feeding, *A. castellanii*. The planktonic bacterial abundance of *M. phyllosphaerae* (A), *S. japonica* (B), *D. donghaensis* (C) and *A. lwoffii* (D) were expressed as colony-forming units (CFU ml^{-1}). Day 3, 6 and 8 represents the length of exposure to *A. castellanii* grazing. Error bars indicate standard deviation of three replicates.

Appendix VI

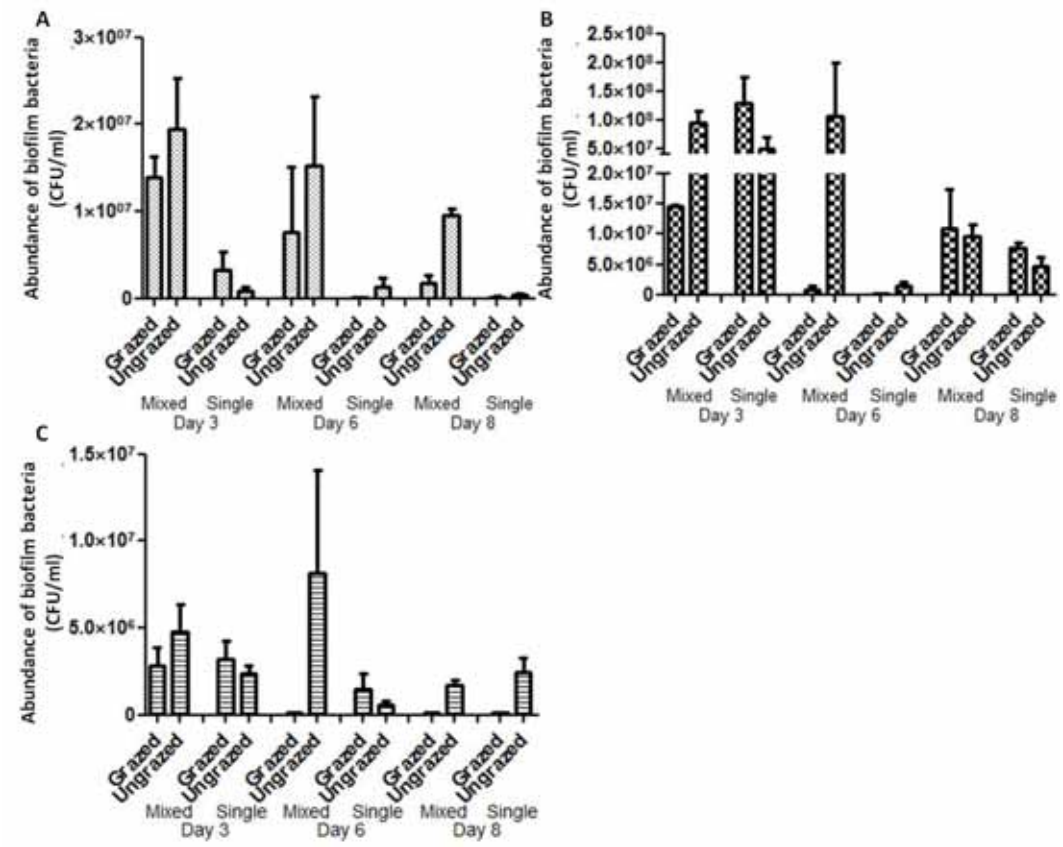


Figure VI: Grazing resistance of each epiphytic isolate grown in single- and mixed-species consortia after removal of *M. phyllosphaerae* under low nutrient conditions in the presence (Grazed) and absence (Ungrazed) of the surface feeding, *A. castellanii*. The biofilm bacterial abundance of *S. japonica* (A), *D. donghaensis* (B) and *A. lwoffii* (C) were expressed as colony-forming units (CFU ml^{-1}). Day 3, 6 and 8 represents the length of exposure to *A. castellanii* grazing. Error bars indicate standard deviation of three replicates.

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