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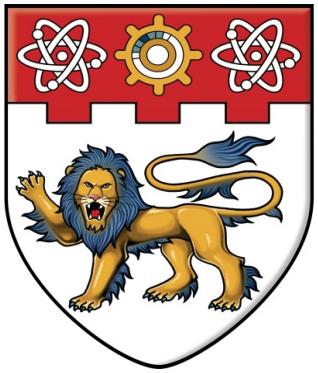

NANYANG TECHNOLOGICAL UNIVERSITY

Impact of *Bdellovibrio*-and-like Organisms (BALOs) on Mixed Species Biofilm Communities

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2016

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ABBREVIATIONS

Abbreviation	Description
ATP	Adenosine Triphosphate
BALOs	*Bdellovibrio*-and-like organisms
BLAST	Basic local alignment search tool
BOD_5	Biochemical oxygen demand
CFU	Colony forming unit
COD	Chemical oxygen demand
DMSO	Dimethyl sulfoxide
DNB	Diluted nutrient broth
EBPR	Enhanced biological phosphorus removal
EPS	Extracellular polymeric substances
FISH	Fluorescence in situ hybridization
HD	Host-dependent
HI	Host-independent
LB	Luria-Bertani
MBR	Membrane bioreactor
MDS	Multi-dimensional scaling
MLVSS	Mixed liquor volatile suspended solids
OTU	Operational taxonomic unit
PAOs	Polyphosphate accumulating organisms
PBS	Phosphate buffered saline
PCO	Principal coordinates
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PFU	Plaque forming unit
PUB	Public Utilities Board
SBR	Sequencing batch reactor
TAE	Tris-Acetate-EDTA
UP	Ulu Pandan

ABSTRACT

Predation has been found to play essential roles in ecosystems, where predatory activity may play an important role in helping to maintain the diversity of population and communities by preventing a single species from becoming dominant. Therefore, predators are considered as integral components of the food webs within the whole ecosystem. Predation is important not only at macro-scale, but also for single celled microorganisms. Microorganisms, especially bacteria, are generally preyed upon by viruses, protozoa and a groups of specific bacterial predators defined as *Bdellovibrio*-and-like organisms. Microbial predators are found in various environments where bacteria are inhabited. Activated sludge in wastewater treatment plants is comprised of highly diverse bacterial species, which carry out essential functions for wastewater treatment. Given that the microbial predators may prey upon diverse bacteria, it is of great interest to investigate the impact of predation on microbial community composition and function.

In this study, one member of the Genus *Bdellovibrio* was isolated from activated sludge collected at the Ulu Pandan Wastewater Reclamation Plant (Singapore). Based on the 16S rRNA gene sequencing, this isolate was 99% identical to '*Bdellovibrio bacteriovorus* strain Tiberius' and was designated here as '*Bdellovibrio bacteriovorus* UP'. The growth pattern of this *Bdellovibrio* isolate on specific prey species was shown to be similar with the well-known predator-prey interaction described by the Lotka-Volterra equation. The prey range and potential specificity of *B. bacteriovorus* UP was tested by quantifying growth in the presence of several model species, including *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas protegens* Pf-5 and the results showed that all of the tested model species were vulnerable to *B. bacteriovorus* UP predation. To further explore the potential impact of *B. bacteriovorus* UP on microbial communities in wastewater treatment plant, 78 isolates from activated sludge samples, were grown as single-species biofilms in the presence of this predator. As expected, none of the Gram-positive (14 isolates) or fungi (4 isolates) supported the growth of the predator. In contrast, with the exception of one species, *O. anthropi*, all of the Gram-negative species tested, represented by 18 Alpha-*Proteobacteria* isolates and 22 Gamma-*Proteobacteria* isolates were sensitive to predation by *B. bacteriovorus* UP irrespective of whether they were present as

biofilms or as planktonic cells. Biofilms and planktonic cells were reduced by 20% – 80% and 10 – 1000 fold, respectively.

It has been suggested that growth in a mixed species community can protect sensitive species from stressors, including predation. To test this possibility, two-membered mixed species communities were developed that included one species, *O. anthropi*, which was shown to be resistant to predation and the second species, which was shown to be sensitive to predation. The results indicated that the predator was able to significantly reduce the biomass of dual-species biofilms and planktonic cultures, suggesting that there was no cross-protection against predation. These results also suggest that the mechanism by which *O. anthropi* is protected is not a diffusible molecule, but is more likely cell associated. To further explore the role of biofilm based predation protection and feeding preference, mixed species communities with high density (> 3500 OTUs) represented by floccular or granular sludge were exposed to the predator and alterations in biomass, viability and community composition were quantified. The results showed significant reductions in the viability and total biomass of both floccular and granular sludge, although the floccular sludge was more sensitive to predation. Due to predation, the microbial community compositions of both floccular and granular sludge were also significantly affected. For example, the microbial diversities of floccular and granular sludge were reduced by 11.5% and 23.1% in their contribution to the total community as a consequence of predation.

The results presented in this study suggest that *B. bacteriovorus* UP isolated from a wastewater treatment plant has a broad spectrum of prey species and whilst it does not exhibit a specific feeding preference amongst the Gram-negative bacteria, some species are more sensitive to predation, especially when grown as biofilms. This suggests that whilst there is no explicit feeding preference for *B. bacteriovorus* UP, it may nonetheless impact specific communities members and hence reduce their contribution to overall system performance more than others. Both floccular and granular sludges were significantly impacted by the predator, although the granular sludge showed slightly less sensitivity to predation pressure. In aggregate, the data demonstrate that the *B. bacteriovorus* UP is a generalist predator and has the potential to strongly impact the performance of activated sludge communities.

Chapter I. LITERATURE REVIEW

1.1 INTRODUCTION

In ecology, the predation is usually defined as an interaction that a predator feeds upon its prey. Predation often leads to the death of prey, either directly or indirectly, where the prey is wounded or suffers energy exhaustion. In the case of direct killing, the prey will be consumed to provide the predator with vital nutrients. Additional categories of consumption have also been characterized, such as herbivory (feeding on parts of plants), mycophagy (feeding on parts of fungi) and detritivory (use dead organic materials) [1]. Grazing organisms may, but less likely so, kill their prey species. For example, the herbivorous animals usually only feed upon parts of the plants [2]. Parasitic organisms are somewhat similar to grazers in that they typically do not kill their host. Whilst the feeding behavior of these two types of predators is similar in many ways, parasites are more closely associated with their host species. Through this close association, the parasites are capable of extracting nutrients from their host species, which significantly reduces the fitness of the host, but normally does not result directly in the death of the host. Similar to parasitic organisms, parasitoids live in or on their host, which eventually results in the death of the host. Like those grazing and parasitic predators, the parasitoid predators do not cause their host death immediately. However, they are, different from parasites, more resembled to the real predators in that their predatory behavior eventually results in death of their prey [3, 4].

Predation plays significant ecological roles, within ecosystems, where predators are likely to enhance the diversity of populations and communities by hindering a single species from growing predominant. Predation not only impacts prey numbers, but also ultimately impacts the predators when prey numbers decline sufficiently to no longer to be capable of supporting the predator populations. The outcome of such predation is ultimately to establish and maintain a balance of organisms that could be supported by the ecosystem and this process has significant impacts on the evolution of organisms within the ecosystems [5, 6].

The effects of predation in ecosystems are generally based upon broad observations at the macro scale, and there is considerable literature on this subject [7-9]. Those

studies have been the basis for the formulation of a range of theories and models aimed at generally describing how predation influences the ecology of ecosystems. One such model is represented by the Lotka-Volterra equation (Figure 1.1) [10]. This equation elegantly describes the relationship between predator and prey as repeating patterns of oscillating population numbers. At the macro scale, this equation has not only provided a fundamental framework to explain the intertwined relationship between predator and prey, such as carnivorous and herbivorous animals, but also provided a mathematical basis for verifying ecological hypotheses. However, in studies of microbiology, fewer efforts have focused on generating such broad based ecological models. This is primarily for the reason that most microorganisms have been studied as pure monocultures under laboratory conditions, rather than as interacting mixed species communities, which are dominant in the natural environment. Bacteria have been shown to inhabit a vast range of highly diverse natural environments on the planet, and this is largely due to their ability to adapt to various conditions. Like higher organisms, microorganisms are always found in communities composed of diverse species, in which they compete or cooperate with each other to access limited resources. Such interaction and limited-resource pressure are similar to those exerted upon higher organisms. As contemporary studies of microbiology ecology begin to explore the complex interactions between organisms, it is increasingly appealing to draw on and apply ecological theories derived from macro ecology. This has the advantage of providing a framework for developing and testing of hypotheses for microbial ecology. Further, results based on microbial systems, arguably easier to replicate and to establish controls than for higher organisms, can be used to refine existing ecological models. The examination of such models can also be used to determine whether the macro-scale ecological principles/models could be applied equally to the microbial world.

Microorganisms display distinct features that may influence whether the same ecological principles could be applied to bacteria and higher organisms. For example, in the absence of any overt stress, they do not undergo natural death. Furthermore, unlike higher organisms, many bacteria can survive for extended periods even in the absence of nutrients [11, 12]. Whilst there is some evidence of a limited life span in microorganisms, the main factors contributing to microbial mortality are bacterial cell lysis mediated by viruses and grazing by protists [13-15]. The predation effects of viruses (bacteriophages) and protists on microorganisms have been extensively

described, but the effects of bacteria that prey on other bacteria, the bacteriovorous bacteria, are less well understood. Considering that predation, by viruses, protozoa or bacteria, represents the primary factor leading to microbial mortality, it is highly likely that predation has a significant effect in shaping microbial communities, both in natural and engineered environments. Similarly, access to suitable prey is likely to drive population dynamics for these predators.

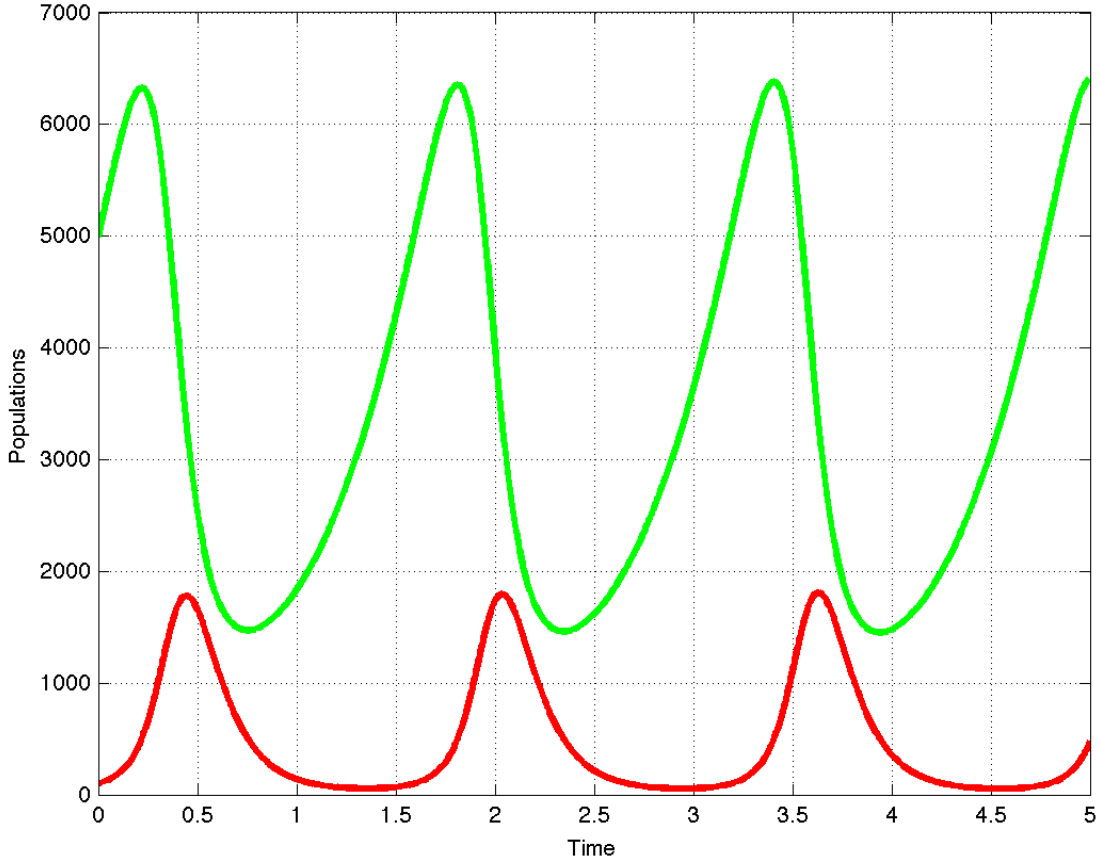


Figure 1.1 Image illustrating the classic predator-prey model based upon the Lotka-Volterra equation. This model demonstrates the interaction between two species (predator-prey), with some inherent assumptions, such as prey populations have adequate food, and food supply for predator is solely dependent upon the prey population. The figure is adopted from [10].

1.2 MICROBIAL PREDATORS

Broadly, bacteria are preyed upon by viruses, protozoa and the group of predatory bacteria defined as *Bdellovibrio*-and-like organisms (BALOs). These groups of predators are found in all habitats where bacteria are present, including wastewater. Their predatory characteristics, presence within wastewater, ecological impacts upon the microbial communities and wastewater, etc., will be presented here.

1.2.1 Virus (bacteriophages)

Viruses are infectious agents, typically about 20 – 200 nm in size with a core containing single or double strand RNA or DNA equipped with a protein cover, and under some circumstances with a lipid envelop. Bacteriophages are the viruses that specifically infect bacteria. Similar to viruses of higher organisms, bacteriophages are strictly intracellular predators having no metabolism of their own, which requires them to rely upon the metabolism of their host cells for proliferation [16].

Through passive diffusion, these predators make contact with the host cells and their adsorption onto and entry into cells is affiliated by specific receptors, usually lipopolysaccharides and proteins, on the host cell's surface. Therefore, bacteriophages are highly specific for their host, which is regulated by the receptors that mediate recognition and interaction [16]. Generally, the host range is narrow, but there are examples of some cyanophages that display relatively broad host ranges within the cyanobacterial group [17, 18].

Bacteriophages are highly abundant in aquatic environments, ranging from 10^4 to 10^8 per milliliter [19], and the numbers of bacteriophages are typically three to ten times higher than their bacterial counterparts [20]. Studies have shown that the addition of bacteriophages to bacterial communities are responsible for 20% – 40% of all bacterial mortality [21, 22]. It has therefore been proposed that aquatic bacteriophages may play a significant role in determining the diversity of microbial communities through controlling the numbers of the selected bacterial species [23].

Some studies have reported that bacteriophages are broadly present within wastewater treatment systems at high abundances and have proposed that the bacteriophages could be utilized as tracers of the occurrence of specific bacteria, especially pathogens [24]. However, the ecological impacts of bacteriophages on microbial communities within wastewater treatment systems are still not well understood. This is particularly reflected in a general scarcity of recognition of the total diversity of bacteriophages present and the correlated bacterial hosts, which is essential for understanding the effect of such bacteriophages on the community species composition [25, 26]. Currently, a limited number of studies have been undertaken and these studies have suggested that bacteriophages may be active constituents of the activated sludge systems. For example, Ewert and Paynter (1980) reported an increased total concentration of bacteriophages during the activated

sludge treatment process, indicating an active proliferation [25]. The total concentration of bacteriophages in the mixed liquors in the activated sludge reactor was significantly higher (4 – 5 times) than those in the influent sewage. They also found that the numbers of bacteriophages detected using direct scanning electron microscopy were much higher than those detected through plaque forming unit (PFU) assays, highlighting the limitation of culture based methodologies for bacteriophage detection.

The observation of high numbers of bacteriophages, reflecting active infection of target bacteria in the wastewater treatment process, also suggests that infectious bacteriophages may influence the numbers of important functional bacterial populations [27]. By manipulating the abundance of essential and functional bacterial groups, bacteriophages may even influence the treatment performance. Several wastewater treatment processes employing activated sludge, such as nitrification and phosphorus removal, are shown to be unstable. The nitrification process could be difficult to maintain because of either the slower growth of the nitrifying bacteria or their inherently low numbers. However, it is also probable that the low abundance of these nitrifying bacteria can be attributed to predation by bacteriophages [28].

Most of the active microorganisms in the activated sludge would be characterized as a suspended biofilm community, for which a key feature is the production of a protective extracellular matrix. Some bacteriophages have been found to produce polysaccharide depolymerase enzymes during attachment of phage to their host cells. These enzymes could break down the bacterial polysaccharide capsules, allowing for the binding of phages to receptors on outer membrane of the host. It has been suggested that the ability of phage to breakdown the bacterial capsule could be exploited to control biofilm formation [29, 30]. Based on this concept, it has been shown that the total amount of microbial EPS after bacteriophage treatment can be significantly reduced. It has been suggested that bacteriophages could be developed for the control of filamentous bacteria and the non-phosphate accumulating bacteria, which may otherwise inhibit nutrient removal. Settlement of activated sludge during the treatment processes is required for the production of clean effluent. Activated sludge bulking and foaming caused by over-reproduction of filamentous organisms is a universal issue in wastewater treatment plants [31, 32]. Growth of filamentous bacteria may yield floccular sludge with poor settling properties causing bulking problems. Furthermore, hydrophobic cell surfaces combined with the overproduction

of EPS would render stable bubbles containing air, water and microbial cells, causing foaming problems [33, 34]. Efforts have been made to isolate specific bacteriophages from sludge samples targeting the filamentous and foaming bacteria, respectively [35]. As for control of the non-phosphate accumulating bacteria, bacteriophage could be developed that only target this group of microorganisms and that avoid non-specific inhibition of the desired community members [28, 36, 37].

The application of bacteriophages in the wastewater treatment process has been broadly investigated for many years, however, there remain some general limitations to the application of bacteriophages in wastewater systems [38, 39]. Host specificity is one such limitation. Studies on the efficacy of bacteriophages in controlling bulking problems or competition from non-phosphate utilizing organisms will necessarily require bacteriophages that target the correct organisms and monitoring of the community composition and function to ensure that non-target species are unaffected. This is particularly important since some polyvalent phages have been shown to exhibit a relatively broad host spectrum [40]. Therefore the application of these bacteriophages should be approached carefully, as they may also infect some beneficial and functional bacteria in the wastewater sludge [35]. The architecture of complex multi-species biofilms could also serve as a refuge for diverse bacteria, where the target species are masked or protected by other microorganisms within the biofilm. Bacteria embedded within complex biofilms have been observed much less vulnerable to phage attack than suspended cells [41, 42]. In addition, the phage-borne enzymes have high specificity, as a result of which, slight changes of EPS structure and composition may inhibit phage infection [43].

An additional challenge to the application of bacteriophages for microbial control applications is that bacterial hosts can rapidly evolve resistance towards phage infection [44]. Long-term infection studies have shown that a stable equilibrium is reached where most bacteria in the population become resistant to the co-inoculated bacteriophages and only a small fraction of bacteria remain sensitive [45]. Studies of bacteriophage infection of activated sludge isolates showed that no more than 20% of dominant bacterial isolates were sensitive to bacteriophages. Thus, this could suggest that these isolates have already evolved resistance, which further implies that they have been recently exposed to the relevant bacteriophages [26, 44]. Therefore, whilst there is significant potential to exploit bacteriophage to improve wastewater treatment systems operation, there remain many hurdles to their full implementation.

This highlights the need for further research to have a better comprehension of bacteriophages and their application for the control of inhibitory bacteria in wastewater treatment systems.

1.2.2 Protozoa

Protozoan predators of bacteria are defined here as unicellular eukaryotic microorganisms, between 2 – 200 μm in size [46]. Based on differences in morphologies, they are typically divided into three groups: ciliates, flagellates and amoebae [47]. These predators, in general, are heterotrophic and graze mainly upon other smaller microorganisms (not only bacteria, but also fungi and algae). For the purpose of this review, only the grazing effect of protozoa upon bacteria and bacterial communities will be discussed.

Protozoan grazing upon free-swimming bacteria has been shown to be primarily driven by size-selectivity of the predators where most predators preferred medium-size bacteria, whilst the efficiency of grazing on smaller (< 0.4 μm) or larger (> 2.4 μm) bacteria was lower [48, 49]. Not surprisingly therefore, such grazing pressure can select for the evolution of changes in the cell morphology of individual bacterial species. For example, studies have shown a partial shift in the cell-size of bacterial populations, from smaller, edible cells to larger grazing-resistant forms [50].

The shifts in cell morphology and size were also observed at the community level. Experimented bacterial communities enriched from activated sludge displayed altered morphologies when exposed to increased protozoan grazing activity. Changes to those bacterial communities were characterized by a shift from suspensions of single cells that were rod-shaped, into long-spiral shaped cells as well as filaments. The elongated cells were shown to be resistant to grazing [51]. Several studies also investigated the impacts of protozoan grazing on the species composition of bacterial communities. Although the results differ between studies, the conclusions were relatively consistent in that the taxonomic compositions were significantly altered as a consequence of protozoan grazing pressure [51-53].

Most studies of protozoan grazing effects have typically focused on aquatic environments. By comparison there are fewer studies on the effects of grazing on wastewater microbial communities. Protozoa have been found in almost all stages of the biological wastewater treatment process [38, 54], suggesting that, as for aquatic

environments, protozoa may also significantly impact wastewater systems. Bacterial communities are the primary organisms involved in wastewater treatment processes, in both their dominance over other groups, as well as their metabolic capability, especially in relation to the removal of organic and inorganic nutrients [55, 56]. Protozoan predators mainly graze upon bacteria present as single cells in suspension, small bacterial aggregates or larger floccular particles that exceed the size limit for grazing by protozoa. Therefore, the flocs are thought to be protective of the bacteria from being grazed, and protozoa may be important in wastewater the treatment process by driving the selection for floc formation, which results in a reduction of suspended bacteria and therefore aids the process of effluent clarification.

Although these predators were initially considered harmful because their grazing effects may remove functional bacterial groups, it was subsequently determined that the presence of protozoa could contribute to the enhanced reduction of BOD_5 and higher-level removal of organic carbon and mixed liquor suspended solids [54, 55]. Later findings indicated that protozoa also excreted mineral nutrients that could enhance organic carbon usage by bacteria. Protozoa have been further demonstrated to excrete growth-stimulating compounds that enhance the activity of bacteria. It has also been suggested that the polymers produced by protozoa may directly contribute to the formation of flocs in wastewater treatment plants [57, 58].

1.2.3 *Bdellovibrio*-and-like organisms (BALOs)

The BALOs are a group of predatory bacteria with similar predation characteristics. The first member of this group to be described belongs to the Genus *Bdellovibrio*, and was first identified in 1962 through experiments aimed at isolating bacteriophages from soil [59]. In the course of their experiments, they observed plaques with morphologies distinct from typically formed by bacteriophages. *Bdellovibrio* are described as bacteria that are small, highly motile with a single sheathed flagellum and an average size of 0.2 – 0.5 × 1.2 – 1.5 μm [60]. Subsequent research determined that the swimming speed of these bacteria could reach 60 – 160 μm/s (or 100× body-length per second). This high motility makes these predators effective at chasing down their prey bacteria [61].

Members of this genus prey upon other Gram-negative bacteria by penetrating into the periplasm of their prey cells. These predators shed their flagellum upon entering

the periplasm where they enter a parasitic phase of their life-cycle feeding on the biopolymers of their prey (host) cells to obtain energy and biomaterials to produce more progeny cells. Because of their predatory properties and life cycle, BALOs are described as predators or parasites analogous to bacteriophages and protozoa [62].

1.2.3.1 Habitat and ecology

BALOs are generally found soils as well as other wet, aerobic environments, and they were also isolated from a wide range of aquatic systems, including estuaries, seacoasts, oceans, rivers, sewage, fishponds, and man-made water supplies [63-65]. Although these bacteria are aerobic, they can also be found in soils, in sediments, on submerged surfaces and in the rhizosphere of plants, indicating that they could also survive and adapt to micro-aerophilic and some anoxic conditions [66]. Therefore, the spectrum of potential niches for BALOs might not be constrained solely to stringently aerobic habitats.

BALOs have also been found associated with biofilms on various surfaces (refer to section '1.3 Biofilms' for detailed information about biofilms). Biofilms in natural environments are typically composed of high-density microbial communities and this feature may favor predation by *Bdellovibrio* spp. given their dependence on high numbers of prey bacteria for growth [67]. It had been shown that biofilms formed by *Escherichia coli* on the surface of stainless steel were effectively removed by exposure to BALOs infection [68]. In some instances, BALOs have been isolated from marine biofilms but have not been constantly detectable in the proximate water suggesting that biofilms may provide BALOs with relatively stable conditions and sufficient prey numbers that support their growth [66, 69]. Within biofilms, BALOs are likely to be entrapped in the gel-like matrix produced by biofilms, where they benefit from the high concentration of potential prey cells, resulting in an enhanced proliferation and physical protection. For example, surface-associated BALOs have been shown to withstand and survive chemical stresses, such as phenol and urea, whilst their free-swimming counterparts were rapidly killed [70].

The high cell density of biofilms has been shown to enhance the survival of BALOs in the environment. Various studies have addressed that prey cell concentrations of at least $10^5 – 10^6$ CFU/mL are required for BALOs survival [71]. Calculations based on the Lotka-Volterra equation have indicated that no less than 3×10^6 prey cells were necessary to provide the *Bdellovibrio* spp. approximately a 50% chance to survive

[72]. Because *Bdellovibrio* spp. are not stringently host-range specific, survival of these predators may be successful in natural habitats because the total numbers of bacteria are sufficient to sustain BALOs' growth. It has been found that 70% to 85% of the bacterial isolates recovered from estuarine environments were susceptible to BALOs isolated from those sites. Considering that only a small portion of total bacteria (around 1% to 10%) could be successfully recovered and cultivated, the numbers and species of the bacterial prey may be adequate to sustain BALOs [73].

One thing should be noted that the least prey cell density calculation was based on experiments where the BALOs preyed upon defined bacteria species in pure culture [68]. Thus these minimum numbers of cells necessary to support BALOs applied to the number of potential prey species within the community and not the total number of bacteria present.

The requirement for a minimum cell density is partly a consequence of the mechanism by which they acquire prey [74]. BALOs spend a large amount of energy to maintain their high motility and rapidly lose viability if they do not encounter suitable prey cells. Whilst these predators rapidly run out of energy in the absence of prey, there is no clear evidence on how fast they lose viability in the absence of suitable prey species [75, 76]. However, there is evidence that they could survive under low-nutrient conditions suggesting they either have the capacity to take up and utilize nutrients at a low level or that they have a starvation survival program, analogous to other heterotrophic bacteria. One additional mechanism of survival is the formation of an inactive growth phase, termed as 'bdelloplast' (this will be described in detail in below section) [77].

1.2.3.2 General isolation of *Bdellovibrio* spp.

Bdellovibrio spp. are generally isolated using methods similar to those used to isolate bacteriophages. The samples, are blended with a selected prey bacterial species in melted soft agar (0.5%) and plated onto the top of an already prepared plate composed of a diluted growth medium. The bacterial predators form plaques that will be different from those formed by protozoa and bacteriophages based on the size and their expansion over time [65]. Usually, BALOs can be found at relatively low abundances in most environmental sources and their isolation can be quite difficult. For example, plaque formation can be obscured by over growth of fast growing

bacteria or the BALOs may be inhibited by metabolites of the potential prey bacteria. To overcome some of these limitations, protocols based on the differential separating BALOs from the rest of microorganisms in samples have been developed so that the BALOs cells could be enriched to a relatively high level, reducing unwanted protozoa, bacteria and viruses [78].

The isolation of BALOs also depends on the choice of prey bacteria, the pre-treatment of samples and the specific protocol used. It has been shown that most *Bdellovibrio* spp. have preferential prey ranges and no single bacterium is ideal to support the growth of all *Bdellovibrio* spp. [65]. Nevertheless, some bacterial species have been used for the general isolation of BALOs, such as *Vibrio parahaemolyticus* for estuarine samples, whilst *Pseudomonas* spp., *Aquaspirillum serpens* or *Pseudomonas syringae* for soil and water samples [79, 80]. After isolation, the BALOs are confirmed by examination under phase-contrast microscopy for small, fast moving bacteria at speeds up to 100 times of body-length per second.

1.2.3.3 Life cycle of *Bdellovibrio* spp.

As stated above, the BALOs in attack-phase growth would rapidly lose activity and viability if they cannot successfully find and attach to prey cells. Therefore, the process of prey identification and penetration is crucial [76]. The mechanisms of how these predators find their prey remains unclear and no chemotactic responses to potential preys, excretion products or lysates of preys have been identified [81]. The results from chemotaxis experiments testing various compounds, such as carbohydrates and amino acids, have generated conflicting results without a clear demonstration of a chemotaxis response among different *Bdellovibrio* strains. However, all of the tested strains have shown aerotaxis [82, 83]. It is thus generally thought that the aerotaxis coupled to limited chemotaxis towards chemical signals/clues (such as amino acids, carbohydrates etc.) enable the BALOs to find optimal niches, however it is not understood how this aids in tracking down prey bacteria [83].

In the absence of clear evidence of chemotaxis as a mechanism to discover prey species, it appears that the BALOs rely upon random collision to find their food, and therefore the concentration both of predator and prey are important for successful predation [72]. If this random process is important for finding prey cells, then flagellum-based activity is also important in encountering prey cells. *Bdellovibrio*

mutants with a single mutation in each of the *fliC* flagella genes have been created, and those mutants were slower in swimming speed and less efficient in predatory action on *E. coli* in liquid culture. Moreover, when all of the *filC* genes were interrupted, the mutant was still predatory when directly applied onto prey lawns on agar plates, but could not survive in liquid culture. Therefore, flagella activity is required in encountering prey cells, but not required for entry into prey cells [84, 85].

The unique life cycle of these predators has been demonstrated using the model strain *Bdellovibrio bacteriovorus* HD100 (Figure 1.2). The free-swimming, attack-phase *Bdellovibrio* cells first randomly collide with the prey cells (Figure 1.2 A). Attachment occurs after colliding with the prey cell and is reversible in the first few minutes, but becomes irreversible after 20 to 30 min [86] (Figure 1.2 B). Although the recognition sites on the surface of prey cells towards BALOs cells remains uncharacterized, it was found that some components were required by different *Bdellovibrio* strains for irreversible attachment. For example, to irreversibly attach to prey cells, *Bdellovibrio bacteriovorus* 109D interacted with specific core sugar of the prey cell's lipopolysaccharide, whilst strain *Bdellovibrio stolpii* UKi2 required particular surface proteins of the prey cells [87, 88].

After irreversible attachment, penetration occurs within 20 min (Figure 1.2 C). It is believed that penetration is achieved by the combination of physical squeezing ('drilling') at the non-flagellum end of the BALOs cell and the secretion of lytic enzymes [86]. The mechanism for entry remains unclear, however, it was proposed that, upon prey infection, the surrounding liquid would enter the infected prey, causing differential expansion or swelling of the prey cytoplasm and cell wall, which would cause their separation. During this process, the BALOs cell, which has already attached to the cytoplasmic membrane of prey, will be passively dragged into the periplasm, shedding the flagellum [89].

Once penetration is complete, the *Bdellovibrio* spp. initiates growth within the periplasm (Figure 1.2 D). This phase is characterized by *Bdellovibrio* cell elongation into a filamentous cell (Figure 1.2 E). During growth in the periplasm, the predator and prey cell together form a structure termed as the 'bdelloplast' [90, 91]. The bdelloplast has been found to be more withstanding than the vegetative counterparts to a number of stresses, such as desiccation, high temperature, sonication, as well as attacks from other microbial predators [85]. The predator within the bdelloplast then

synthesizes large amounts of a variety of hydrolytic enzymes, such as nucleases and proteases etc., to degrade all of the contents of the prey cell.

After contents of prey cell have been exhausted, the elongated filamentous-like predator cell divides into several progeny cells, each of which will synthesize a new flagellum (Figure 1.2 F). The envelop of the prey cell is then broken down by hydrolytic enzymes produced by the predator, releasing the new progeny to restart their life-cycle (Figure 1.2 G). The numbers of progeny yielded are simultaneously proportional to the size of the prey cells [92]. For example, 4 – 6 progeny cells are produced when *E. coli* is used as the prey [93], whilst 20 – 30 progeny cells are yielded when *Aquaspirillum serpens* is used as the prey [94]. Under laboratory conditions, in planktonic culture, the life cycle usually is finished within 2 to 4 h [94]. Whilst *Bdellovibrio* spp. are obligate predators that are dependent on prey for growth, a host-independent (HI) growth phase of *Bdellovibrio* spp. was discovered where the predator could obtain energy and nutrients by heterotrophic growth on media constituents (Figure 1.2 H). In an actively growing population of *Bdellovibrio* this mutation occurs at a rate of approximately 10^{-6} to 10^{-8}. The HI growth form is thus considered to be the result of a single mutation, although the specific mutation has not been identified. Interestingly, *Bdellovibrio* cells could enter HI growth if high concentrations of amino acids and cofactors are present, given that cells tend to lose the HI growth ability, the phenotype may be unstable or rapidly selected again [95].

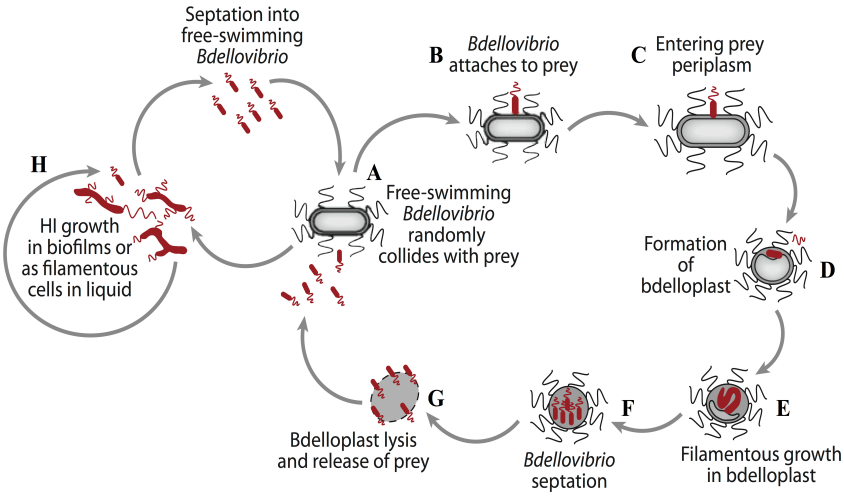


Figure 1.2 Host-dependent and host-independent (HI) life cycles of *Bdellovibrio bacteriovorus*. The life cycle on the right shows different stages of growth of host-dependent bdellovibrios. Whilst the life cycle on the left shows the HI, axenic, replication life cycle. Figure reproduced from [95].

1.2.3.4 Fundamental understanding based on genome sequence analysis

To date, several *Bdellovibrio* strains have been studied and their genomes have been sequenced. One of the best-studied model strains is *B. bacteriovorus* HD100, the entire genome of which has been completely annotated and published [85]. The genome of this strain is fairly large, approximately 4 Mbp, and lacks any plasmids or phage-derived elements (BX842601, http://www.cbs.dtu.dk/services/GenomeAtlas/). Also it has almost no repeat elements, which contribute to diversity in many other bacterial species [96]. Moreover, there is little evidence suggestive of horizontal gene transfer from prey to predator, although prey and predator are closely associated during predator's periplasmic growth. Nine RNases and twenty DNases have been detected in the genome sequence, inferring that prey nucleic acid would be degraded rapidly into nucleotides for use as building blocks for *Bdellovibrio*'s DNA synthesis, rather than the uptake of whole genes for horizontal gene transfer. The enzyme pool is additionally supplemented with approximately 15 genes encoding lipases, 10 encoding glycanases, 150 encoding proteases, and around 90 encoding other hydrolytic enzymes [85]. Therefore, the *Bdellovibrio* genome encodes a significantly higher numbers of hydrolytic enzymes relative to other bacteria. For example, *E. coli* (MG1655, K12) encodes three DNases, eight RNases, two lipases as well as about 60 proteases. This high numbers of enzymes is congruent with the predatory properties of the *Bdellovibrio*, where they are used for the total degradation of prey cell to provide precursors for the growth of the predator.

In addition, the genome contains multiple sets of gene encoding surface proteins, such as pili, flagella and outer membrane proteins. Compared with a non-predatory bacterium, such as *E. coli* K12, where *E. coli* K12 has one gene set encoding flagella, *Bdellovibrio* has six sets; where *E. coli* K12 has one pair of genes encoding the flagella motor, *Bdellovibrio* has three pairs. The excess gene products associated with motility and surface proteins may highlight the importance of surface proteins, such as pili and flagella, for *Bdellovibrio* to be successful as a predatory bacterium [97, 98]. Type IV pili systems have first been described in relation to their role in twitching and gliding motility in some Gram-negative and Gram-positive bacterial species, such as *Myxococcus*, *Neisseria* and *Pseudomonas* [99, 100]. In addition to their role in motility, type IV pili also have been shown to be essential for adherence and invasion, gliding motility, intracellular interactions, DNA uptake, as well as biofilm formation [101]. The occurrence of multiple gene sets encoding type IV pili has led

to speculation that *Bdellovibrio* may likely use pili to facilitate the entry into prey cells, probably by adherence to the cell wall or for the subsequent process of entry into the periplasm of the prey. Electron microscopy images of *Bdellovibrio* during attachment to the pore formed on outer membrane of prey cells have shown that the pore is smaller that the invading *Bdellovibrio* cell, which infers that, by squeezing into the prey, a great force would be required [102]. Type IV pili can generate retractile force as great as more than 100 pN [103], which would provide *Bdellovibrio* cell with sufficient force to facilitate entry into prey cells. This is supported by experiments where type IV pili mutants were unable to enter the prey cells [102].

1.2.3.5 Genetic relatives

Bdellovibrio isolates were initially considered to assign to the unique genus *Bdellovibrio*, which was assigned to the Delta-*Proteobacteria* group based on 16S rRNA sequence similarity. However, subsequent reports have identified isolates outside this group that are capable of predation and now includes members within the Delta-*Proteobacteria* order *Bdellovibrionales*, as well as within the Alpha-*Proteobacteria* subdivision, *Micavibrio* spp. [104]. *Micavibrio* spp. are a group of recently characterized predatory bacteria that are not only phylogenetically different from the delta bdellovibrios, but they also exhibit physiological differences. They have a single, non-sheathed polar flagellum and shows an epibiotic predatory action. Instead of entering into the periplasm of the prey cells, these predatory bacteria only attach to the surface of prey cells to uptake the nutrient resources from the prey. When the nutrients of the prey cell are completely exhausted, the predator would divide by binary fission and seek out new prey cells. One model species of this group is *Micavibrio aeruginosavorus* ARL-13, which yields two progeny cells after the epibiotic growth life cycle upon a prey cell [104].

1.2.4 Defense strategies by bacterial prey species against microbial predators

Predation imposes a significant selection pressure on the prey species and thus exerts a strong evolutionary selection for the development of resistance. Most bacteriophages have narrow prey ranges, targeting specific bacterial species. This specificity is often mediated by recognition of specific receptors on the prey cells' surfaces, followed by attachment of viral particles to the prey cell surface. Prey

species could thus avoid being infected by bacteriophages via modification of the surface receptors through mutation. This would result in the bacteriophage not being able to recognize the bacterial host and hence protects the bacteria.

Protozoan predators engulf their prey cells and digest them intracellularly in a food vacuole. Previous studies have already shown that bacteria can avoid predation through a range of strategies including cell-surface modification, production of toxic metabolites, increased swimming speed, elongation, aggregate formation and the ability to survive in the food vacuole to avoid being digested [105]. Given that the bacteria have shown the ability to generate mutations that allow them to avoid two of the key predators, it is also possible they can use similar strategies to avoid predation by the BALOs. However, few studies to date have reported any effective defense strategies developed by bacterial prey species evading attack by *Bdellovibrio* spp.

One possible defensive strategy may be the formation of matrix encased multi-cellular structures, called biofilms, which are more likely to survive predation. Some studies have demonstrated that biofilms are protected from predation by some protozoa. Biofilms in natural environments are often comprised of a mixture of diverse species and it has also been demonstrated that multi-species biofilms display enhanced resistance relative to single species biofilm. Therefore, formation of biofilms is thought to be an effective strategy protecting bacteria from predation.

1.3 BIOFILMS

The conventional view is that bacteria grow exclusively as unicellular organisms in suspension. As such, their characteristics and physiology have broadly been studied using planktonic cultures of single species populations. However, direct observation of various natural habitats has revealed that microorganisms primarily co-exist in mixed communities, attached to surfaces, embedded into a self-produced matrix, to form biofilms [106, 107]. Biofilms could also be surface independent, where they are present as suspended aggregates, such as floccular and granular sludges formed at different stages during the wastewater treatment process [107]. Biofilms have also been observed in a variety of environments, such as dental plaque, surfaces of river stones, sticky coatings on boat hulls and as activated sludge in wastewater treatment systems [107]. The microorganisms within biofilms produce extracellular polymeric substances, which make up the cohesive matrix and also serves to protect the bacteria

from a number of stresses, such as high temperature, ultra-violet radiation, extreme pH value, nutrient limitation and protection from other microbes [108, 109]. Given their predominance in the environment, there is an increasing interest in investigating the molecular mechanisms involved in the formation and maintenance of these communities, as well as approaches that could be employed to control and modulate biofilms [110].

1.3.1 Biofilm communities and structure

The bacterial communities in natural environments are essential for many biogeochemical processes, such as biodegradation of organic matter, recycling of sulfur, nitrogen and the reduction of heavy metals [111]. Studies on bioreactors have demonstrated that biofilms are the key drivers of the remediation of wastewater and groundwater pollution, as well as being responsible for nitrification and the removal of phosphate [112, 113]. Biofilms have also been found in extreme environments, such as acid mine drainage [114], thermal springs [115, 116] and the Antarctic [117].

In up-flow anaerobic sludge reactors, bacteria usually form floating aggregates of different sizes (floccular or granular sludges) composed of complex microbial communities [118]. Briefly, the formation of this biofilm is favored by degradation of organic matter into carbon dioxide and methane [119, 120]. From an engineering perspective, these floccular or granular aggregates are desirable since their density is greater than water resulting in their spontaneous sedimentation, which facilitates separation of biomass from the clean water. The biological process that drives the formation of these high-density granules is not clear. One hypothesis is that granules may form in response to strong predation pressure, whereby the granule community is physically less accessible to the predators.

1.3.2 Biofilms as protective niche

Bacteria form biofilms in almost all habitats and there are several hypotheses to explain this phenomenon. The most prevalent explanation is that biofilms provide the bacteria with protection from stresses within the environment. Indeed, it has been demonstrated that biofilms are significantly more resilient to chemical stresses, such as antibiotics, than their planktonic counterparts. This protective feature is partly due to production of extracellular polymeric substances matrix, which is comprised of

mixed components, such as polysaccharides, nucleic acids, protein and other substances [109]. EPS plays a variety of roles, from structural to functional in different microbial communities.

The EPS is clearly an integral and vital component for the structural organization of biofilms. Viega [121] found that the polysaccharides generated by two species, *Methanosarcina mazeii* and *Methanobacterium formicium* isolated from a granulating anaerobic bioreactor, had a similar composition as the EPS extracted directly from the granules. It was suggested that EPS produced by these two methanogens were the main contributors to the polymers found in mature granules. In addition, it was proposed that EPS produced by these two organisms could serve as the polymeric backbone that other bacteria use for subsequent incorporation into the growing aggregate. This sequential development can be a strategy for a community with physiologically diverse organisms to cooperate. For example, the interior of granules may be anaerobic as a consequence of respiration by bacteria on the exterior of the granule surface. Thus, anaerobes could be highly active in the interior and such organisms are required for the nutrient removal process. In this case, the EPS functions both in the formation of granules and as a protective barrier for microbes within the core of the granules [122]. EPS may also be protective by preventing the entry of various antibiotics into biofilm by physically binding to such compounds. The effectiveness and efficiency of this function is largely decided by the nature of both antibiotic agent and matrix [123]. In addition, EPS matrix has been found to sequester metal ions, cations and toxins [124]. EPS could also function as a defensive barrier against predation. For example, a mucoid isolate of *P. aeruginosa* was more resistant to grazing by amoeba than the non-mucoid counterpart [125].

1.3.3 Mixed-species biofilms

Natural environments are typically complex and dynamic in contrast to laboratory-based conditions. Biofilm communities associated with surfaces in natural environments, such as the plant rhizosphere, the oral cavity and many other natural settings, are occupied by diverse microbial species in close proximity. Studies have shown that active interactions between species are needed for the establishment of mixed-species biofilms. For example, *Streptococcus mutans* functions as the starting colonizer attached to the surface of tooth along with *Actinomyces* species to facilitate

subsequent colonization by *Lactobacillus* species [126], all of which together promote formation of mixed-species biofilms. Alternatively, when *Streptococcus gordonii* is the first colonizer, the interspecies interactions change and favor growth of the pathogens *Porphyromonas gingivalis* resulting in oral disease.

Another example of synergistic interactions in mixed-species biofilm is cooperation. Biofilms comprised of *Acinetobacter* and *Pseudomonas putida* were shown to change in spatial organization depending on the carbon source available. When the biofilms comprised of these two species were subjected to benzyl alcohol, *Acinetobacter* first generates benzoate, which could then be used by *P. putida* [127]. Cooperative interactions within mixed-species biofilms are well illustrated in bioremediation and biodegradation processes such as denitrification by the combined activities of *Nitrosomonas* and *Nitrobacter* species [128, 129]. Often, these cooperative interactions are achieved through successive biological reactions from diverse bacteria with a close spatial location in biofilms [130].

Synergistic interactions among microbial communities have also been documented, for example, increased resistance to antimicrobial agents [131]. For example, it was demonstrated that in a mixed-species biofilm comprised of *Candida albicans* and *Staphylococcus epidermidis*, extracellular polymers produced by *S. epidermidis* could prevent the infiltration of the antimicrobial drug fluconazole whilst *C. albicans* could defend *S. epidermidis* from vancomycin [132].

The mixed microbial communities could cooperate forming biofilms to better protect community members from hostile environmental conditions. Besides protecting from biochemical and physical hostile environments, it is also possible that mixed species biofilms also show increased resistance to predation relative to mono-species systems.

1.4 WASTEWATER TREATMENT

The continued rapid expansion of the human population, especially associated with increased urbanization, results in an ever-growing demand for clean water and to recycle used wastewater. One of the main techniques used to clean wastewater is the activated sludge treatment process [133]. As early as the 1900s, wastewater treatment focused on the removal of pathogenic microorganisms to prevent waterborne diseases, however after half a century, the focus switched to prevent eutrophication, through the removal of nutrients, such as nitrogen and phosphorus [134].

Activated sludge is usually comprised of numerous and diverse categories of microorganisms, such as bacteria, viruses (bacteriophages), eukaryotes, as well as some rotifers. Among these microorganisms, bacteria are the most dominant in numbers, and also the most important in terms of function (nitrogen, phosphorous removal, etc.) due to their versatile metabolic activities [135-137]. Specific bacterial communities have been correlated with certain processes of wastewater treatment and therefore, an efficient wastewater treatment system is highly dependent on the presence of these key microorganisms [129, 138-140].

1.4.1 Important bacteria in the wastewater treatment process

Specific bacterial groups are important to wastewater treatment process as they carry out important biological removal processes, such as reducing the organic matter as well as removal of nitrogen and phosphorus. The most frequently detected bacteria in wastewater treatment systems are from the classes of Alpha-, Beta-, and Gamma-*Proteobacteria*, as well as *Actinobacteria* and *Bacteroidetes* [134]. Organic matter in wastewater, such as proteins, polysaccharides and fats, are mainly degraded by heterotrophic bacteria under aerobic conditions, which generates ammonia, carbon dioxide and new biomass. Whilst, under anaerobic conditions, Achaea species would partially oxidize organic matter to produce methane and carbon dioxide [129, 130, 141].

Biological removal of nitrogen, more important and complex than carbon recycling, is carried out by combined processes of nitrification, oxidizing ammonia to nitrate, denitrification and the transferring nitrite to nitrogen gas. Nitrifying bacteria are chemolithotrophic, using inorganic nitrogen as electron donors. Ammonia oxidizing bacteria, such as *Nitrosomonas* and *Nitrosospira*, will transform ammonia to nitrite. Whilst nitrite oxidizing bacteria, such as *Nitrobacter*, *Nitrospira* and *Nitrospina*, will subsequently convert nitrite to nitrate [135, 142]. The denitrification step is to reduce nitrates into nitrogen gas, therefore releasing nitrogen from the wastewater. The denitrification pathway is widely spread amongst different heterotrophic bacteria, which makes it hard to confirm the crucial bacteria species crucial for in situ denitrification during wastewater treatment process and also implies there may be some physiological redundancy in the system. Based on culture-dependent methods, species of *Pseudomonas*, *Acinetobacter*, *Paracoccus*, *Bacillus* and *Hyphomicrobium*

are generally considered to be the primary denitrifying groups in wastewater treatment plants [137, 141, 142]. Thus, if one organism is removed from the system, denitrification can still be achieved through the metabolism of any of these other species.

Phosphorus removal is mainly completed by intracellular polyphosphate accumulation and partially through bacteria uptake for cell growth. Those bacteria capable of most efficiently removing phosphate are known as polyphosphate accumulating organisms (PAOs). By switching between aerobic and anaerobic conditions, PAOs uptake of phosphorus. PAOs uptake phosphorus under aerobic conditions, and after the aerobic step, the phosphorus would be simultaneously removed from the wastewater system along with the removal of the biomass,. A limited number of bacterial species, such as *Acinetobacter calcoaceticus*, *Acinetobacter iwoffi* and *Aeromonas hydrophila*, have been demonstrated to enhance phosphorus uptake under aerobic conditions [141, 142].

Many of the key functional bacterial groups responsible for the efficient function of the wastewater treatment process belong to Alpha-, Gamma-*Proteobacteria* and *Bacteroidetes*. Studies have shown that prey species of *Bdellovibrio*-and-like predators mainly belong to *Proteobacteria*, such as *Pseudomonas*, *Acinetobacter* and *Aeromonas*. Therefore, it is possible that those important bacterial communities could also be sensitive to predation by BALOs. Given that these bacteria may be sensitive to predation by BALOs, it is also highly possible that predation could eventually deteriorate the functions of wastewater treatment process, resulting in significantly increased public health risk and economic loss.

1.4.2 Improved wastewater treatment using granular biomass

The influent into wastewater treatment systems is a mixture of various planktonic microorganisms and suspended solid particles usually termed as flocs. Before wastewater can be discharged, the biomass must be separated from the clean water. Floc formation is thus important in this process as the floccular biomass or activated sludge has better settling property, which would allow for settling to the bottom of the wastewater tank. Once settling is complete, the clean water can be decanted and discharged. However, due to their relatively small size, the floccular sludge typically requires long settling time and this step represents a current bottleneck in the water

recycling process. Further, the settling tanks are quite large and constitute a significant capital expenditure for the plant.

An alternative to floccular sludge has recently been described, called granular sludge or aerobic granules [143]. Granular sludge was first addressed in anaerobic wastewater treatment systems in the 1980s [144], and in the 1990s studies had reported the development and application of aerobic granules [145-147]. A number of studies have demonstrated the efficiency and effectiveness of granular sludge for water purification, and the utilization of aerobic granular sludge is considered to be one of the most favorable new technologies in wastewater reclamation [148-150]. Some unique properties of granular sludge have been described, such as excellent settling ability, dense microbial communities, high retention on biomass, and increased ability to resist various chemical stresses [151-153].

Aerobic granules have shown great efficiency in wastewater treatment with high concentrations of organic materials, and they have also been demonstrated to successfully degrade toxic organic compounds, such as phenol and pyridine in wastewater. In addition, aerobic granules could be used for the dairy industry wastewater processing with high efficiencies in removing total COD, total nitrogen and phosphorous [154]. Biological removal of organic materials, nitrogen and phosphorous via aerobic granules had also been investigated. Nitrification and COD removal rates as high as over 95% were reported, because of the co-existence of heterotrophic, nitrifying and denitrifying bacterial population as well as cooperative metabolic activities within the aerobic granules [152]. Granular sludge is also effective in the removal of phosphorus [155], and can act as bio-adsorbents for heavy metal ions in wastewater. It had been observed that highly toxic heavy metal ions like zinc (II) and copper (II) were removed from wastewater by bio-adsorption of granules [156, 157].

The microbial communities in aerobic granules contain the basic and functional bacterial groups necessary for waster remediation, such as nitrifying, denitrifying, phosphorous accumulating bacteria as well as glycogen accumulating bacteria. Studies on the microbial taxonomic diversity have figured out that the *Proteobacteria* members are the dominant populations, which could also be indicative that this clade of bacteria would be affected by predation of BALOs, even when grown in the form of granular sludge [158-161].

1.5 AIM OF THIS STUDY

Species of *Bdellovibrio*-and-like organisms (BALOs) are ubiquitous in engineered and natural environments, including a wide range of aquatic systems, the bulk soil, the rhizosphere, extreme environments and wastewater treatment plants. Due to their predatory nature, these bacteria may play important roles in modifying microbial communities and influencing their associated functions. Further, given that bacteria predominantly occur as biofilms, the BALOs may significantly impact biofilm formation by bacteria.

Wastewater is a unique man-made environment, containing various organic materials as well as high concentrations of nutrients from different sources. In this unique system, diverse bacterial communities develop into highly complex, structured networks to optimize carbon and nutrient utilization. Within the networks, predation by different microbial predators could influence the microbial communities and potentially deteriorate specific functions, such as nutrient removal process to collapse. In wastewater, bacteriophages and protozoa have been extensively studied with a focus on their impacts on system performance. However, fewer studies have focused on the role of BALOs in modifying the microbial communities and influencing their functions in wastewater treatment systems. Therefore, the aim of this study was to investigate the existence and functions of BALOs, as well as their predatory effects upon the microbial communities in wastewater. This aim was addressed through the following specific objectives:

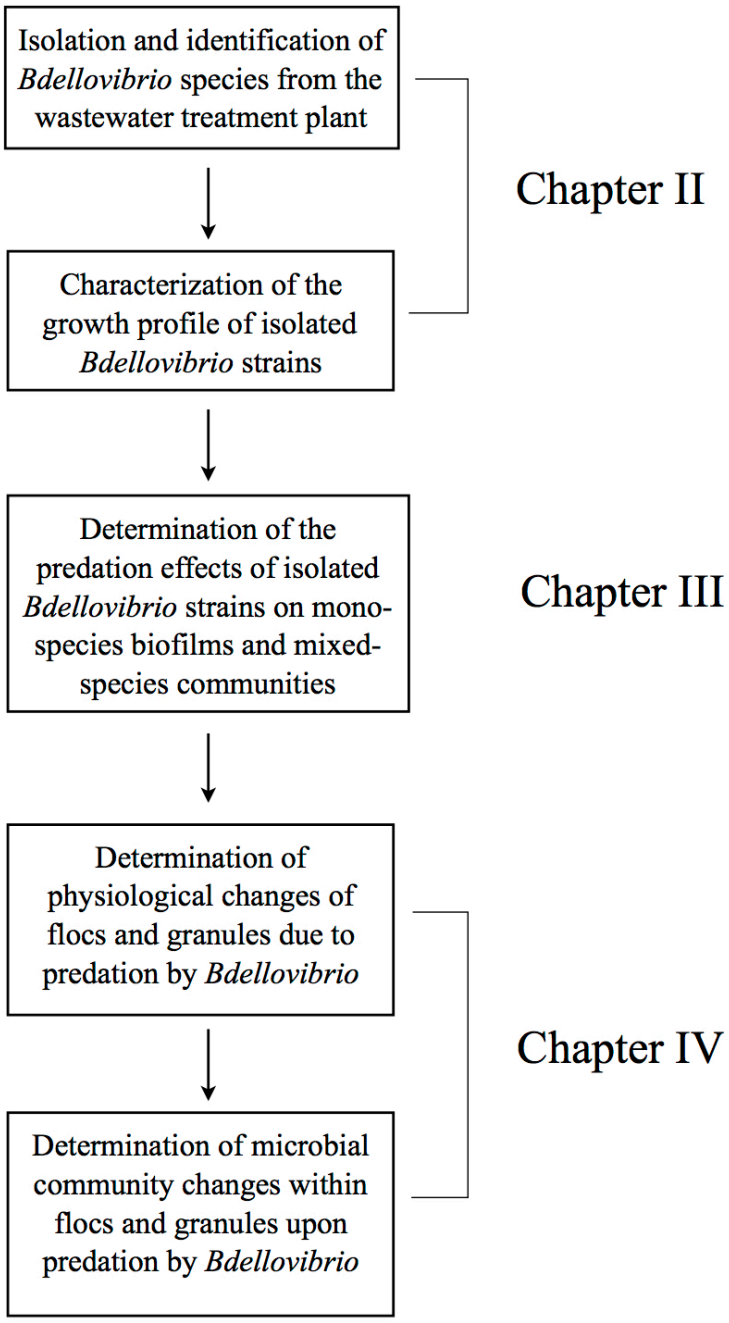
A. Determine the prevalence of BALOs in a wastewater treatment system, identify those BALOs isolates and to characterize their predatory properties including studies of growth rates and prey range.

B. Characterize the ability of these predators to prey upon bacterial biofilms, either in single species populations or mixed species communities and to determine if they exhibit prey species specificity in mixed species communities.

C. To address the predatory effects of BALOs isolates on microbial communities in floccular and granular sludge.

1.6 WORKFLOW OF THIS STUDY

According to the aims addressed above, the workflow of this study was briefly shown as follows.


Isolation and identification of *Bdellovibrio* species from the wastewater treatment plant
Characterization of the growth profile of isolated *Bdellovibrio* strains
Chapter II
Determination of the predation effects of isolated *Bdellovibrio* strains on mono-species biofilms and mixed-species communities
Chapter III
Determination of physiological changes of flocs and granules due to predation by *Bdellovibrio*
Determination of microbial community changes within flocs and granules upon predation by *Bdellovibrio*
Chapter IV

Chapter II. ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF *BDELLOVIBRIO*-AND-LIKE ORGANISMS FROM WASTEWATER

2.1 INTRODUCTION

Bdellovibrio-and-like organisms (BALOs) have been isolated from almost every habitat investigated, including various natural and man-made systems [63, 64]. Their ubiquitous presence and unique predation characteristics suggest that they can greatly affect the species composition and related functions of the microbial communities within those habitats [65]. Whilst they have significant potential to impact microbial communities, the microbial ecology of these predators, as well as their impacts on community functions and diversity, have not been systematically studied since their first isolation and identification. This is in part because their small sizes and fast swimming speeds make them difficult to observe by traditional microscopy and they typically do not grow as colonies on agar plates due to their dependence on prey bacteria for nutrients. They are generally suggested to be present in limited numbers in the various habitats studied and the BALOs were originally thought to be restricted to a single domain of the *Proteobacteria*, the Delta-*Proteobacteria*. Even when BALOs are the focus of investigation, few of those studies provide quantitative descriptions of the numbers of predatory bacteria present and this is further confounded by the recent description of a novel clade of predators that belongs to the Alpha-*Proteobacteria*, suggesting that the diversity of this group of predators may be higher than originally considered [104]. Interestingly, the mechanisms of predation by these two groups of different isolates are distinct [87, 104]. Additionally, these two groups of BALOs can be readily identified through 16S rRNA gene sequencing or through fluorescence in situ hybridization (FISH), and whilst the methods are quantitative, they are not often used to directly determine the cell numbers of BALOs in natural and engineered habitats. Therefore, the population diversity and abundance of these predators may be substantially underestimated as a consequence of their limited study.

Whilst, BALOs prey on a broad spectrum of bacteria, including *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas* spp. [162], isolates tend to display some prey preference and thus no individual prey species is able to sustain the growth of all *Bdellovibrio* species [163, 164]. Therefore, studies of BALOs tend to use a range of potential prey species for the isolation of predators and these include *Vibrio parahaemolyticus* for *Bdellovibrio* isolates from estuarine environments, whilst *Pseudomonas* spp., *Aquaspirillum serpens*, *Erwinia carotovora*, *Erwinia amylovora*, *E. coli*, *Xanthomonas oryzae* have been used for isolation from soil and freshwater samples [89]. Studies comparing the prey range of model predators both of Alpha-BALOs (*M. aeruginosavorus*) and Delta-BALOs (*B. bacteriovorus* 109J) have shown that these two predators both were capable of attacking bacteria from the genera *Acinetobacter*, *Aeromonas*, *Escherichia*, *Klebsiella*, *Pseudomonas* and *Vibrio*, etc. [162]. Additionally, there are no published studies of BALOs demonstrating that these predators can feed on Gram-positive bacteria or fungal species, and this may be due to the fundamental differences in the arrangements of the outer membrane or the cell structures of these microorganisms and the requirement for the BALOs to access the periplasmic space to establish the 'bdelloplast'.

Due to their dependence on prey bacteria and the limited numbers of progeny produced per prey cells [165] (for example, 4 – 6 progeny cells are produced when *E. coli* is used as the prey, whilst 20 – 30 progeny cells are yielded when *A. serpens* is used as the prey), the change in population density of the BALOs and their prey species in planktonic culture tend to reflect the well-defined Lotka-Volterra equation, which has been universally used to express predator-prey interactions for higher organisms [10]. This equation is mainly used to describe the predator-prey interactions in simplified systems, where a predator has a limited prey range or conversely where the prey have few predators. In the case of bacteria, this relationship might not be suitable to demonstrate the BALOs-prey interactions in far more complex systems, such as activated sludges in wastewater treatment systems, which are composed of highly diverse, dense microbial communities where several potential prey species will be present. Whilst the changes in BALOs populations in wastewater treatment systems have not been well defined, the broad prey spectrum of these predators suggest that they can significantly impact the species that are important for the wastewater remediation process. For example, the nitrite oxidizing bacteria in activated sludge, such as *Nitrospira* and *Nitrospina*, have been shown to

be sensitive to predation by the Alpha-proteobacterial predator *Micavibrio* spp. [166]; whilst denitrifying bacteria, such as species of *Pseudomonas* and *Acinetobacter*, were subjected to the predation by *Bdellovibrio* spp. [162, 164].

In this study, representative BALOs isolates were collected from the activated sludge from Ulu Pandan Wastewater Reclamation Plant. Their growth pattern (growth curve) was investigated along with their prey range. This was achieved by using fluorescence in situ hybridization (FISH) combined with confocal microscopy examination. The specific growth curves of the BALOs were determined using model prey bacteria to identify the peak numbers of the predators during the growth cycle. The prey range of the predators was determined using a range of model laboratory bacterial strains and were identified by a combination of FISH as well as by sequencing on 16S rRNA gene amplified by PCR.

2.2 MATERIALS AND METHODS

2.2.1 Sampling description

The activated sludge samples were collected from the aeration tanks of the membrane bioreactor (MBR) systems located at the Ulu Pandan Wastewater Reclamation Plant, Singapore (Figure 2.1 A). Sludge samples were shaken vigorously to disrupt the floccular biomass and 200 mL of sludge was segregated into four separate 50 mL test tubes (Falcon® Corning Inc.), which were kept on ice for transportation back to the laboratory (Figure 2.1 B). Twenty milliliters of the evenly shaken samples was used for the isolation of BALOs isolates.

2.2.2 Bacterial strains utilized in this study

In this study, BALOs were isolated from the activated sludge samples using two model laboratory bacteria as prey species, *K. pneumoniae* and *P. protegens* Pf-5 [167, 168]. Cell suspensions of these bacteria were prepared by streaking out prey bacteria from -80°C frozen stocks onto Luria-Bertani (LB, Bacto™, tryptone 10 g/L, yeast extract 5 g/L, NaCl 5 g/L, pH 7.4) agar plates. Single colonies were inoculated into 5 mL of LB liquid medium at 30°C with shaking at 200 rpm overnight. These bacterial cultures were further transferred into fresh LB liquid medium after dilution 1:100 at 30°C with shaking at 200 rpm for 24 h. After 24 h inoculation, bacterial cultures were

collected by centrifugation (8,000 *g*, 5 min), and re-suspended in diluted nutrient broth (DNB, 'Lab-Lemco' powder 0.1 g/L, yeast extract 0.2 g/L, peptone 0.5 g/L and NaCl 0.5 g/L) amended with 3 mM $MgCl_2$, 2 mM $CaCl_2$, pH 7.4 for subsequent experiments.

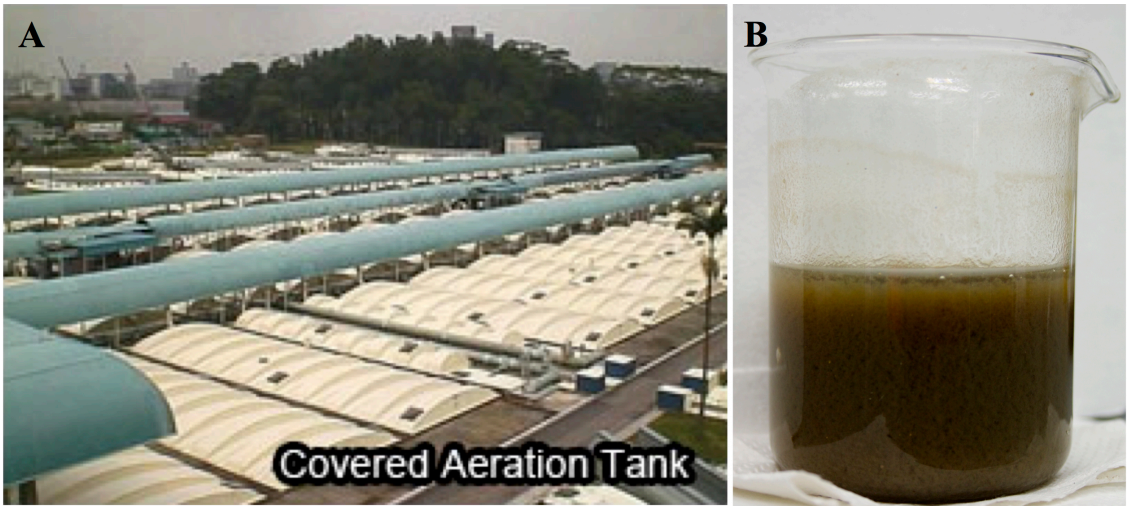


Figure 2.1 Images showing (A) the covered aeration tank at the Ulu Pandan Wastewater Reclamation Plant, and (B) the activated sludge sample collected. Figure A was taken from the website of Public Utilities Board (PUB) (http://www.pub.gov.sg/PRODUCTS/USEDWATER/Pages/WaterReclamationPlants.aspx), whilst Figure B was taken using digital camera (Canon 400D, 18 – 55 mm lens, f/3.5 – 5.5).

2.2.3 Isolation of *Bdellovibrio*-and-Like Organisms (BALOs)

The small size of BALOs, relative to other bacterial species, enables their partial separation by differential centrifugation [89]. In brief, 20 mL of the fresh activated sludge samples were transferred into a clean 50 mL tubes (Falcon®, Corning Inc.) and incubated at room temperature (24 – 26°C) with shaking at 200 rpm overnight. These cultures were subsequently centrifuged at 500 *g* at 4°C for 10 min (Centrifuge 5810R, Eppendorf®) and the supernatants that contained enriched BALOs were collected into two sterile 250 mL conical flasks containing cell suspensions of overnight-grown *K. pneumoniae* and *P. protegens* Pf-5 in 100 mL DNB medium with cell densities of approximately 10^9 CFU/mL. The conical flasks were incubated at 30°C with shaking at 200 rpm for 24 h. This step allows for the putative BALOs cultures to increase in overall numbers prior to quantified and isolated on the double layer plates. Subsequently, the cultures were centrifuged at 2,000 *g* at 4°C for 10 min, and the

supernatants were passed through a series of membrane filters of 1.2, 0.8, and 0.45 µm. The filtrate was then 10 fold serially diluted in liquid DNB.

The BALOs were then isolated using the double-layer plating technique. The bottom layer was prepared with DNB medium with the addition of 1.5% agar. The bottom layer was poured into a petri dish and allowed to solidify at room temperature. To prepare the top layer, prey bacteria, *K. pneumoniae* and *P. protegens* Pf-5, which were selected as prey species because they have been shown generally to be sensitive to predation by BALOs [162], were first inoculated in LB medium at 30°C with shaking at 200 rpm for overnight growth, collected by centrifugation (8,000 *g*, 5 min) and subsequently re-suspended in DNB medium to final cell densities of 10^9 CFU/mL. The re-suspended prey cells were added (4 mL) to 5 mL of DNB containing 1.0% molten agar maintained at 48°C (to keep the agar in liquid form prior to pouring). To this mixture, 2 mL of the diluted BALOs samples, as prepared above, were added, vortexed briefly to homogenize the samples and poured onto the bottom agar plates and allowed to cool at room temperature. After the top agar solidified, the plates were incubated upside-down for 3 to 5 d at 30°C and the formation of lytic plaques on the prey lawns was monitored daily and recorded using digital camera (Canon 400D with 18 – 55 mm lens of f/3.5 – 5.5). The images were subsequently processed with software (iPhoto®, Apple Inc.) to adjust the contrast of the images for characterization of the plaques.

Individual plaques were picked using a sterile pipette tip and re-suspended in 0.5 mL DNB liquid in 1.5 mL microfuge tubes (Eppendorf Tubes®, Singapore) at room temperature. A drop of the suspension was placed on a clean glass slide, covered with a cover slip and examined by phase-contrast microscopy (Axio Vert. A1, ZEISS), and the presence of fast-moving bacteria suggested the presence of BALOs in the cultures. Multiple plaques formed on the plates were individually picked and re-suspended 1 mL of DNB in separate 1.5 mL microfuge tubes with vigorous vortexing to release BALOs from the soft agar. The supernatants, 0.5 mL in each tube, were subsequently incubated with 2 mL of the corresponding prey cell suspension. The co-culture was incubated at 30°C with shaking at 200 rpm for 2 d before plating on DNB agar for further plaques formation. This process of transferring putative BALOs from the plaques was repeated at least five times to increase the purity of the BALOs isolates. The purity of the BALOs isolates was investigated by FISH with specific probes for

BALOs, as well as sequence analysis targeting 16S rRNA gene of BALOs amplified by polymerase chain reaction (PCR).

2.2.4 Identification of the BALOs isolates

Genomic DNA was extracted from each of the BALOs enrichments, using the PureLink™ Genomic DNA extraction Mini Kit (Invitrogen). The extracted genomic DNA was used as a template for PCR using general primers 27F and U1492R (Table 2.1) targeting the 16S rRNA gene in a first round of PCR, which consisted of (final concentration): 1 × DNA polymerase buffer, 1 mM $MgCl_2$, 0.02 mM nucleotide mixtures, 0.4 µM of each primer, 0.01 U/µL of *Taq* DNA polymerase (Fermentas, Thermo Scientific Inc.), 5% DMSO, and 40 ng of the template DNA. The reaction cycles were as follows: 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 45 s and 72°C for 1 min 45 s, subsequently followed by 72°C for 10 min. PCR products were visualized by loading samples on a 1 × TAE agarose gel (0.8%), electrophoresed at 10 V/cm for 35 min and stained with ethidium bromide (100 ng/mL). The gels were analyzed using Quantity-One 1-D Analysis software (Version 4.0, BioRad®, the USA.)

The DNA products were purified using the PCR products PureLink™ PCR Purification Kit (Invitrogen) and the concentration of the purified products was quantified using a NanoDrop 2000 (Micro-Volume UV-Vis Spectrophotometer for Nucleic Acid and Protein Quantitation, Thermo Scientific Inc.). The purified products were subsequently used as templates for the second round of PCR using the same reaction mixture as above and the following PCR program: 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 56°C (for Alpha-*Proteobacteria* specific primers) or 57°C (for BALOs specific primers) for 45 s, and 72°C for 1 min, subsequently followed by 72°C for 10 min. The PCR products were purified as above and sent for sequencing.

Table 2.1 Primers used and their targets

Primers	Sequence 5'-3'	Target group	References
27F	AGA GTT TGA TCM TGG CTC AG	Bacterial Universal	[169]
U1492R	ACC TTG TTA CGA CTT	Bacterial Universal	
28F	ARC GAA CGC TGG CGG CA	Bacterial Universal	[170]
684R	TAC GAA TTT YAC CTC TAC A	Alpha-*Proteobacteria*	
63F	CAG GCC TAA CAC ATG CAA GTC	Bacterial Universal	[171]
842R	CGW CAC TGA AGG GGT CAA	Delta-BALOs	

2.2.5 Classification of *Bdellovibrio* isolates

Enrichments containing putative BALOs along with their prey were investigated by fluorescence in situ hybridization (FISH) to verify that the BALOs were present. As controls, the individual prey species were also used in the FISH experiments. Four probes were used in this study (Table 2.2), in which EUB338 is a universal eubacterial probe that detects both prey and predator species, whilst BDE525 was used for detection of all known Delta-*Bdellovibrio* members and ALF968 and GAM42a targets Alpha- and Gamma-*Proteobacteria*, respectively. It should be noted that the prey bacteria were neither Alpha- nor Delta-*Proteobacteria*, and hence binding of these group-specific probes would likely be attributed to predatory bacteria in Alpha- and Delta-*Proteobacteria*, respectively.

Table 2.2 FISH probes and their targets

Probe	Sequence 5'-3'	Fluorophores at 5'-end	Targeted bacteria groups	References
EUB338	GCTGCCTCCCGTAGGAGT	Alexa-405	Universal bacteria	[172]
BDE525	GATCCCTCGTCTTAC	Alexa-488	Delta-BALOs	[173]
GAM42a	GCCTTCCCACATCGTTT	Cy 5	Gamma-*Proteobacteria*	[174]
ALF968	GGTAAGGTTCTGCGCGTT	Cy 3	Alpha-*Proteobacteria*	[175]

All of the cultures were hybridized with the EUB338 probe along with two additional probes, either GAM42a and BDE525, or GAM42a and ALF968. FISH hybridization was performed by collecting the prey-predator co-cultures by centrifugation at 20,000 *g* and 4°C for 10 min (Centrifuge 5427R, Eppendorf), followed by re-suspension in 8% paraformaldehyde (PFA) at 4°C for 2 h. The fixed samples were centrifuged and washed twice in a 1×PBS-Ethanol buffer (50% final concentration) followed by re-suspension in the same PBS-Ethanol buffer. The re-suspended samples were briefly vortexed and 3 µL of the sample was loaded onto an acid-washed Teflon-coated glass slide and dried in a fume hood [172].

The slides were sequentially dipped for 3 min each in solutions of 50%, 80% and 100% ethanol. After drying in a fume hood, the samples were hybridized with probes described above at 46°C in the dark for 4 h. The hybridization master mixture consisted of 50 ng of labeled probes in 100 μL of hybridization buffer (20 mM Tris-HCl, 0.9 M NaCl, 0.1% sodium dodecyl sulfate, with different concentrations of formamide). The optimal working concentration of formamide for EUB338, BDE525 and GAM42a ranged from 25% – 35% [172-174], whilst the optimal concentration for ALF968 was 20% [175]. The master mixture was applied to each sample on the multiple-well Teflon-coated slide, which was kept horizontal in a 50 mL tube for hybridization.

After 4 h hybridization, the slides were then placed in wash buffer (20 mM Tris-HCl, 0.1% sodium dodecyl sulfate, 5 mM EDTA, and the appropriate concentration of NaCl corresponding to the formamide concentration was used in the previous step) and incubated at 48°C in a water bath in the dark for 15 min. Slides were then rinsed in cold, distilled water for several seconds, followed by air drying in the dark. Samples were either imaged immediately once dry, or were stored in the dark at -20°C for future examination. Imaging and image quantification were performed as described below.

2.2.6 Synchronization of predator cultures and growth patterns of the BALOs isolates

To characterize the growth of the BALOs isolates (BALOs #1 and BALOs #2), cultures were grown in DNB liquid medium at 30°C with shaking at 200 rpm with the enriched prey cultures. In order to obtain consistent numbers of predator cells for subsequent experiments, experiments designed to optimize and synchronize predator cells were undertaken [176]. The 2 d old, two-membered cultures of BALOs cells with corresponding prey species were centrifuged at 2,000 *g* for 10 min to collect the supernatant. Low-speed centrifugation enables the removal of prey cell debris caused by predation, leaving mostly free-swimming BALOs cells in the supernatant. The supernatant was subsequently passed through 0.45 μm filters twice to further separate BALOs cells from the prey, the filtrate of which was then mixed in 1:9 (v/v) with a freshly prepared prey cell suspensions prepared as described above (Section 2.2.2).

The two-membered cultures were subsequently incubated at 30°C with shaking at 200 rpm for 24 h and then mixed with sterile glycerol (final concentration, 25%) to prepare -80°C predator-prey stocks for long-term storage. BALOs cells were revived from -80°C stocks by thawing on ice and then pipetted into freshly prepared prey cell suspensions for growth. The co-cultures were inoculated at 30°C with shaking at 200 rpm for 24 h, followed by centrifugation at 2,000 *g* for 10 min to remove any debris of the prey cells. The supernatants were collected for plaque formation assay on DNB agar plates, and fixed by PFA for in situ hybridization with group-specific probes for confocal microscope examination to verify the re-viability of predators from the frozen stocks.

Subsequently, to determine the growth pattern of the BALOs species with specific prey species, the BALOs cells from frozen stocks (approximately 10^9 cells/mL) were mixed 1:24 (v/v) with prepared prey cell suspensions (approximately 10^9 cells/mL) (total volume, 50 mL) in DNB medium and incubated for 24 h. The cultures were subsequently centrifuged at 2,000 *g* for 10 min. The supernatant (15 mL) was then inoculated in 1:9 (v/v) with freshly prepared prey cell suspensions (total volume, 150 mL). The two-membered cultures were then incubated in conical flasks at 30°C with shaking at 200 rpm. Every 4 h, 1 mL of the co-cultures were collected and fixed with PFA (final concentration 4%) for FISH assay. Growth experiments were conducted for 96 h, which was determined to cover the entire growth cycle of the BALOs. The fixed samples were then subjected to FISH assay as described above (specificity of FISH probes was tested and the protocols used here were as established above). Fluorescently labeled probes, BDE525 (specific for Delta-BALOs) and GAM42a (specific for prey species, *K. pneumoniae* and *P. protegens* Pf-5), were used for hybridization with the predator and prey cells in the samples, respectively.

Hybridized samples were imaged using confocal scanning laser microscopy (LSM780, ZEISS). For each sample at each time point from an individual growth experiment, 30 images were randomly taken to calculate the cell numbers using software IMARIS (Version 7.6.4, Bitplane, Oxford Instrument Inc.). Mean numbers of both predator and prey cells on all of these 30 images were calculated to determine the numbers of both predator and prey cells (cell numbers per milliliter). The cell numbers of both predator and prey cells from each sampling time point were used to reflect the dynamic shifts of predator and prey numbers.

2.2.7 Quantification of cell numbers based on FISH images

The FISH images were processed using IMARIS to quantify the cell numbers of both the predator and prey in each image. The module 'Spots' of IMARIS was used to enumerate the cell numbers of both the predator and prey species based on their cell sizes of 0.8 μm and 1.5 μm, respectively. These values were consistently used for all of the FISH images of the predator with prey cells. Afterwards, the cell numbers of predator and prey were automatically and separately calculated, and the cell counts (cell numbers per milliliter) of both predator and prey species were extracted to generate the growth pattern of BALOs incubated with specific prey species.

2.2.8 Preparation of *Bdellovibrio* isolate and model bacteria for test of *Bdellovibrio* predation

One of the isolated predatory bacteria was identified as a member of the genus *Bdellovibrio* (refer to section 2.3.3 below), which was used as the bacterial predator for the experiments. This *Bdellovibrio* species was routinely grown and enriched in DNB medium with *P. protegens* Pf-5 as prey. Briefly, the predators were at the highest concentration (cell numbers per milliliter) after 48 h when cultivated with prey cells at 30°C with shaking (200 rpm) (refer section 2.3.4). *Bdellovibrio* cells were collected through low-speed centrifugation (2,000 *g* for 10 min) to remove prey cells debris, followed by filtration of the supernatant through 0.45 μm membrane filters (Acrodisc® syringe filters, PALL Corporation) twice. The filtrate contained the enriched *Bdellovibrio* cells. Part of the filtrate was further passed through 0.2 μm membrane filters (Acrodisc® syringe filters, PALL Corporation) three times, and the subsequent filtrate contained no *Bdellovibrio* cells, which was used as a negative control.

The predation effects of the isolated *Bdellovibrio* species upon additional prey species were initially assessed with a number of well-studied laboratory model bacterial species, such as *Acinetobacter* spp., *E. coli*, *K. pneumoniae* [167], *Pseudomonas aeruginosa* PAO1 [167], *Pseudomonas protegens* Pf-5 [167] and *Vibrio cholerae* A1552 [177]. These species have all been previously reported to be vulnerable towards *Bdellovibrio* attack during both planktonic and biofilm growth.

Prey bacterial species were grown and kept on agar plates containing LB medium. To assess the predatory effects of *Bdellovibrio* isolate on these model bacterial species

during planktonic growth, a single colony of the prey species was first inoculated into ~2 mL LB liquid medium at 30°C with shaking at 200 rpm for 18 h. Cultures were then diluted in 1:50 (v/v) into fresh LB liquid medium (total volume 20 mL) at 30°C with shaking at 200 rpm for 24 h. The enriched prey cells were collected by centrifugation (8,000 *g*, 5 min), and were subsequently re-suspended in 20 mL DNB medium.

The re-suspended bacterial cultures were evenly divided into two 50 mL test tubes (Falcon®, Corning Inc.). *Bdellovibrio* cells that had been passed through 0.2 μm (negative control) and 0.45 μm (treatment) membrane filters as described above were incubated in 1:10 (v/v) with the prey cell cultures at 30°C with shaking at 200 rpm. At each time point 0, 24 and 48 h, 0.5 mL of the cultures were collected for determination of colony forming units (CFU) of experimented bacterial species on LB agar plates. Experiments were conducted three times.

To assess the effects of predation on biofilms formed by these prey species, bacteria were grown in LB liquid medium as described above, and diluted with fresh LB liquid medium to achieve an optical density (OD_{600}) of 0.1. Diluted cells were distributed in 1 mL aliquots into the wells of 24 well micro-titer plates (Costar®, 24 well clear, not tissue culture treated). The micro-titer plates were then incubated on an orbital shaker at room temperature (25 – 26°C) with shaking at 100 rpm for 24 h to form bacterial biofilms [178]. Afterwards, the biofilms were carefully washed twice with DNB to remove any planktonic cells, and 1 mL of prepared *Bdellovibrio* cell suspension (adjusted to 10^8 cells/mL) was added into three wells for each bacterial species. As a negative control, 1 mL of sterilized medium, prepared by passing the *Bdellovibrio* cell suspension through 0.2 μm membrane filters three times, was added into another three wells. The micro-titer plates were subsequently incubated at 30°C with shaking at 100 rpm for 24 h. Preliminary experiments showed that bacteria formed biofilms at the air-liquid interface and on the bottom of the micro-titer wells and these pre-formed biofilms survived in DNB medium over the 72 h period used for predation (data not shown).

Quantification of the biofilm biomass with and without *Bdellovibrio* treatment was performed via crystal violet staining. After 24 h incubation, the wells of micro-titer plates were washed three times with DNB to remove any planktonic cells. The wells were subsequently immersed with 1 mL crystal violet (0.1% in dH_2O) for 15 min. The crystal violet solution was then discarded and the stained wells were carefully

washed twice with saline solution (0.85% NaCl) to remove any unbound crystal violet residue. To dissolve the bound crystal violet, 1 mL of 95% ethanol was added into each well for at least 15 min. The amount of crystal violet, which is a representative measure of the biomass of biofilm, was quantified at 590 nm using a micro-titer plate reader (TECAN, INFINITE® 200, PRO).

2.3 RESULTS

2.3.1 Isolation and visualization of BALOs from wastewater

The pre-treated wastewater samples were initially cultured with *K. pneumoniae* and *P. protegens* Pf-5 separately, prior to performing the double-layer plating technique in order to increase the density of predators to sufficient numbers to be observed by plaque assays. After 3 – 5 d of incubation on the double layer plates, lytic plaques with differences in their morphologies and the time required for formation were observed (Figure 2.2). Based on these observations, selected plaques with different morphologies on *K. pneumoniae* and *P. protegens* Pf-5 (Figure 2.2 A and D, respectively) were selected for further characterization. The predatory species on different prey lawns were referred as 'BALOs #1' (initially isolated on lawns of *K. pneumoniae*) and 'BALOs #2' (initially isolated on lawns of *P. protegens* Pf-5). Single plaques of 'BALOs #1' and 'BALOs #2' were picked for further enrichment and were maintained with the corresponding prey species on agar plates. After 3 d of incubation, plaques formed by the two predatory species on the prey lawns were measured.

When *K. pneumoniae* was used as the prey lawn, the isolated predator, 'BALOs #1', generally formed round-shaped plaques with a smooth edge and diameters ranging from 0.5 – 1 cm (Figure 2.2 A). These were semi-transparent in the center, and at the edge of the plaques, the top agar surface was recessed to the bottom agar. When the enriched 'BALOs #1' cultures (originally isolated on lawns of *K. pneumoniae*) were incubated on *P. protegens* Pf-5 lawns, similar plaque patterns (semi-transparent, deeply recessed) were also observed, but the central plaques were bigger (1 – 1.5 cm), around which semi-transparent, broad halos were also detected (Figure 2.2 B). The differences in plaque morphologies on these two prey lawns may suggest that the predation patterns by this predator were different against these two prey species.

When *P. protegens* Pf-5 was used as the selective prey lawn, the plaques formed by the isolated predator, 'BALOs #2', were also generally observed as round-shaped plaques with smooth edge and diameters ranging from 0.8 – 1.2 cm (Figure 2.2 D). These plaques were more transparent, and the plaques expanded along the surface of the prey lawns with no penetration to the bottom agar. This isolated predator, 'BALOs #2' (originally isolated on lawns of *P. protegens* Pf-5), was also incubated

with *K. pneumoniae*, and similar plaque patterns (transparent with clear area in the center, no alcove-shaped, similar sizes) were also observed on the *K. pneumoniae* lawns (Figure 2.2 C). The similarity of the plaques formed on these two prey lawns may indicate the non-preferential predation by this predatory bacterial species on these two prey species. Therefore, based on the differences in plaque morphology, it was preliminarily considered that 'BALOs #1' and 'BALOs #2' represented different types of bacterial predators.

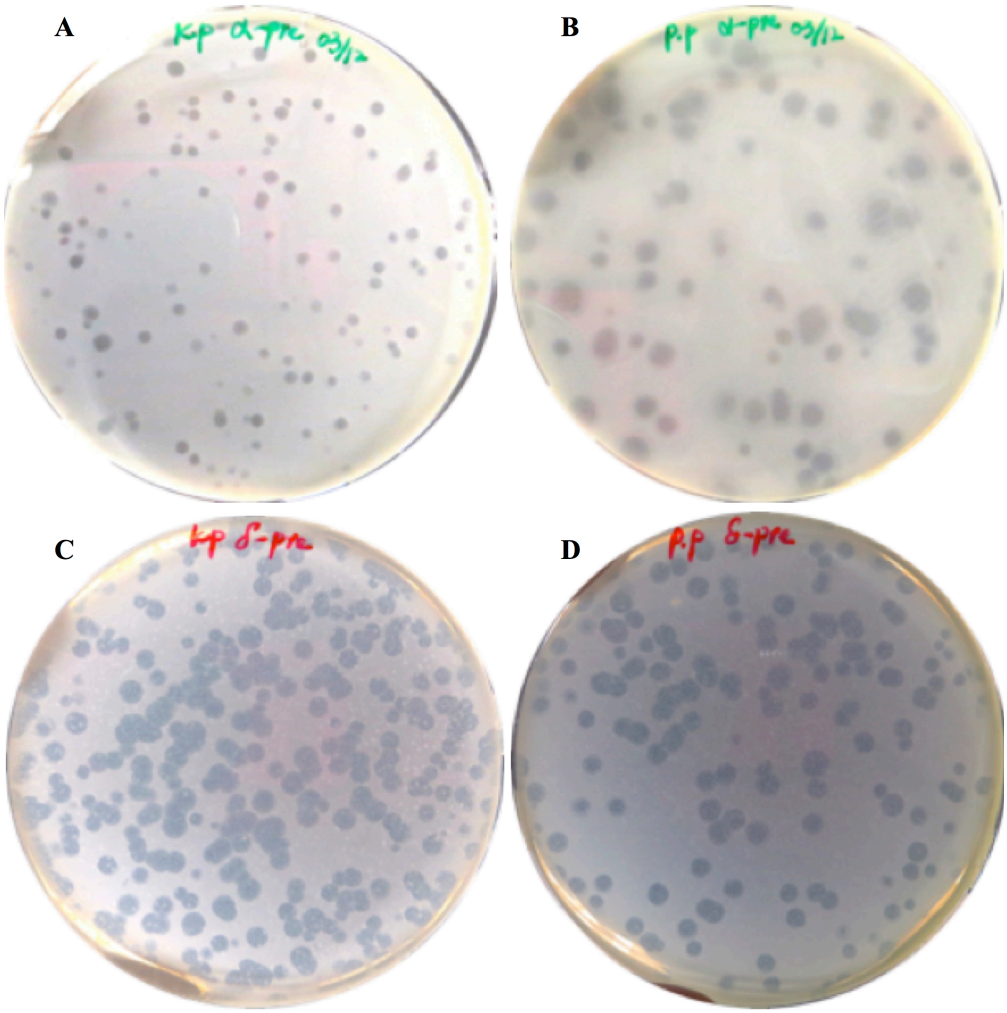


Figure 2.2 Plaque formation by BALOs species from wastewater treatment activated sludge. Lytic plaques formed on *K. pneumoniae* (A and C) and *P. protegens* Pf-5 (B and D) lawns, respectively, by different predatory bacterial isolates after 3 d of incubation at 30°C. The plaques formed on plates were imaged by digital camera, and for each prey species, representative images were shown above. Images A and B showed plaques formed by predator 'BALOs #1' on *K. pneumoniae* and *P. protegens* Pf-5 lawns, respectively. Images C and D show plaques formed by a second predator 'BALOs #2' on *K. pneumoniae* and *P. protegens* Pf-5 lawns, respectively.

The plaques formed by 'BALOs #1' and 'BALOs #2' predators were both observed to expand upon longer incubation (after 3 d of incubation). And the plaques of 'BALOs #2' eventually covered the entire prey lawn (data now shown). This observation that the plaques expanded over time suggested that these predators (both 'BALOs #1' and 'BALOs #2') were motile and actively predatory within the soft agar, which differentiated them from the types of plaques typically associated with bacteriophages. When both the plaques and the corresponding co-cultures were examined by phase-contrast microscopy, tiny (smaller than prey cells) but highly motile cells (predators) were detected along with larger and rod-shape cells (prey).

2.3.2 Classification of isolated bacterial predators

To confirm the isolation of BALOs, group-specific oligonucleotide probes (here after referred to as FISH probes, for the method, fluorescence in situ hybridization, according to the convention in molecular microbial ecology) labeled with different fluorescent markers were used. The FISH probes were first tested for their sensitivity and specificity to confirm there was no cross-hybridization.

In this study, the predator 'BALOs #1' was inoculated together with *K. pneumoniae*, whilst 'BALOs #2' was maintained with *P. protegens* Pf-5. Both co-cultured isolates were fixed with PFA and then hybridized with the FISH probes GAM42a and EUB338. Both of the co-cultures were additionally hybridized with the FISH probes ALF968 and BDE525, respectively. In addition to the predator-prey co-cultures, the mono-specific cultures containing only the prey species (*K. pneumoniae* or *P. protegens* Pf-5) were used as hybridization negative controls with the corresponding FISH probes. Specifically, *K. pneumoniae* cultures were hybridized with GAM42a and ALF968, whilst *P. protegens* Pf-5 cultures were hybridized with GAM42a and BDE525. Both *K. pneumoniae* and *P. protegens* Pf-5 are Gamma-*Proteobacteria*, and therefore it was expected that they would hybridize with the group-specific probe GAM42a targeting Gamma-*Proteobacteria*.

The results showed that *K. pneumoniae* mono-species cultures hybridized with probes of GAM42a but gave no fluorescence when hybridized with ALF968 (Figure 2.3 A – C), and the *K. pneumoniae* cultures were observed as single, spherical-shaped cells in these FISH images (Figure 2.3 A). Similarly, *P. protegens* Pf-5 mono-species cultures only showed a fluorescent signal when hybridized with the GAM42a probe

and were negative when hybridized with the BDE525 probe (Figure 2.4 A – C). The *P. protegens* Pf-5 mono-species cultures were observed as single, rod-shaped cells (Figure 2.4 A).

In contrast to the mono-species cultures, co-cultures of 'BALOs #1' predator with *K. pneumoniae* hybridized with the probes ALF968, GAM42a and EUB338 (Figure 2.3 D, E and F, respectively). Since *K. pneumoniae* did not cross-hybridize with ALF968 (Figure 2.3 B), the positive fluorescence signals attributed to the ALF968 probe was likely to be due to the presence of bacteria associated with the Alpha-*Proteobacteria* clade in the co-cultures. Careful observation of 'BALOs #1' (Figure 2.3 D) showed that the ALF968 hybridization signal was associated with small cells, about 0.5 – 0.8 μm wide and 1 – 1.2 μm long, distinct from the prey cells, which were typically spherical-shaped with diameter about 2 μm (Figure 2.3 F).

The co-cultures with the 'BALOs #2' predator and *P. protegens* Pf-5 hybridized with the FISH probes BDE525, GAM42a and EUB338 showed positive hybridization signals for all of these probes (Figure 2.4 D, E and F, respectively). It was demonstrated that *P. protegens* Pf-5 did not hybridize with the BDE525 probe (Figure 2.4 B), and therefore the positive fluorescence signals were likely to be due to the presence of a member of the Delta-proteobacterial *Bdellovibrio*. The hybridization signals associated with the BDE525 probe correlated with cells that were smaller than *P. protegens* Pf-5 prey cells (Figure 2.4 D), which were observed to be short rod-shaped of about 0.8 μm wide and 1 – 1.2 μm long. Interestingly, some of the BDE525 probe signals overlapped with the GAM42a probe signals, and the larger cells with the overlapped signals may represent the cell structure 'bdelloplast'. The results from in situ hybridization using the group-specific oligonucleotide probes suggested that predatory isolates 'BALOs #1' and 'BALOs #2' belonged to the classes of Alpha-*Proteobacteria* and the Delta-*Bdellovibrio* clades, respectively.

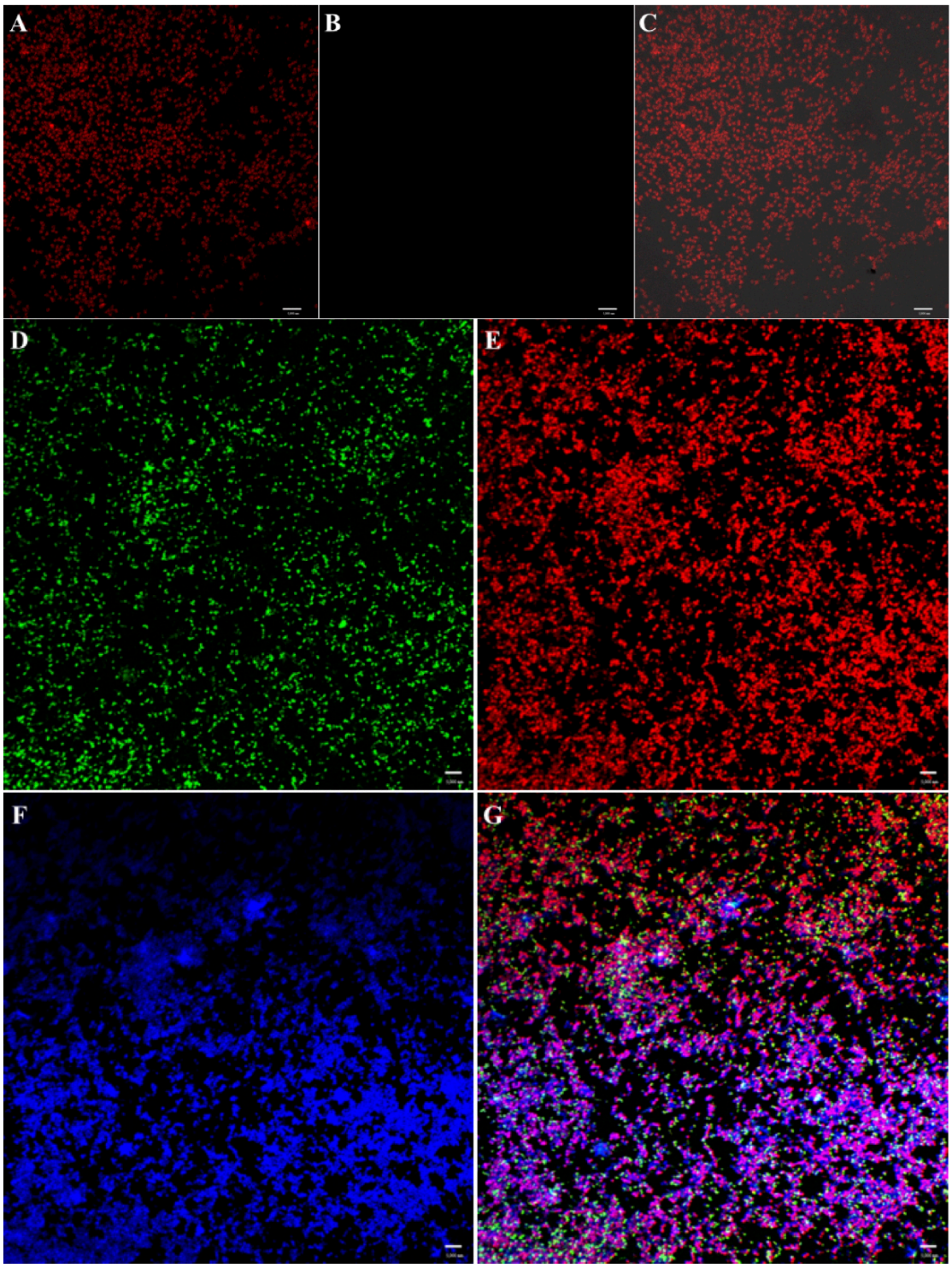


Figure 2.3 FISH images of *K. pneumoniae* cultures and co-cultures of 'BALOs #1' predator and *K. pneumoniae*. Images showed the *K. pneumoniae* cultures hybridized with fluorescently labeled oligonucleotide probes of (A) GAM42a, (B) ALF968 and (C) the combination of (A) and (B); and predator-prey co-cultures hybridized with fluorescently labeled oligonucleotide probes of (D) ALF968, (E) GAM42a, (F) EUB338 and (G) the combined of D, E and F. Magnification 400×, scale bars are 5 μm.

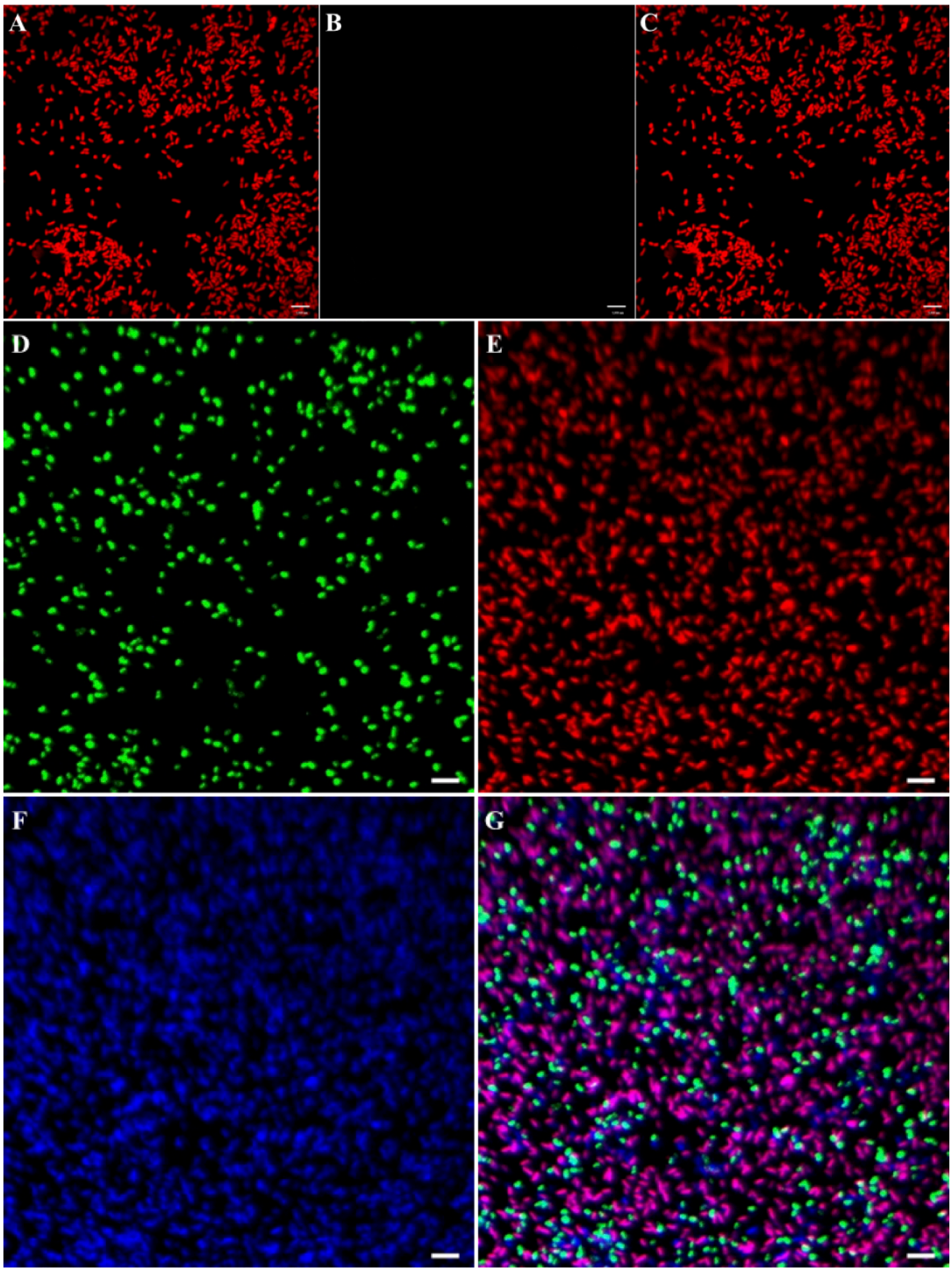


Figure 2.4 FISH images of *P. protegens* Pf-5 cultures and co-cultures of 'BALOs #2' predator and *P. protegens* Pf-5. Images showed the *P. protegens* Pf-5 cultures hybridized with fluorescently labeled oligonucleotide probes of (A) GAM42a, (B) BDE525 and (C) the combination of (A) and (B); and predator-prey co-cultures hybridized with fluorescently labeled oligonucleotide probes of (D) BDE525, (E) GAM42a, (F) EUB338 and (G) the combined of D, E and F. Magnification 600×, scale bars are 5 μm.

2.3.3 Identification of isolated bacterial predators

To identify these bacterial predators, genomic DNA was extracted from cultured samples, and amplified using the primers 27F plus 1492R (the amplified products will cover the full-length of the 16S rRNA gene sequences). The gel electrophoresis profiles showed that the PCR reactions yielded single bands of the expected sizes (~1500 bp, data not shown). The PCR products were purified from the reaction mixture and then used for sequencing using the 27F and 1492R primer (Figures 2.5 and 2.6, respectively).

For both 'BALOs #1' and 'BALOs #2' predators, the 16S rRNA gene of several individual cultures (n > 5) originating from single plaques were sequenced. These sequenced 16S rRNA genes were aligned and compared, and one representative 16S rRNA sequence was presented showing all of the nucleotides, and two more representative sequences were compared with the first representative one. Dots indicated positions where the nucleotides sequences were identical, whilst nucleotide differences were shown in the individual sequences. By comparison with the representative gene (Figure 2.5, BALOs #1 A and Figure 2.6, BALOs #2 A), it was shown that one representative sequence of 'BALOs #1' was slightly different (Figure 2.5, BALOs #1 C, 8 out of 1426 nucleotides) from the representative sequence (Figure 2.5, BALO #1 A), whilst all of the 'BALO #2' sequences showed identical 16S rRNA gene sequences (Figure 2.6).

The representative sequence of each BALOs isolate was searched using the basic local alignment search tool (BLAST) against existing GenBank of microorganisms for those predators' identities. The identification was based upon comparison of the 16S rRNA gene sequence similarity, where the accepted cut-off for 'species' was >97% nucleotide identity [179]. The closet matched bacterial species for each 16S rRNA gene search were presented in Table 2.4 and Table 2.5, respectively. The search results of the representative 16S rRNA gene sequence of 'BALOs #1' showed that the closest match was to an 'uncultured bacterium' with 99% nucleotide identity (Table 2.3). Two of the 'BALOs #1' isolates (isolate A and B) were 100% identical to each other and isolate C showed 99.4% nucleotide identity, suggesting it may be a related, but different strain or species. All three 'BALO #2' isolates (100% identity to each other) were most closely related to the *Bdellovibrio* genus, with 99% nucleotide identity to the *Bdellovibrio bacteriovorus* str. Tiberius (Table 2.4).

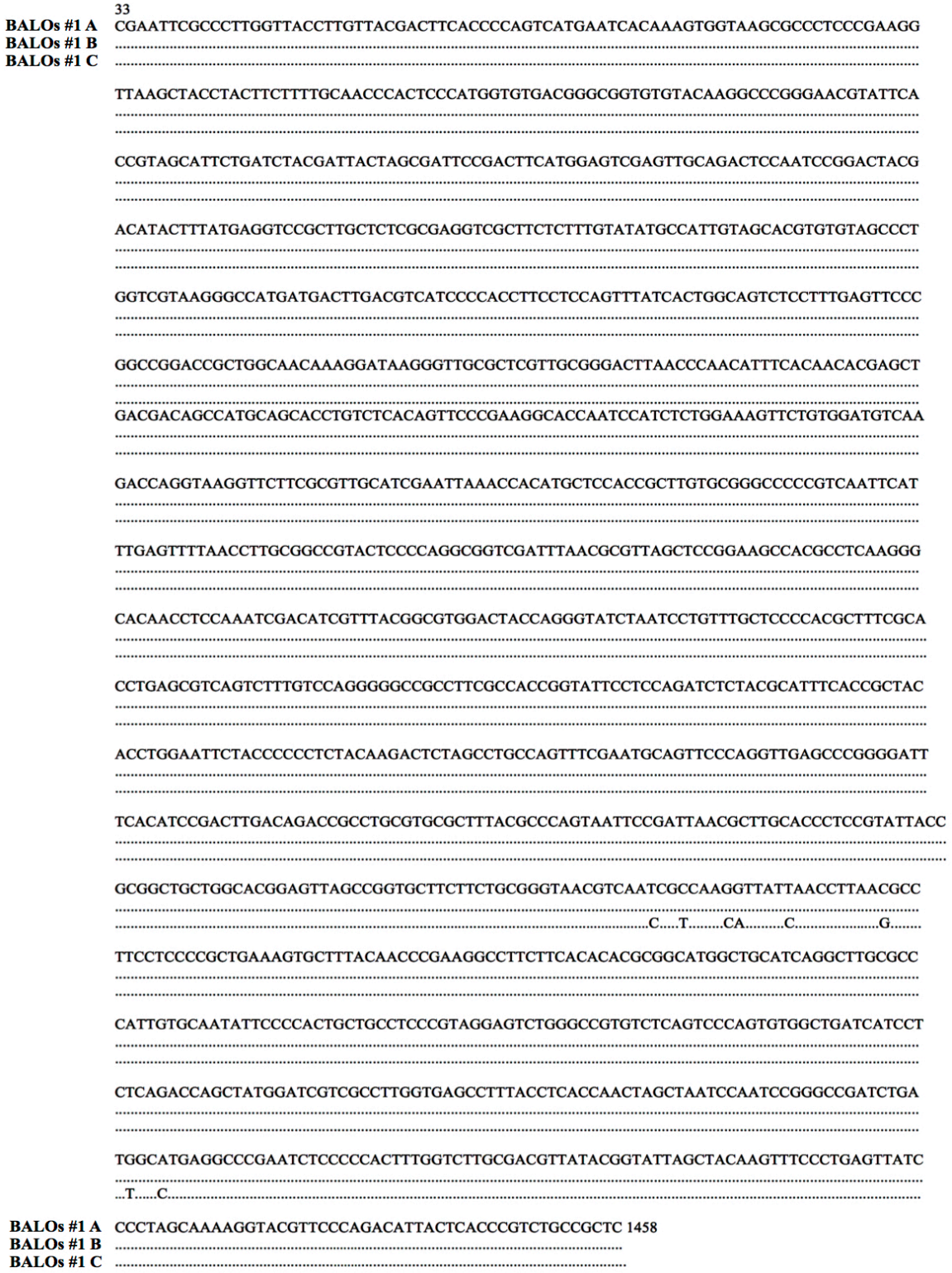


Figure 2.5 Comparison of representative 16S rRNA sequences of 'BALO #1'. Dots indicated that the nucleotides from all sequences were identical to the representative sequence, whilst nucleotide differences were presented in individual sequence.

```
                11
BALOs #2 A      ATGCAGTCGACGGGGTAGCAATACCTAGTGGCGCACGGGTGAGTAACGCGTGGATAATCTGCCTTAGAGTGGGGG
BALOs #2 B      ..........................................................................
BALOs #2 C      ..........................................................................

                ATAACTAGTCGAAAGATTAGCTAATACCGCATAAGACCACAAGAGCTGCGGCTCAAGGGGTCAAAGGTTTTTCGC
                ..........................................................................
                ..........................................................................

                TCTAAGATGAGTCCGCGTAAGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCTTTAACTGGTCTG
                ..........................................................................
                ..........................................................................

                AGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGCAC
                ..........................................................................
                ..........................................................................

                AATGGAGGAAACTTCTGATGCAGCGACGCCGCGTGAGTGATGAAGGCCTTCGGGTCGTAAAAGCTCTGTCGGCAG
                ..........................................................................
                ..........................................................................

                GGGAAATAACACAATGAATGTACCCTGTAAGAAAGGATCGGCTAACTTCGTGCCCAGCAGCCGCGGTAAAGACGA
                ..........................................................................
                ..........................................................................

                GGGATCCTAGCGTTGTTCGGAATCATTGGGCGTAAAGCGGATGTAGGTGGCTTTGTAAGTCAGGTGTGAAAGCCTG
                ..........................................................................
                ..........................................................................

                GGGCTCAACCCCAGAAGTGCATTTGATACTGCGAAGCTTGAGTGTCGGAGAGGTTACTAGAATTGTTGGTGTAGTG
                ..........................................................................
                ..........................................................................

                GTGAAATACGTAGATATCAACAGGAATACCGGAGGCGAAGGCGGGTAACTGGCCGAACACTGACACTGAGATCCG
                ..........................................................................
                ..........................................................................

                AAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTTGTTGTTGGAGGTA
                ..........................................................................
                ..........................................................................

                TTGACCCCTTCAGTGACGAAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAG
                ..........................................................................
                ..........................................................................

                AAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTAGGCTT
                ..........................................................................
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                GACATGTACTGGAAGATTGGGCAGAAATGTCGTCGGCCCGCAAGGGTCGGTACACAGGTGCTGCATGGCTGTCGTC
                ..........................................................................
                ..........................................................................

                AGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTGCATTTAGTTGCCAGCATTCAGTTGGG
                ..........................................................................
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                CACCTCTAGATGGACTGCCGGTGTTAAACCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTATGCCTAG
                ..........................................................................
                ..........................................................................

                GGCTACACACGTGCTACAATGGTAGTCACAGAGCGAAGCTAAGCCGCGAGGTAGAGCAAATCGCTTAAAAGCTATC
                ..........................................................................
                ..........................................................................

                TAAGTTCAGATTGATCTCTGCAACTCGAGATCATGAAGTTGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTG
                ..........................................................................
                ..........................................................................

                AATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGAAAGTCGGCTGTACCAGAAGTCGCTGCGCTAACCG
                ..........................................................................
                ..........................................................................

BALOs #2 A      CAAGGAGGCAGCGC 1390
BALOs #2 B      ..............
BALOs #2 C      ..............
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Figure 2.6 Comparison of representative 16S rRNA sequences of 'BALO #2'. Dots indicated that the nucleotides from all sequences were identical to the representative sequence.

Table 2.3 Sequence comparison of 16S rRNA genes for the 'BALOs #1'

Accession No.	Description	Identity	Sources
GQ359981.1	Uncultured bacterium clone IC31 16S ribosomal RNA gene, partial sequence	97%	Environmental samples [180]
KC305868.1	Uncultured bacterium clone 6-12H34 16S ribosomal RNA gene, partial sequence	95%	Environmental samples (Direct submission to NCBI)
AB369187.1	Uncultured bacterium gene for 16S rRNA, partial sequence, clone: CK06-06_Mud_MAS4B-22	96%	Environmental samples [181]
EF509823.1	Uncultured bacterium clone P4D7-404 16S ribosomal RNA gene, partial sequence	99%	Environmental samples [182]

Table 2.4 Sequence comparison of 16S rRNA genes for the 'BALOs #2'

Accession No.	Description	Identity	Sources
NR_102470.1	*Bdellovibrio bacteriovorus* str. Tiberius 16S ribosomal RNA	99%	*Bdellovibrio bacteriovorus* str. Tiberius [183]
CP002930.1	*Bdellovibrio bacteriovorus* str. Tiberius, complete genome	99%	*Bdellovibrio bacteriovorus* str. Tiberius [183]
AF263832.1	*Bdellovibrio bacteriovorus* strain SRE7 16S ribosomal RNA gene, partial sequence	98%	*Bdellovibrio bacteriovorus* [164]

The sequencing results indicated that the closest match for 'BALOs #1' belonged to the Alpha-*Proteobacteria* represented by one 'uncultured bacterium' from environmental samples, whilst 'BALOs #2' most closely matched the strain '*Bdellovibrio bacteriovorus*' isolated from the Tiberius river at Rome [183]. Given the similarity results and accepted cut-off for species (>97%), 'BALOs #2' was suggested to be a strain of a *B. bacteriovorus* (at an E-value of 0.0), which will be referred to here as *B. bacteriovorus* UP, for *Bdellovibrio bacteriovorus*, strain Ulu Pandan. In contrast, the 'BALOs #1' species could not be clearly identified based on the 16S rRNA gene sequencing analysis and likely represents a novel genus of predatory bacteria within the Alpha-*Proteobacteria* clade. Since there was a clear identification of the *B. bacteriovorus* UP species and the availability of genome sequences of related *Bdellovibrio* members, further experiments focused solely on the *B. bacteriovorus* UP species.

2.3.4 Growth pattern of the isolated predators

The co-cultures of isolated *Bdellovibrio* species with prey cells were kept as frozen stocks at -80°C to synchronize the growth phase of these predators. For these assays, three frozen culture-stock tubes were randomly selected, thawed on ice, and then separately incubated with freshly prepared *P. protegens* Pf-5 prey cells. After 24 h incubation, predator cells in the co-cultures were collected as supernatants by centrifugation (2,000 *g*, 10 min), which were subsequently incubated with freshly prepared prey cell suspensions for growth pattern experiments.

The co-cultures of predators (*B. bacteriovorus* UP) with prey cells (*P. protegens* Pf-5) were collected at specific time points and fixed with PFA for FISH hybridization using FISH probes of BDE525, GAM42a and EUB338. At each time point, multiple images (n = 30) of hybridized co-cultures were taken (Figure 2.7). The numbers of the predators (Figure 2.7, green color) continuously increased in the first 48 h, at which point it reached the highest numbers, whilst the numbers of prey cells (Figure 2.7, red color) continuously decreased due to predation by *B. bacteriovorus* UP. After 48 h, the numbers of predators decreased along with the prey cell numbers.

The numbers of *B. bacteriovorus* UP and *P. protegens* Pf-5 at each time point (Figure 2.7) were determined by quantitative image analysis. The numbers of *B. bacteriovorus* UP were initially ~10^7 cells/mL and increased exponentially from 12 h (3.0×10^7 cells/mL) to 48 h (Figure 2.8), reaching the final maximum density of

5.0×10^9 cells/mL. This exponential growth rate can thus be estimated to be 1.15 in the natural exponential index (*e*) increase per hour for *B. bacteriovorus* UP.

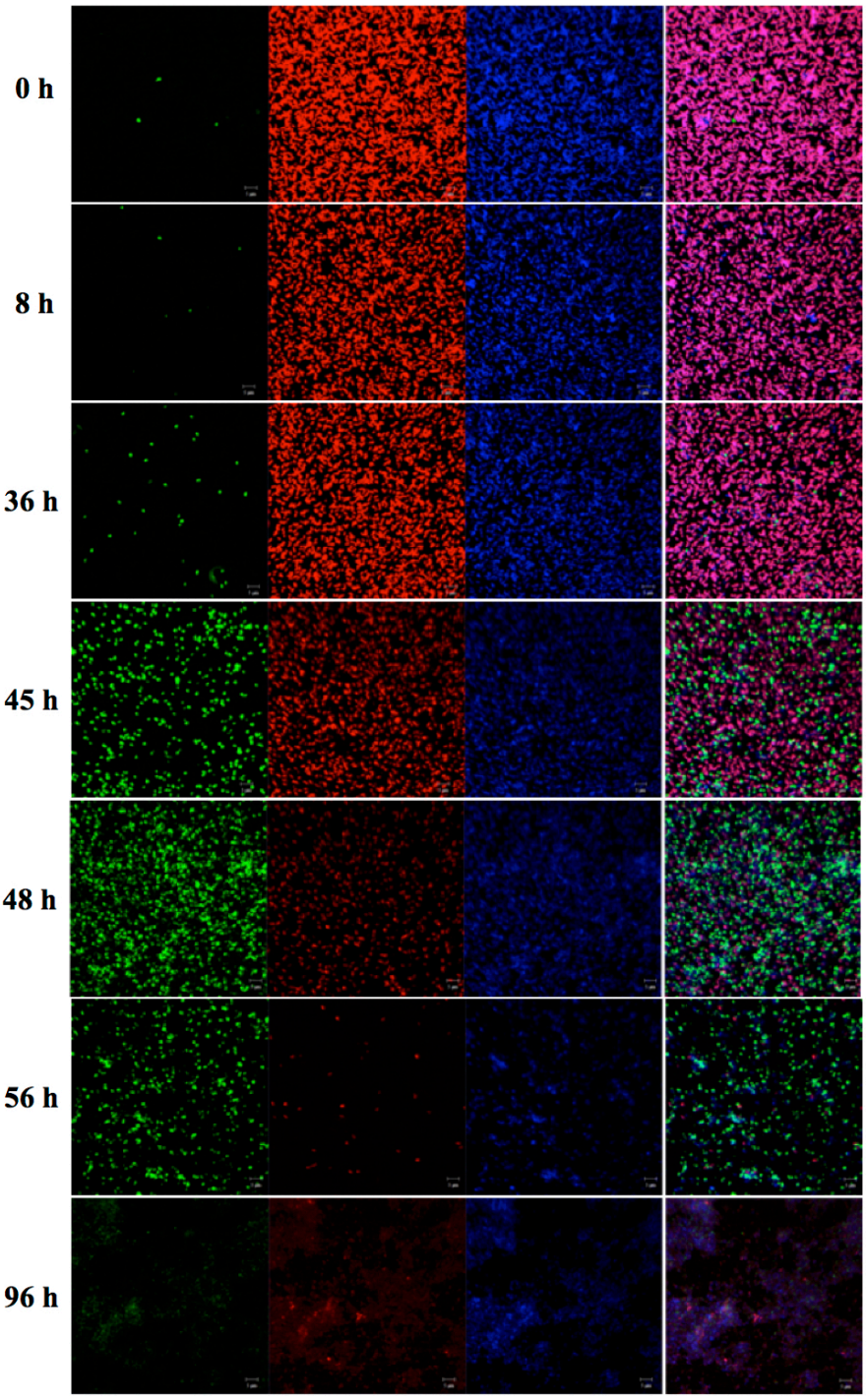

Figure 2.7 FISH images of co-cultures of *B. bacteriovorus* UP and *P. protegens* Pf-5 at selected time points. Representative images demonstrated the dynamic changes of numbers of predator (green color, BDE525 probe) and prey (red color, GAM42a) cells along time. Fluorescence color of green, red and blue represent the fluorescence signals with the oligonucleotide probes of BDE525, GAM42a and EUB338, respectively. Magnification 600×, scale bars represent 5 µm.

The numbers of the *P. protegens* Pf-5 were initially ~7.0×10^9 cells/mL, and the prey numbers gradually decreased to 3.0×10^9 cells/mL at the end of the exponential growth phase for *B. bacteriovorus* UP (Figure 2.8, 48 h). At the time when the *B. bacteriovorus* UP reached its maximum density from 45 h to 48 h, the prey cells began to rapidly decrease from 4.0×10^9 cells/mL at 45 h to 2.0×10^9 cells/mL at 48 h. In addition, the exponential decrease of *P. protegens* Pf-5 cell numbers continued from 48 h (3.0×10^9 cells/mL) to 60 h (2.0×10^8 cells/mL) of incubation, whilst in conjunction with the rapid decrease of prey cell numbers, the cell numbers of *B. bacteriovorus* UP also began to rapidly decrease from 56 h (4.0×10^9 cells/mL) to 72 h (5.0×10^8 cells/mL). Although the prey cell numbers rapidly decreased from 48 h to 56 h (Figure 2.8), the numbers of *B. bacteriovorus* UP remained constant at 5.0×10^9 – 4.0×10^9 cells/mL. Based on these growth dynamics, the *B. bacteriovorus* UP cultures were routinely harvested after 48 h of incubation for subsequent experiments.

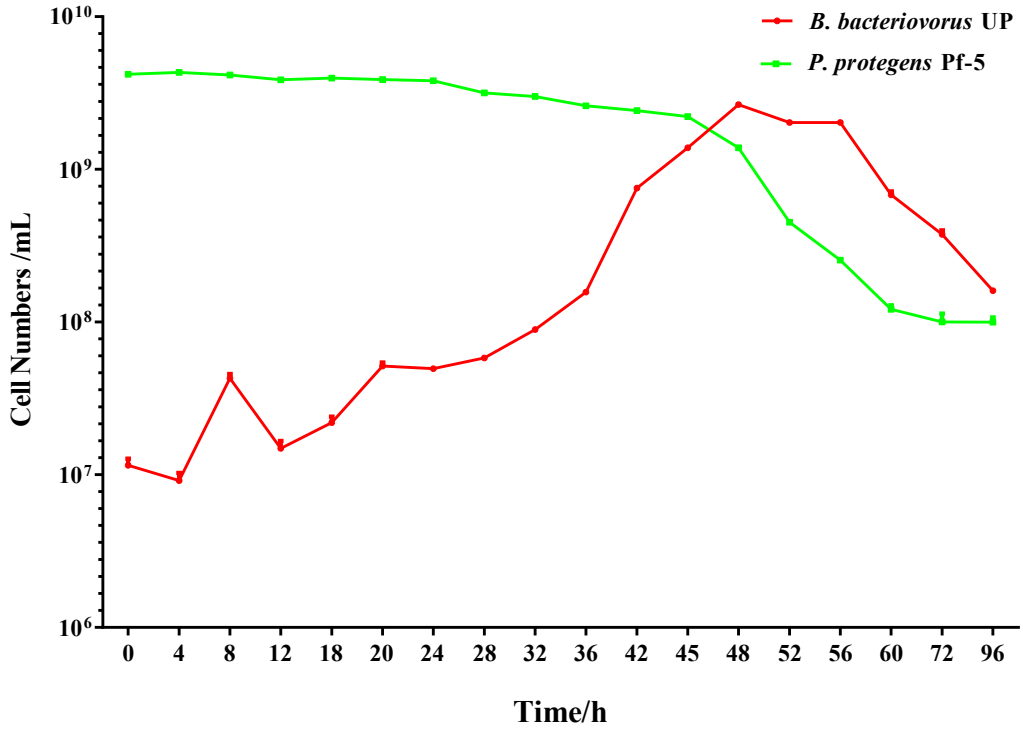

Figure 2.8 Changes in predator and prey numbers during co-culture of *B. bacteriovorus* UP with *P. protegens* Pf-5. Average values of cell numbers/mL of both predator and prey species at each time point were used to yield the growth pattern of the *B. bacteriovorus* UP with the prey species of *P. protegens* Pf-5. Error bars represent the standard error of the mean of the cell numbers at each time point.

2.3.5 The effects of predation on model prey bacteria in planktonic and biofilms

The predatory ability of the *B. bacteriovorus* UP was investigated to determine whether this species from wastewater samples had similar predation effects as other *Bdellovibrio* spp. isolated from soil and marine waters. Therefore, model prey species, including *Acinetobacter* spp., *E. coli*, *K. pneumoniae*, *P. aeruginosa* PAO1, *P. protegens* Pf-5 and *V. cholerae*, were tested both as planktonic cells (Figure 2.9) and biofilms (Figure 2.10) for their sensitivity to predation by *B. bacteriovorus* UP.

The results demonstrated that the prey bacteria, in the absence of the predators, retained viability in DNB medium for at least 48 h, where it was observed that there was no decrease in CFU/mL over this time period (Figure 2.9, 'Control'). The one exception was *V. cholerae* (Figure 2.9 F), which decreased from 10^8 CFU/mL to 10^7 CFU/mL representing a 10 fold decrease in CFUs in the absence of the predators. When *B. bacteriovorus* UP was added to the cultures, all of the prey species showed a statistically significant ($P < 0.05$) decrease in CFUs, indicating that they were highly sensitive to the predation by *B. bacteriovorus* UP.

Although *B. bacteriovorus* UP could prey upon all of these model bacteria in the planktonic phase, the predation efficiency of *B. bacteriovorus* UP differed for each species at the end of the 48 h period. For example, there was a 10 fold reduction in CFUs for *Acinetobacter* (Figure 2.9 A), which was much less than the 1,000 fold reduction observed for *E. coli*, *K. pneumoniae*, *P. protegens* Pf-5, respectively (Figure 2.9 B, C and E). *P. aeruginosa* PAO1 (Figure 2.9 D) was the most resistant towards *B. bacteriovorus* UP predation with less than a 10 fold reduction of viable cells. In contrast, predation by *B. bacteriovorus* UP resulted in a >1,000 fold reduction in *P. protegens* Pf-5 cell numbers. These results suggested that for all of the bacteria chosen, there might either be a preference of *B. bacteriovorus* UP for some strains, such as the *P. protegens* Pf-5, or that some strains were defended from predation such as *P. aeruginosa* PAO1 and *Acinetobacter*.

In addition to differences in overall predation effects, there were also differences between the model prey bacteria in the dynamics of predation. Where the prey cells were present above 10^4 CFU/mL, the rate of predation appeared to be constant (Figure 2.9 B, C, D and E). However, when the prey cell density approached 10^4 CFU/mL or less, the rate of prey cell loss appeared to decrease (Figure 2.9 A and F).

This change in predation rate could indicate a minimum cell density required for active infection and growth of the *B. bacteriovorus* UP.

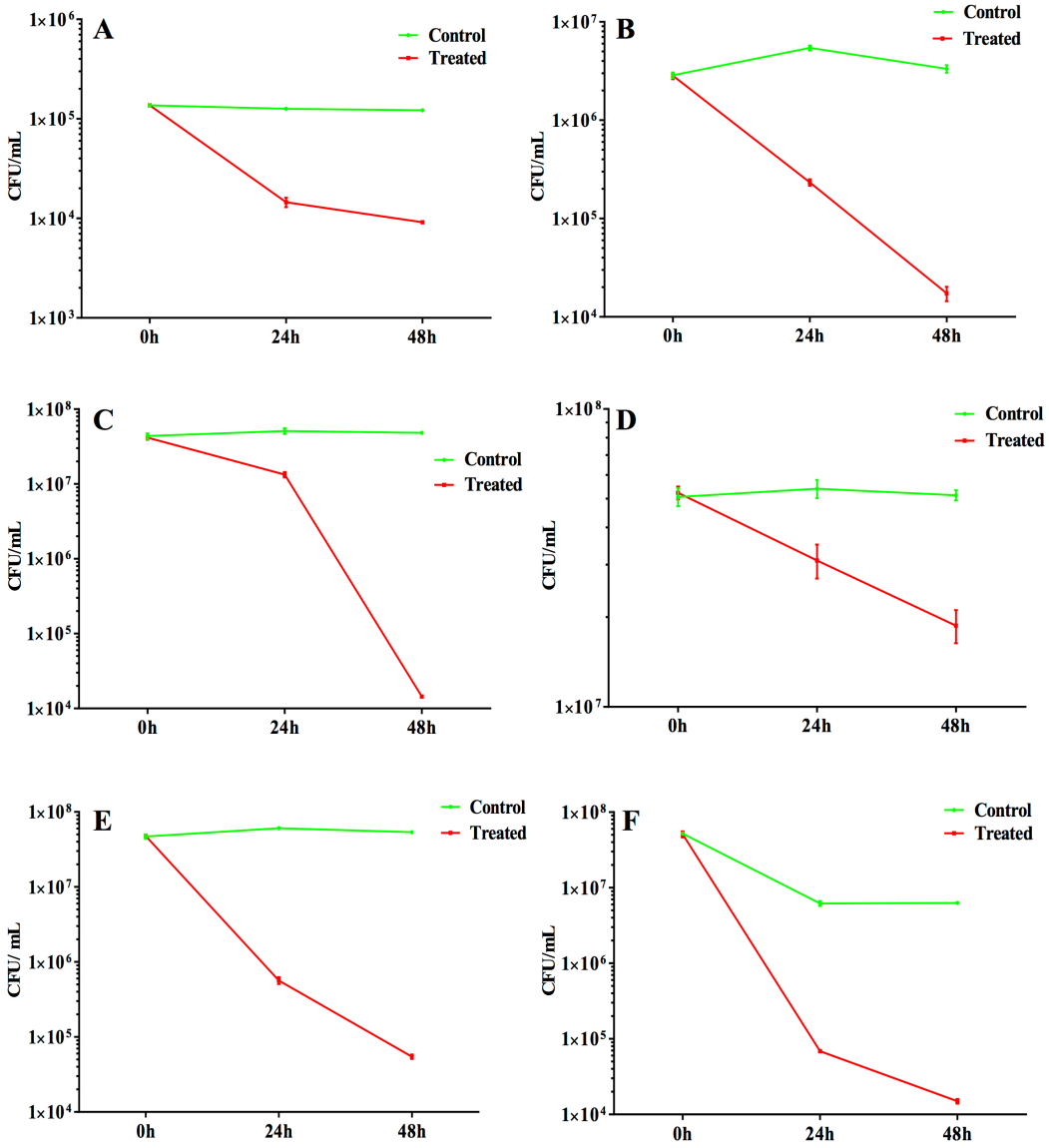

Figure 2.9 Quantification of viable cells of different bacteria incubated with *B. bacteriovorus* UP along time. (A) *Acinetobacter* spp., (B) *E. coli*, (C) *K. pneumoniae*, (D) *P. aeruginosa* PAO1, (E) *P. protegens* Pf-5, and (F) *V. cholerae*, were inoculated in LB liquid medium, followed by incubated with *B. bacteriovorus* UP cells (treated) and *Bdellovibrio*-free medium (control), respectively. Data represent the means of three replicates from one set of representative experiments, with error bars representing the standard error of the mean value. Error bars in some figures were not clearly seen because of the relatively small errors associated with the measurements. The log scales on the Y-axis were slightly different for A – F.

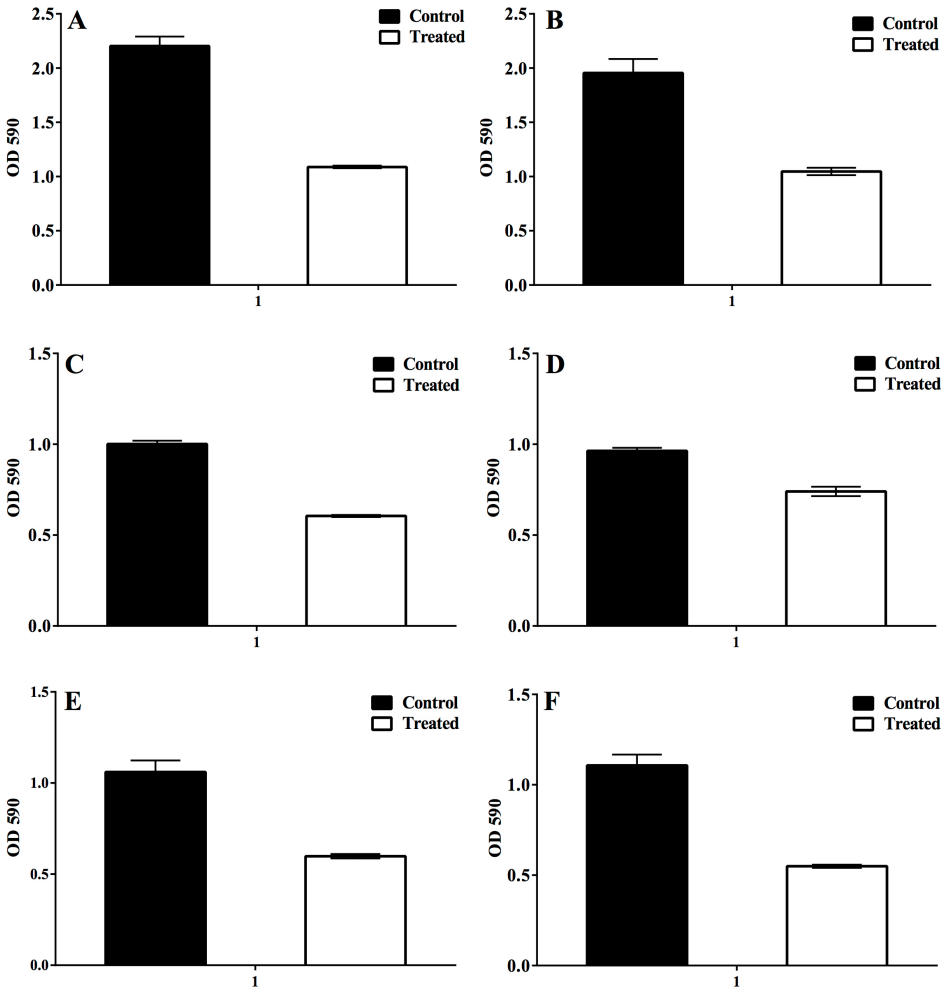

Figure 2.10 The effect of predation by *B. bacteriovorus* UP on mono-species biofilms. Preformed biofilms (24 h) of (A) *Acinetobacter* spp., (B) *E. coli*, (C) *K. pneumoniae*, (D) *P. aeruginosa* PAO1, (E) *P. protegens* Pf-5 and (F) *V. cholerae*, were incubated in the absence and presence of *B. bacteriovorus* UP for 24 h. The biofilm biomass was quantified by crystal violet staining. Each value represents the mean of three replicates from one representative experiment, with error bars representing the standard error of the mean. Experiments were repeated three times with similar results each time.

The effects of predation by *B. bacteriovorus* UP on biofilms formed by these model bacteria were also assessed. Biofilms formed by these bacteria were also found to be vulnerable to predation by *B. bacteriovorus* UP, where most of the biofilms were reduced by >50% after 24 h incubation with the predators (Figure 2.10). Biofilms formed by *P. aeruginosa* PAO1 were again shown to be the most resistant to *B. bacteriovorus* UP, with only about a 30% reduction of total biomass. The reduction in biofilm biomass (~50%) was not as great as was observed for the planktonic cells

(10 to 1,000 fold reduction) suggesting that biofilm growth may protect these species from predation by *B. bacteriovorus* UP relative to growth as free-living, planktonic cells.

2.4 DISCUSSION

Since the first isolation of *Bdellovibrio* from a soil sample [59], *Bdellovibrio* spp. have been isolated from various environments, such as rivers and marine waters, etc., and have also been found in man-made environments, such as activated sludge in wastewater treatment plants [63-65]. Although these predators have been found to be associated with most habitats that have been studied, there are relatively few studies investigating their community abundance or their ecological functions in such environments. In engineered environments, such as wastewater treatment systems, the desired functions are dependent on the selection and maintenance of key microbial species with in the communities [135-137]. Whilst generally robust, such systems are regularly subjected to disturbance and hence lose their desired functions, and in some cases the causes of disturbance are understood, such as the input of industrial contaminations, whilst in other cases, the loss of functions is not well explained. It has been hypothesized that the action of predatory microorganisms such as bacteriophages or microbial predators may account for some of the performance disturbance of wastewater treatment systems [54, 184]. In addition, based on saturation sequencing, it was observed that the microbial predators belonging to the group of BALOs were present in the Ulu Pandan Wastewater Reclamation Plant systems (Prof. Cohen, personal communication). Therefore, to determine the potential impact of BALOs on the microbial communities in wastewater treatment systems, BALOs species were isolated from such systems for further detailed investigation.

Although *Bdellovibrio* spp. have been broadly detected in various environments, it has been shown that these predators are relatively low in abundance due to their unique parasitic growth requirements [65]. The in situ quantification of *Bdellovibrio* spp. in various environments is not commonly examined since they do not survive well as pure cultures without suitable prey species and the direct quantification methods, based on plaque formation, are generally laborious and are not quantitative [89]. The prey species selected for quantification of *Bdellovibrio* spp. as by plaque

formation units may also represent an additional bias against some species of these predators [65], much in the same way that quantification of bacteriophages in dependent on selection of the appropriate hosts. Because of these difficulties, the approaches taken in this study currently cannot be used to accurately quantify the numbers of BALOs in the original activated sludge samples. However, based on total microbial community sequencing and comparison of 16S rRNA gene sequence reads that can be attributed to BALOs, the Ulu Pandan Wastewater Reclamation Plant appears to have relatively low abundance of these predators (Prof. Cohen, personal communication).

The plaques formed by the BALOs species can be distinguished from plaques formed by bacteriophages based on their characteristics. For example, plaques that have irregular borders, in contrast to the typically defined borders formed by bacteriophages, as well as the increase in size of the plaques, which does not happen for bacteriophages, were critical in identifying those plaques as being associated with BALOs. Subsequently, individual plaques typical of those formed by BALOs (based on comparison with published results for known BALOs species [95, 171]) were picked and sub-cultured several times to purify the predatory species. There is no reported methodology for differentiating plaques formed by different species of BALOs and it may be that more careful examination and standardization of timing of plaque characterization could yield useful, morphologically useful descriptions. Here, plaque sizes were measured by a ruler for ~50 plaques for each putative BALOs species. Whilst this measure is probably not sufficient to identify individual BALOs, it is useful, similar to comparing colony morphologies of bacteria grown on agar plates, to differentiate predators with clearly distinct plaque morphologies. For example, despite the limitations of plaque morphologies for the identification of BALOs species, the two isolated predatory species reproducibly formed plaques with distinct features. The possibility that the two different plaque morphologies were the consequence of having isolated two different species was supported by the FISH probe results and the sequencing data, both of which indicated that the two isolated species were different.

Based on the differences in morphologies of plaques formed on the individual prey lawns and the in situ hybridization results, it was primarily concluded that at least two different types of bacterial predators were present in the Ulu Pandan Wastewater Reclamation Plant in Singapore. Whilst the two predatory species were initially

identified on different prey lawns, both predators can form plaques of similar morphology on both *K. pneumoniae* and *P. protegens* Pf-5 lawns. Both of the predators formed plaques on lawns of *K. pneumoniae* and *P. protegens* Pf-5, suggesting that the isolated predators might have prey ranges that extend across multiple bacterial species. The plaques formed by 'BALOs #2' were also consistent with those formed by relatively well-studied *Bdellovibrio* spp., such as *B. bacteriovorus* HD100 and *B. bacteriovorus* 109J [89, 91], suggesting 'BALOs #2' is closely related to the group of *Bdellovibrio* spp.

FISH and 16S rRNA gene sequence analysis was performed to determine the phylogenetic classification and the identities of these predatory species. For the FISH assays, the group-specific probe ALF968 targeting Alpha-*Proteobacteria* and genus-specific probe BDE525 targeting known *Bdellovibrio* spp. of Delta-*Proteobacteria* was individually used to hybridize with the isolated predators [173, 175]. The results suggested that the two isolated predators separately belonged to the Alpha-*Proteobacteria* (BALOs #1) and the genus of *Bdellovibrio* (BALOs #2).

To date, there are two known groups of predatory bacteria belonging to Alpha-*Proteobacteria*. *Ensifer adhaerens* is an isolate from soils, which can attach to and lyse other bacteria [185]. Despite its predatory feeding mechanism, this bacterium is not an obligate predator, as it can also be cultured axenically in different laboratory media. *E. adhaerens* differs from other known predators as it exhibits predatory activity towards *Micrococcus luteus* (Gram-positive) as well as Gram-negative bacteria [185]. Since the predator 'BALOs #1' did not match *E. adhaerens* based on the 16S rRNA gene sequence similarity or prey range (data not shown), this isolated predator is unlikely to represent a strain of *E. adhaerens*. Another group of predators in this class are the *Micavibrio* spp., which are obligate predatory bacteria upon other Gram-negative bacteria. Two species in this genus have been described, both of which have been isolated from wastewater plants [104]. They do not penetrate into the prey's inner compartments, but rather function by adhering to the prey's surface and 'sucking' the nutrients from the attached prey cell. The probe of ALF968, targeting species of Alpha-*Proteobacteria*, is not specific at the genus level; therefore the hybridization results alone cannot identify this Alpha-proteobacterial predator [175]. The closet match to the putative Alpha-proteobacterial predator was an 'uncultured bacterium', neither *E. adhaerens* nor *Micavibrio* spp., which was

detected in environmental samples [182]. Therefore, this isolated species probably represents a novel predatory bacterium.

In contrast, hybridization results using the genus-specific probe BDE525 with the 'BALOs #2' and the 16S rRNA gene sequence data clearly indicated that this isolated species was a member of the genus *Bdellovibrio*. The closest match for the isolated *Bdellovibrio* predator was '*B. bacteriovorus* strain Tiberius', which was isolated from the River Tiberius at Rome [183].

The full-length of the 16S rRNA genes (~1500 bps) for both isolated predatory bacteria were sequenced here, and the representative gene sequences for each predatory species were searched against the GenBank database to identify the closest matching organisms. The percentage match for BALOs #2 was 99% nucleotide identity with *B. bacteriovorus* str. Tiberius and >97% for all other *B. bacteriovorus* strains. Therefore, given that the accepted cut-off for 'species' is >97% [179], the sequence data clearly supported this isolated bacterium as being a member of the *B. bacteriovorus* species. Given the close match to *B. bacteriovorus* str. Tiberius, it was not entirely clear if this was the same strain or if 'BALOs #2' represented a new strain. This will be best resolved by whole genome sequencing, which was attempted for 'BALOs #2'. However, the genome assembly was of poor quality and could not be used for comparison (data not shown). Considering the very different sources of isolation, where *B. bacteriovorus* str. Tiberius was isolated from the River Tiberius (Rome, Italy) and 'BALOs #2' was found in a wastewater treatment plant in Singapore, it was appropriate to give this species a unique identification here, *B. bacteriovorus* UP. A formation strain designation will only be possible upon a more thorough characterization of *B. bacteriovorus* UP with other *B. bacteriovorus* strains including *B. bacteriovorus* str. Tiberius.

The growth pattern of the isolated *Bdellovibrio* species is one of the most important characteristics when study the predation effects of *Bdellovibrio* on bacteria in biofilms of various types. Traditional methods for studying the growth pattern have been based on serial dilution of samples and incubation on plates with prey lawns to form lytic plaques [163, 164]. However, the results may not be accurate because a single plaque can be derived from multiple *Bdellovibrio* cells. Therefore, molecular methods, such as in situ hybridization and image quantification, were used here to study the growth pattern of *B. bacteriovorus* UP on given prey species.

The growth assay was performed for three individual biological replicates, and one representative growth curve was shown. The data were generally consistent, where the growth rates, time to reach maximum numbers and decline phases occurred in the same time frames showing that the growth patterns were consistent across the three individual biological replicates (data not shown). Although there were some variations between experiments as anticipated, these were not considered to be biologically relevant here nor were they discussed since they were not reproducible. The growth pattern of *B. bacteriovorus* UP established using *P. protegens* Pf-5 as prey here was similar with the pattern demonstrated by Lotka-Volterra equation, which describes predator-prey interactions at macro scale [10]. This equation demonstrates the correlated population fluctuation of predator and prey in an open system with adequate nutrients for both predator and prey to survive. However, in this study, the growth pattern of *B. bacteriovorus* UP was assessed with single prey species in an enclosed system (conical flasks) and without continuous nutrient or prey species input [8, 10]. Therefore, it was observed that with the decrease of prey (*P. protegens* Pf-5) cell numbers, the cell numbers of the predator (*B. bacteriovorus* UP) decreased accordingly. The growth pattern of 'BALOs #1' using *K. pneumoniae* as prey was also investigated, and the results showed similar pattern with *B. bacteriovorus* UP incubated with *P. protegens* Pf-5 (data not shown). Similar predator-prey dynamics were reported for *B. bacteriovorus* C–1 when grown on *Aeromonas hydrophila* [186]. During the 90 h experiment, it was also observed that the numbers of *B. bacteriovorus* C–1 showed an exponential increase starting approximately 20 h after inoculation, whilst the numbers of prey began to decrease exponentially at the same time.

Bdellovibrio spp. generally have broad prey spectra consisting of Gram-negative bacteria [163, 164], and *B. bacteriovorus* UP demonstrated the capacity to feed on a broad spectrum of prey, which was shown in the assay of the effects of predation by *B. bacteriovorus* UP on some well-studied laboratory model bacteria in both planktonic and biofilm growth.

For these model bacteria, significant reductions of CFUs (10 to 1,000 fold, $P < 0.01$) in planktonic growth and reductions of biomass (> 50%, expect *P. aeruginosa* PAO1) were observed, which indicated the efficient predation by *B. bacteriovorus* UP. Of these prey species, *E. coli*, *K. pneumoniae* and *P. protegens* Pf-5 were the most sensitive to *B. bacteriovorus* UP, whilst *P. aeruginosa* PAO1 was shown to be the

most resistant of the prey. Additionally, the decrease of prey cells stopped or slowed markedly when the prey species reached < 10^4 – 10^5 cells/mL. Similar results were stated in other studies, in which minimal prey cell concentration of ~10^5 cells/mL was required to sustain the survival of *Bdellovibrio* spp. [71], which suggested that the predators may be able to sense the concentration of prey cells.

The significant reduction of CFUs for the prey species suggested that the biofilms biomass reduction was primarily due to the predation by *B. bacteriovorus* UP lysing prey cells in biofilms and hence reducing the biomass contents, instead of the cell dispersal from biofilms driven by predation pressure [177, 187]. However, at least 50% of the biofilm biomass of each species was retained, suggesting that the biofilm may protect the prey cells to some extent [109, 125]. The mechanism of this biofilm specific protection is not clear, but could be related to physical protection afforded by the biofilm matrix or could be the consequence of expression of biofilm specific genes. For *V. cholerae*, it has been shown that protection from predation by protozoa was induced in biofilms and was likely to be related to the production of quorum sensing regulated gene expression [177].

Whilst two predatory bacteria were isolated here, it is likely that more predatory bacterial species may be present in the activated sludge community [66, 77]. However, because of the methodological challenges, no other predatory bacterial species were isolated in this study. Additionally, due to the relatively low abundance of *Bdellovibrio* populations in the microbial communities, as indicated by the total community sequencing data, it is difficult to accurately quantify the numbers of *Bdellovibrio* spp. in the original sludge samples using the plaque formation assay.

Future work aimed at the quantification of these predators directly in sludge samples should focus on more detailed community sequencing as well as using quantitative PCR methods targeting specific predator groups. Whilst these approaches will be useful to identify and quantify known predators, they are poorly suited to identifying new predators and thus, culture based methods are still essential tools in this process. Additionally, efforts are required for the detailed investigation of the impact of these predators on the communities where they live in, e.g. natural and engineered systems.

The work presented here has isolated two distinct predators and investigated the growth rate of the species *B. bacteriovorus* UP, which demonstrated a classic predator-prey feeding response, where the predators numbers decline in parallel with declining numbers of available prey.

Chapter III. THE EFFECTS OF *BDELLOVIBRIO* PREDATION ON BACTERIAL SPECIES ISOLATED FROM WASTEWATER

3.1 INTRODUCTION

Predation plays an important role in structuring the ecology of biological communities [1, 5]. The impacts of predation have been broadly studied amongst higher organisms [6-8], whilst there have been fewer studies on the role of predation of prokaryotes, despite predation being one of the most important factors contributing to the mortality of bacteria in the environment. Predation of bacteria is primarily by protozoa [15], however it is now understood that at least one specialized group of predatory bacteria, the *Bdellovibrio*, also prey upon bacteria as their primary food sources.

The characteristics, mechanisms and genetics of predation by model member(s) of *Bdellovibrio*, such as *B. bacteriovorus* 109J, on prey bacterial species have been studied for both planktonic and biofilm cells. The results showed that prey cells both in planktonic and biofilms were susceptible to *Bdellovibrio* attack [162, 164]. Whilst there have been many fundamental studies of *Bdellovibrio* mediated predation, most of these studies have only focused on model laboratory bacterial strains [73, 178]. Such studies have demonstrated that bacterial strains, such as *E. coli*, *K. pneumoniae*, *Pseudomonas* spp. and *Vibrio* spp. are susceptible to predation by *Bdellovibrio* spp. [162]. Whilst the *Bdellovibrio* spp. were shown to prey on a broad spectrum of prey bacteria, these predators do exhibit some preference for specific bacteria when offered an option. For example, five different *Bdellovibrio* strains were tested against 22 different bacterial species and it was found that no single species could support the growth of all five *Bdellovibrio* strains. In that study, *B. bacteriovorus* 109J was found to mainly target species of *Pseudomonas* and *Enterobacter*, whilst *B. bacteriovorus* strain TRA2 mainly preyed upon species of *Rhizobium* and *Sinorhizobium* [164].

While there is a good understanding of how *Bdellovibrio* spp. prey upon model bacteria, the effects of such microbial predators on co-occurring bacterial species are much less understood, especially in complex communities. The most relevant studies

concerning prey bacteria of environmental origin have primarily investigated the prey range for different *Bdellovibrio* species using diverse bacterial prey species directly isolated from natural environments, such as soil and marine waters [163]. However, few studies have focused on wastewater environments. Previous prey range studies showed that *Proteobacteria* were generally sensitive to predation by *Bdellovibrio* spp. [162]. Given the functional bacteria in wastewater activated sludge, such as nitrifying bacteria and phosphorus accumulating bacteria [128, 138] are primarily dominated by members of this phylum, it is reasonable to predict that such predators could play a significant role in modifying the microbial communities of wastewater treatment systems. This is important because the influence on abundance and viability of members of *Proteobacteria* by predation of *Bdellovibrio* may significantly affect the related performance of the wastewater treatment process.

Previous studies using model *Bdellovibrio* strains have demonstrated that single model species both in planktonic and biofilm mode of growth were susceptible to these predators [68, 73]. However, the predation efficiency and effects of the *B. bacteriovorus* UP strain upon the prey species in activated sludge, either as planktonic cells or biofilms are poorly understood. Biofilm growth might provide protection from predation by the *B. bacteriovorus* UP strain whilst their planktonic counterpart might not be protected.

To understand the significance of *Bdellovibrio* based predation on the microbial community from activated sludge, experiments were designed to first examine the effect of these predators on individual species (78 individual species, including some fungal species). Biofilms and planktonic cells were evaluated to compare their response to predation by the *B. bacteriovorus* UP. Subsequently, dual-species communities were also tested to investigate their sensitivity to predation. Such experiments aim to determine if microbial species in communities of mixed-species can offer protection to members of the communities sensitive to predation compared with mono-species populations.

3.2 MATERIALS AND METHODS

3.2.1. Growth conditions for *Bdellovibrio bacteriovorus* UP

B. bacteriovorus UP was routinely grown and enriched in DNB medium with *P. protegens* Pf-5 as prey as described in Chapter II. Briefly, the predators were at the highest concentration (cell numbers per milliliter) after 48 h when cultivated with prey cells at 30°C with shaking (200 rpm). *B. bacteriovorus* UP cells were collected through low-speed centrifugation (2,000 *g* for 10 min) to remove prey cell debris, followed by filtration of the supernatant through 0.45 μm membrane filters (Acrodisc® syringe filters, PALL Corporation) twice. The filtrate contained the enriched *B. bacteriovorus* UP cells. Part of the filtrate was further passed through 0.2 μm membrane filters (Acrodisc® syringe filters, PALL Corporation) three times, and the subsequent filtrate contained no *B. bacteriovorus* UP cells, which was used as a negative control.

3.2.2. Preparation of planktonic and biofilm prey cells

Prey bacterial species were originally isolated by SCELSE researchers from activated sludge collected from Ulu Pandan Wastewater Reclamation Plant, Singapore [188] (Table 3.1). These species were initially collected, identified for the breadth of microorganisms present and their quorum sensing behaviours, which was not relevant to the study presented here [188]. These microorganisms were, however, relevant for defining the prey range of *B. bacteriovorus* UP as they represent a significant portion of the culturable microbial community co-occurring with *B. bacteriovorus* UP and also represent a broad range of taxa, including members from all of the *Proteobacteria* domains, *Firmicutes* and fungi. These species were grown and maintained on LB (Bacto™ LB) nutrient agar plates. To assess the predatory effects of *B. bacteriovorus* UP on these species in biofilm growth, a single colony of the prey species was first inoculated into 2 mL LB liquid medium at 30°C with shaking at 200 rpm for 18 h. Cultures were subsequently diluted in 1:50 (v/v) into fresh LB liquid medium (total volume 10 mL) at 30°C with shaking at 200 rpm for 24 h. The cultures were then diluted with fresh LB liquid medium to achieve an optical density (OD_{600}) of 0.1.

Diluted cells were distributed in 1 mL aliquots into the wells of 24 well micro-titer plates (Costar®, 24 well clear, not tissue culture treated). The micro-titer plates were then incubated on an orbital shaker at room temperature (25 – 26°C) with shaking at 100 rpm for 24 h to form bacterial biofilms [177]. Afterwards, the biofilms were carefully washed twice with DNB to remove any planktonic cells, and 1 mL of

prepared *B*. *bacteriovorus* UP cell suspension (adjusted to 10^8 cells/mL) was added into three wells for each bacterial species. As a negative control, 1 mL of sterilized medium, prepared by passing the *B*. *bacteriovorus* UP cell suspension through 0.2 µm membrane filters three times, was added into another three wells. The micro-titer plates were subsequently incubated at 30°C with shaking at 100 rpm for 24 h [73, 178]. Preliminary experiments showed that bacteria formed biofilms at the air-liquid interface and on the bottom of the micro-titer wells and these pre-formed biofilms survived in DNB medium over the 72 h experimental period (data not shown).

Quantification of the biofilm biomass with and without *B*. *bacteriovorus* UP treatment was performed by crystal violet staining. After 24 h incubation, the wells of micro-titer plates were washed three times with DNB to remove any planktonic cells. The wells were subsequently filled with 1 mL crystal violet (0.1% in dH_2O) for 15 min. The crystal violet solution was then discarded and the stained wells were carefully washed twice with saline solution (0.85% NaCl) to remove any unbound crystal violet residue. To dissolve the bound crystal violet, 1 mL of 95% ethanol was added into each well for at least 15 min. The amount of crystal violet, a representative measure of the biomass of biofilm, was quantified at 590 nm using a micro-titer plate reader (TECAN, INFINITE® 200, PRO). Each treatment (negative control and treatment) had three replicates in each experiment and each experiment was conducted three times (three biological replicates).

Table 3.1 Microorganisms used in this study

Class of Microorganisms	Identities of microbial strains *
Alpha-*Proteobacteria*	*Agrobacterium tumefaciens*
	Bosea sp. strain FF6
	Bosea sp. CRIB-12
	Bosea sp. dv-3
	Bosea sp. LJY4
	Brevundimonas sp. SGJ
	Brevundimonas sp. NBRC 101767
	Mesorhizobium sp. IV-48
	Ochrobactrum anthropi
	Rhizobium borbori
	Rhizobium Unc. Bacterium clone
	Rhodobacter gluconicum
	Rhodobacter maris
	Rhodobacter sp. XJ-1

(Table 3.1 continued) Alpha-*Proteobacteria*	*Roseomonas* Unc. Bacterium clone
	Shinella fusca
	Sphingomonas Unc. Bacterium clone
	Sphingobium sp. DSP-1
Beta-*Proteobacteria*	*Achromobacter xylosoxidans*
	Achromobacter sp.
	Acidovorax delafieldii
	Acidovorax facilis
	Alcaligenes sp. LSBA1977
	Delftia tsuruhatensis
	Diaphorobacter nitroreducens
	Unc. *Hydrogenophaga* sp.
	Paludibacterium ongneupense
	Thauera selenatis
	Variovorax paradoxus
Gamma-*Proteobacteria*	*Acinetobacter* sp. 409
	Acinetobacter schindleri strain M1-2
	Acinetobacter sp. 8A18N1
	Aeromonas caviae
	Citrobacter freundii
	Dokdonella enrichment clone phytdeg60
	Enterobacter sp. VITNC1
	*Frateuria*sp. Ni-H2-1
	Klebsiella sp. Unc. Clone
	Lysobacter brunescens
	Pantoea ananatis
	Pantoea stewartii
	Pseudomonas otitidis
	Pseudomonas sp.
	Pseudoxanthomonas japonensis
	Pseudoxanthomonas Unc.Bacterium clone
	Rheinheimera Unc. Bacterium clone
	Stenotrophomonas sp.
	Stenotrophomonas maltophilia YSP48
	*Stenotrophomonas maltophilia*SPd
	Stenotrophomonas maltophilia CCF0025
	Stenotrophomonas sp. strain M2
Bacteroidetes	*Chryseobacterium* sp.
	Dyadobacter fermentans
	Elizabethkingia anophelis
	Flavobacterium sp. NL124

(Table 3.1 continued) *Bacteroidetes*	*Flavobacterium* sp.WG1
	Sphingobacterium multivorum strain IAM14316
	Sphingobacterium multivorum strain DW-1
	Sphingobacterium mizutaii strain NBRC 14946
	Sphingobacterium mizutaii
Actinobacteria	*Agromyces* sp.
	Brevibacterium aureum
	Microbacterium flavum
	Microbacterium laevaniformans
	Microbacterium oxydans
	Microbacterium sp.
	Pimelobacter simplex
	Rhodococcus erythropolis
	Tsukamurella tyrosinosolvens
Firmicutes	*Bacillus megaterium*
	Bacillus sp. NB22
	Bacillus sp. TSWCSN16
	Staphylococcus condimenti
	Staphylococcus haemolyticus
Fungus	*Cryptococcus curvatus strain* ATCC 10567
	Candida tropicalis
	Trichosporon montevideense
	Candida sojae

* The identities of listed microorganisms were based upon comparison of 16S/18S rRNA sequences using basic local alignment searching tool (BLAST) against sequences present in GenBank. Sequence identities of > 99% were used as the cutoff for the species assignments. The list is adopted from [188].

3.2.3. Vulnerability of mixed-species communities to predation by *Bdellovibrio bacteriovorus* UP

The vulnerability of dual-species communities towards predation by *B*. *bacteriovorus* UP was assessed for both the planktonic and biofilms cells. These dual species biofilms were composed of an Alpha-*Proteobacteria* species, *Ochrobactrum anthropi*, which was found to be resistant to predation by *B*. *bacteriovorus* UP (Section 3.3, Figure 3.1), whilst the other species were sensitive (Table 3.2).

The bacteria were individually prepared as described above. Briefly, bacteria were grown in fresh LB liquid medium and collected by centrifugation (8,000 *g*, 5 min). The pellet was re-suspended in DNB liquid medium and these cells were diluted to an

OD_{600} of 1.0 with DNB for subsequent experiments. Planktonic cultures of dual-species communities were then prepared by evenly mixing cell suspensions of two bacterial species in equal volumes. The mixed planktonic cells were evenly divided as 10 mL aliquots into two sets of 50 mL tubes. One set was also incubated with *B. bacteriovorus* UP cells (final concentration 10^8 cell/mL) and another set was incubated with *Bdellovibrio*-free medium as negative controls. Each treatment had three replicates for each experiment and each experiment was repeated three times.

Dual-species biofilms were also developed in the same manner. The bacterial species were individually grown in LB liquid medium, and diluted to an OD_{600} of 0.1 with fresh LB [189]. The diluted cells of the two bacterial species were then mixed evenly in equal volumes. The mixed cell suspensions were then pipetted into 24 well micro-titer plates (1 mL aliquot for each well). The micro-titer plates were incubated at room temperature (25 – 26°C) with shaking at 100 rpm for 24 h to form dual-species biofilms. The preformed, dual-species biofilms were also subsequently incubated with (treatment) and without (negative control) *B. bacteriovorus* UP cells. Each treatment (negative control and treatment) had three replicates for each experiment and each experiment was also repeated three times.

Table 3.2 Combinations of bacterial species used for mixed-species biofilms

	Bacterial species	Phylogenetic Class
Each pair-wise combination included this resistant species	*Ochrobactrum anthropi*	Alpha-*Proteobacteria*
The other one member was chosen from the following:	*Roseomonas* Uncul. clone	Alpha-*Proteobacteria*
	Rhizobium borbori	
	Shinella fusca	
	Chryseobacterium spp.	*Bacteroidetes*
	Sphingobacterium multivorum	
	Delftia tsuruhatensis	Beta-*Proteobacteria*
	Achromobacter spp.	
	Stenotrophomonas maltophilia	Gamma-*Proteobacteria*
	Acinetobacter sp. 409	
	Pantoea stewartii	
	Stenotrophomonas spp.	

3.3 RESULTS

3.3.1 The effects of predation by *Bdellovibrio bacteriovorus* UP on bacterial species isolated from wastewater samples

All of the model prey bacteria were determined to be vulnerable to predation by *B. bacteriovorus* UP (Chapter II). To better understand the effects of predation by *B. bacteriovorus* UP on ecologically relevant microorganisms and microbial communities, it is necessary to use relevant prey species isolated from the same habitat of *B. bacteriovorus* UP. Therefore, 51 species of *Proteobacteria* (18 Alpha-, 11 Beta- and 22 Gamma-*Proteobacteria*), 9 species of *Bacteroidetes*, 9 species of *Actinobacteria*, 5 *Firmicutes*, and 4 fungal species isolated from activated sludge collected from the Ulu Pandan Wastewater Reclamation Plant were also investigated for their susceptibility to predation when grown as mono-species biofilms. The results showed that all of the *Bacteroidetes* and almost all of the *Proteobacteria* species were sensitive to predation where the amount of biofilm biomass after predation treatment was statistically significantly ($P < 0.05$) lower than the biomass in the negative controls (Figure 3.1, 3.3 – 3.5).

Overall, almost all of the species tested formed biofilms ($OD_{590} > 0.5$), although the actual amount of biofilm biomass differed between species. Generally, strains of the same species produced similar amounts of biofilm. For example, in the absence of the predator, the biofilm biomass of *Acinetobacter* sp. 409 was high ($OD_{590} > 3.0$), whilst biofilm biomass of *A. schindleri* strain M1-2 and *Acinetobacter* sp. 8A18N1 was approximately 1.0 (Figure 3.4). Different strains of the same species tended to form similar amounts of biofilm biomass, although this was not the case for members of the genus *Strenotrophomonas*, where the biomass differed markedly amongst strains (Figure 3.4).

Twenty (out of 51) bacteria showed strong biofilm production ($OD_{590} > 1.0$ in controls), including *Brevundimonas* sp. SGJ, *O. anthropi*, *R. borbori* (Figure 3.1), as well as *Acinetobacter* sp. 409, *Aeromonas caviae*, *P. otitidis*, *Rheinheimera* sp. and *Stenotrophomonas* sp. strain M2 (Figure 3.4). In contrast, more than 20 species were relatively poor biofilm formers ($OD_{590} \leq 0.5$ in controls), such as *Bosea* sp. strain FF6, *Sphingobium* sp. DSP-1 (Figure 3.1), *Achromobacter xylosaxidans* (Figure 3.3)

and *Dyadobacter fermentans* (Figure 3.5). Bacteria with high levels of biofilm biomass were selected for subsequent dual-species biofilm assays.

Significantly, an Alpha-proteobacterial species, *O. anthropi*, was resistant to predation by *B. bacteriovorus* UP (Figure 3.1). This bacterium was the only tested Gram-negative species to be completely resistant to predation during growth as a biofilm in this assay. Further, when tested in the dual-species community assays, *O. anthropi* was also resistant to predation (see section 3.3.2). Whilst the rest members of this phylum, including genera such as *Bosea*, *Brevundimonas*, *Rhizobium*, *Rhodobacter* and *Sphingomonas*, were sensitive to predation when grown as biofilms. Strains of *Bosea* not only showed varied biofilm biomass production, but also showed differential sensitivity to predation by *B. bacteriovorus* UP. For example, *Bosea* sp. dv3 showed ~40% biomass reduction, whilst biofilms of *Bosea* sp. CRIB-12 were reduced more than 60% of biomass. Whilst the biofilm biomass production by *Brevundimonas* strains differed considerably amongst species, the biofilm biomass reduction upon predation was similar across the different species, ranging from 50% – 55% reduction in biofilm biomass. Similar results were also obtained from the different species of *Sphingomonas* and *Rhodobacter* tested in this study. In general, there was a significant effect of predation on biofilm cells for the Alpha-proteobacterial species, which suggested that the members of Alpha-*Proteobacteria* could be one of the main food sources for *B. bacteriovorus* UP in wastewater.

To further explore the resistance of *O. anthropi* to predation, biofilms of four strains of *Ochrobactrum* were tested for their resistance to predation. In addition, these experiments included *Shinella fusca* (sensitive to predation, Figure 3.1) and *Microbacterium oxydans* (Gram-positive, resistant to predation, Figure 3.6) as controls. The four *Ochrobactrum* strains all showed resistance to predation by *B. bacteriovorus* UP, as no biomass reduction was observed, whilst a significant biomass reduction was observed for *S. fusca* biofilms and no biofilm reduction was observed for the Gram-positive species, *M. oxydans* as expected (Figure 3.2).

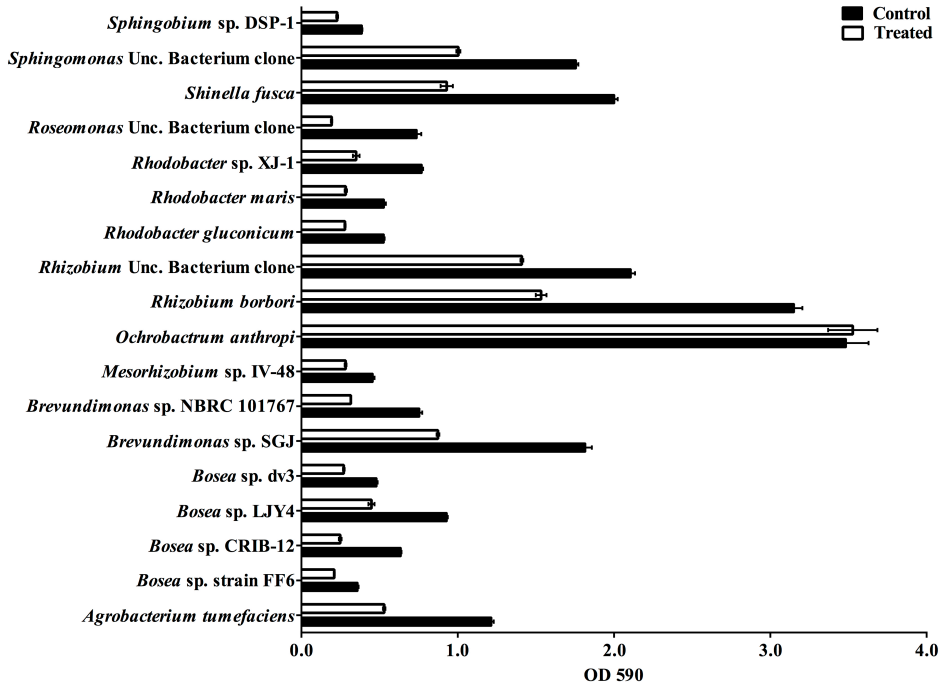

Figure 3.1 The effects of predation by *B. bacteriovorus* UP on Alpha-*Proteobacteria*. Preformed biofilms were incubated with *B. bacteriovorus* UP cells (treated) and *Bdellovibrio*-free medium (control) for 24 h. Biofilms were quantified by crystal violet and measured at 590 nm. Data represent the means of three replicates of one representative experiment, with error bars representing the standard error of the mean.

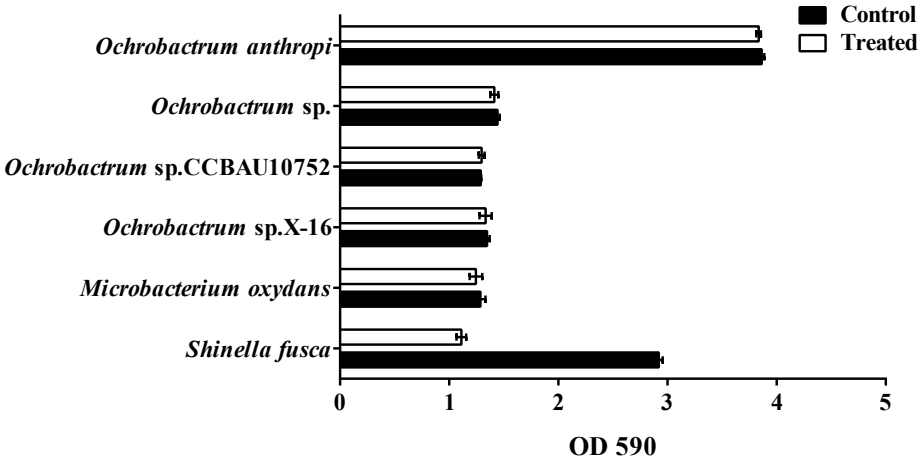

Figure 3.2 The effects of predation by *B. bacteriovorus* UP on *Ochrobactrum* species and *Microbacterium oxydans* and *Shinella fusca*. Preformed biofilms were incubated with *B. bacteriovorus* UP cells (treated) and *Bdellovibrio*-free medium (control) for 24 h. Biofilms were quantified by crystal violet and measured at 590 nm. Data represent the means of three replicates of one representative experiment, with error bars representing the standard error of the mean.

Eleven species of Beta-*Proteobacteria* were tested for their responses to predation by *B. bacteriovorus* UP (Figure 3.3). Except for *Hydrogenophaga* sp. and *Acidovorax facilis*, most of the Beta-proteobacterial species (9 out of 11) formed weak or little biofilm biomass (with OD_{590} of 0.4 – 0.5 in controls) under the experimental conditions used here. In addition to making poor biofilms, these species were also generally sensitive to predation by *B. bacteriovorus* UP (40% – 60% biomass reduction), with the exception of *Paludibacterium*, which showed only an 18% reduction of biofilm biomass. Two species of *A. facilis* and *Acidovorax delafieldii* showed similar vulnerability to *B. bacteriovorus* UP and were the most vulnerable to predation by *B. bacteriovorus* UP, where their biofilms were reduced by more than 55%.

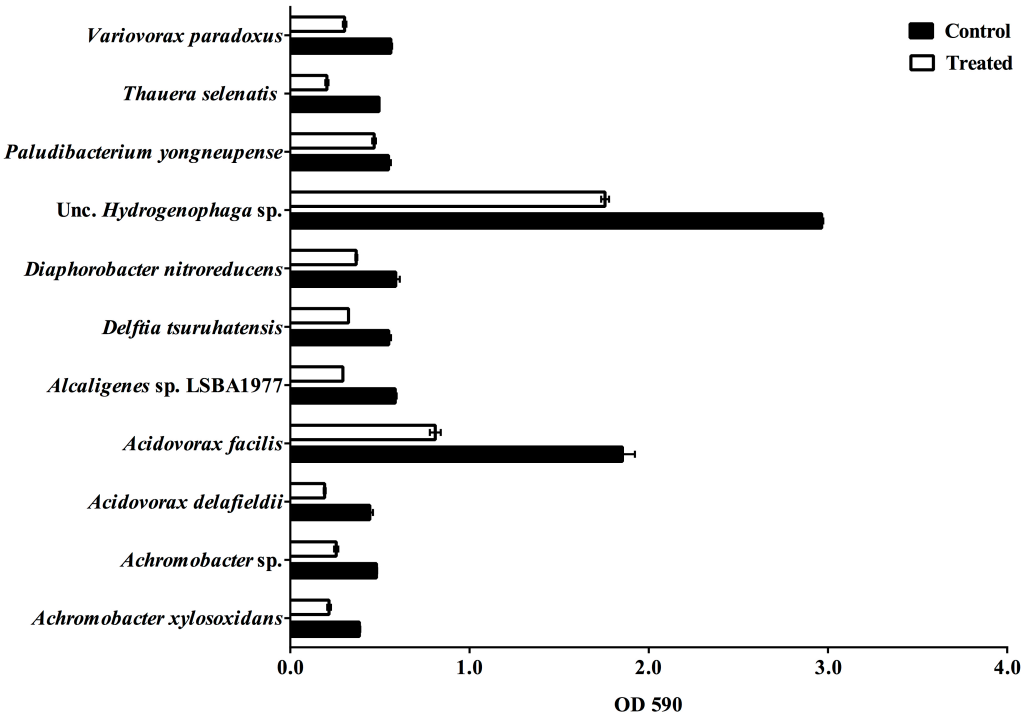

Figure 3.3 The effects of predation by *B. bacteriovorus* UP on Beta-*Proteobacteria*. Preformed biofilms were incubated with *B. bacteriovorus* UP cells (treated) and *Bdellovibrio*-free medium (control) for 24 h. Biofilms were quantified by crystal violet and measured at 590 nm. Data represent the means of three replicates of one representative experiment, with error bars representing the standard error of the mean.

Twenty-two species of Gamma-*Proteobacteria* were tested for their sensitivity to predation by *B. bacteriovorus* UP. The majority of these species, such as

Acinetobacter sp. 409, *Aeromonas caviae*, *Pantoea ananatis*, *P. otitidis* and *Stenotrophomonas* sp. strain M2, were strong biofilm formers (Figure 3.4, with OD_{590} > 2.0 in controls). All of these species tested were vulnerable to predation by *B. bacteriovorus* UP with biomass reductions ranging from 30% (*Stenotrophomonas maltophilia* CCF0025) to more than 80% (*Acinetobacter* sp. 409). Most of these species showed >40% biomass reduction of biofilms, with some biofilms being reduced by more than 70% (*Citrobacter freundii* and *Stenotrophomonas* sp. strain M2). Amongst these species, *Acinetobacter* sp. 409 with > 80% biofilm reduction, was the most vulnerable to predation by *B. bacteriovorus* UP (Figure 3.4). The biofilm biomass produced by *Stenotrophomonas* sp. strain M2 was the highest amongst members of this genus (OD_{590} > 2.5 in controls), and these biofilms were reduced ~70% when incubated with *B. bacteriovorus* UP. In contrast, *S. maltophilia* strain CCF0025 produced relatively less biofilm (OD_{590} ~0.6 in controls) and showed approximately a 30% biofilm reduction upon incubation with *B. bacteriovorus* UP.

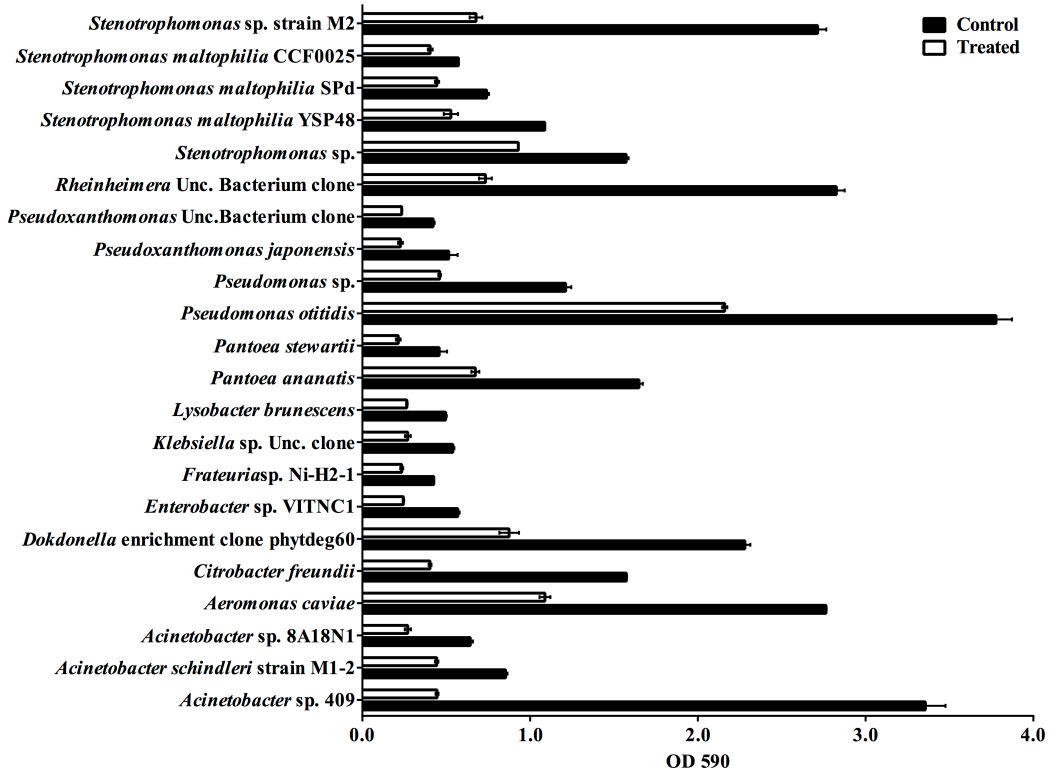

Figure 3.4 The effects of predation by *B. bacteriovorus* UP on Gamma-*Proteobacteria*. Preformed biofilms were incubated with *B. bacteriovorus* UP (treated) and *Bdellovibrio*-free medium (control) for 24 h. Biofilms were quantified by crystal violet and measured at 590 nm. Data represent the means of three replicates of one representative experiment, with error bars representing the standard error of the mean.

Nine species of *Bacteroidetes* were tested for predation by *B. bacteriovorus* UP (Figure 3.5). Members of this group were largely associated with the genera of *Sphingobacterium* and *Flavobacterium*. None of these species formed substantial amounts of biofilm (with OD_{590} < 0.6 in controls), and all of these species were vulnerable to predation by *B. bacteriovorus* UP. Biofilm biomass reductions ranged from 30% (*S. multivorum* strain DW-1 and *S. mizutaii*) to 55% (*Chryseobacterium* sp.). The biofilm biomass production by species of *Sphingobacterium* ranged from 0.5 – 0.6 (OD_{590} in controls) with biomass reductions ranging from 30% – 45%. Similar patterns were also observed between species of *Flavobacterium*.

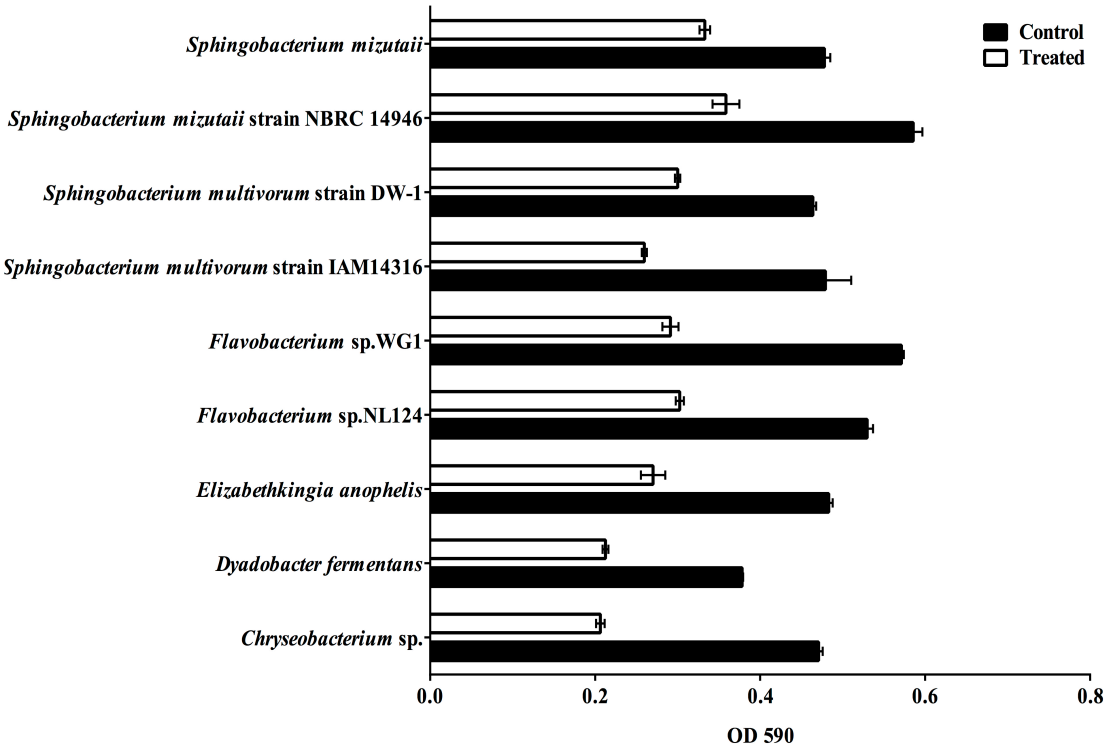

Figure 3.5 The effects of predation by *B. bacteriovorus* UP on *Bacteroidetes* species. Preformed biofilms were incubated with *B. bacteriovorus* UP cells (treated) and *Bdellovibrio*-free medium (control) for 24 h. Biofilms were quantified by crystal violet and measured at 590 nm. Data represent the means of three replicates of one representative experiment, with error bars representing the standard error of the mean.

Additionally, 4 fungal species, 5 species of *Firmicutes* and 9 species of *Actinobacteria* were also tested for their biofilm formation and response to predation by *B. bacteriovorus* UP (Figure 3.6, from top to bottom). Some of these species formed high amounts of biofilm biomass, including *Brevibacterium aureum*,

Microbacterium oxydans, *S. condimenti*, *S. heamolyticus* and *Candida sojae*. In contrast to the results described above, none of the biofilms formed by these microorganisms were affected by predation of *B. bacteriovorus* UP.

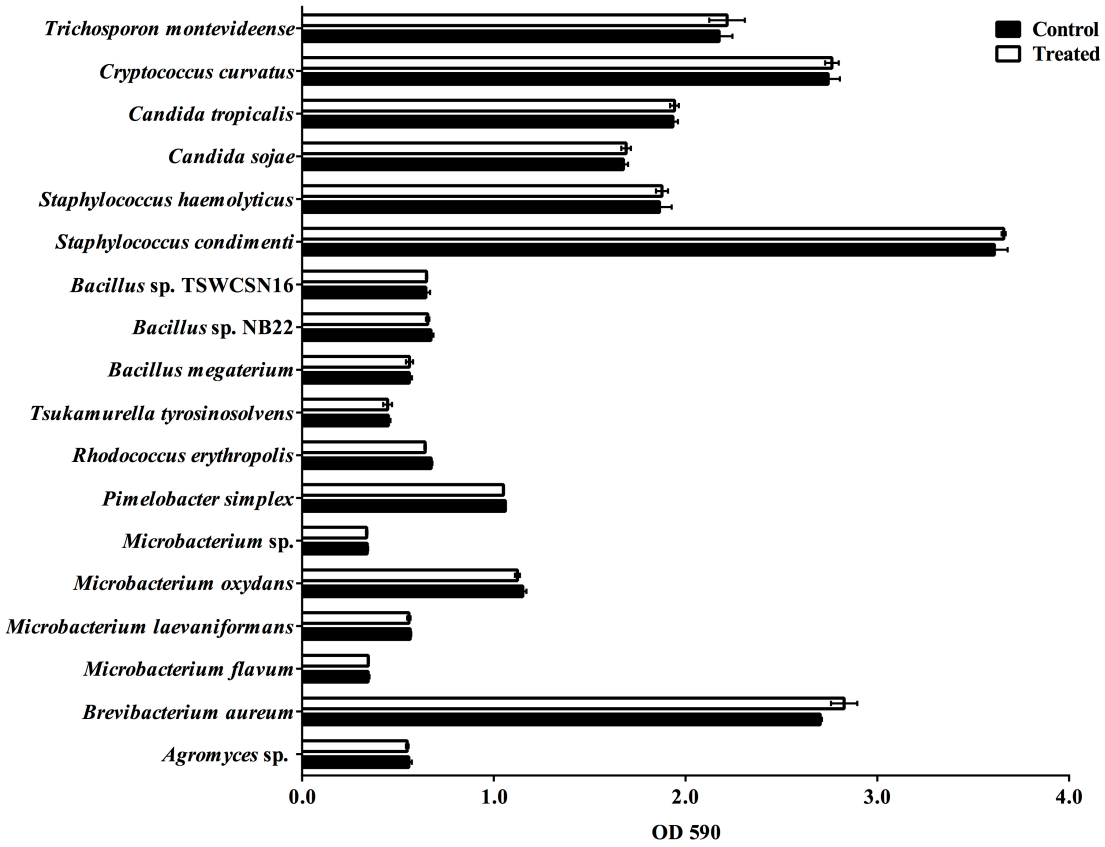

Figure 3.6 The effects of predation by *B. bacteriovorus* UP on species of fungi, *Firmicutes* and *Actinobacteria*. Preformed biofilms were incubated with *B. bacteriovorus* UP cells (treated) and *Bdellovibrio*-free medium (control) for 24 h. Biofilms were quantified by crystal violet and measured at 590 nm. Data represent the means of three replicates of one representative experiment, with error bars representing the standard error of the mean.

Because of the significant differences in biofilm formation and biomass reduction amongst species, it is difficult to compare the overall effects of predation by *B. bacteriovorus* UP. Therefore, the ratio of the 'treated' biofilm biomass to the corresponding 'control' (no predator biofilms) was determined to normalize the data for comparison of the effects of predation (Figure 3.7). To further simplify the data, the ratios were subsequently plotted based on their distribution for each phylogenetic cluster. For the Gram-positive and fungal organisms, the ratios were between 0.95 and

1, indicating no biomass reduction by predation. In contrast, the ratio distributions for the *Bacteroidetes* or sub-classes of *Proteobacteria* (Alpha-, Beta- and Gamma-*Proteobacteria*) tended to show a normal distribution pattern, where very few of the biofilms were reduced by < 20% (*Paludibacterium*, Figure 3.3) or > 80% (*Acinetobacter* sp. 409, Figure 3.4).

For the Alpha-*Proteobacteria* (Figure 3.7 A), the biofilm biomass reduction of sixteen species out of nineteen ranged from 30% – 60%, with eight species in the range of 40% – 50%. A similar distribution was also observed for the Gamma-*Proteobacteria* (Figure 3.7 C), with fifteen out of twenty-two species in the biomass reduction range of 30% – 60%. Additionally, a portion of the Gamma-*Proteobacteria* (6 out of 22) showed biofilm reductions of > 60%. The biomass reduction distribution patterns for the Beta-*Proteobacteria* (Figure 3.7 B) and *Bacteroidetes* (Figure 3.7 D) were similar, where nine out of eleven and eight out of nine isolates, respectively, showed biofilm reductions ranging from 30% – 60%. For these two groups, the maximum biofilm reduction was < 60%.

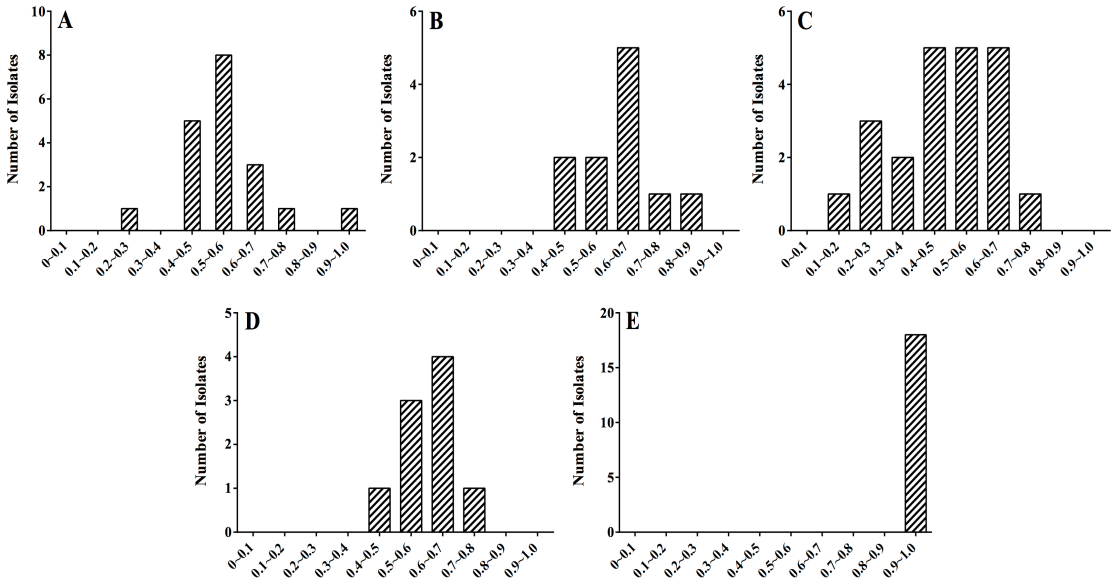

Figure 3.7 Distribution of the sensitivity of biofilms to predation. The X-axis shows the ratio (treated/control) of the effect of predation on biofilms whilst the Y-axis represents the numbers of species within each ratio distribution, showing (A) Alpha-, (B) Beta-, (C) Gamma-*Proteobacteria*, (D) *Bacteroidetes* and (E) the collective ratio distribution for *Actinobacteria*, *Firmicutes* and fungi.

3.3.2 Effects of predation by *Bdellovibrio bacteriovorus* UP on dual-species communities

As shown above, biofilms of the bacterium *O. anthropi* were resistant to predation. Since the wastewater microorganisms were derived from floccular activated sludge, where all of the species would be present as a mixed species community, it is possible that this species could protect other species when co-cultivated as mixed species biofilms. Therefore dual-species biofilms, as well as corresponding planktonic cultures comprised of *O. anthropi* and selected other species, were tested for sensitivity to predation by *B. bacteriovorus* UP. Sensitivity was quantified for each species in the mixed culture based upon CFU counts where isolates could be distinguished from *O. anthropi* based on colony morphology.

The effects of *B. bacteriovorus* UP on dual-species communities in planktonic cells were assessed based on CFU quantification of the communities in the absence (control) and presence (treated) of *B. bacteriovorus* UP. The strain *O. anthropi* was evenly mixed with the other species, which were chosen from Alpha-, Beta-, Gamma-*Proteobacteria*, and *Bacteroidetes*, respectively. In these dual-species planktonic communities, it was found that *O. anthropi* could co-exist with other bacteria with neither synergistic nor antagonistic effects (Figure 3.8, refer to the 'control' group), with the exception of *D. tsuruhatensis* (Figure 3.8 F). There was no clear reduction for *D. tsuruhatensis* incubated with *O. anthropi* for the first 24 h, however, a significant CFU reduction for *D. tsuruhatensis* (80% CFU decrease) was observed for the subsequent 24 h period, with no changes in CFUs for *O. anthropi*.

For the dual species, planktonic communities grown in the presence of the predators, there were no significant changes in the numbers of viable cells for *O. anthropi*. In contrast, in all cases, the number of viable cells for the other species, such as *Rhizobium borbori*, *Shinella fusca* and *Chryseobacterium* sp., decreased significantly ($P < 0.05$) (Figure 3.8 B, C and D). Whilst the decrease in CFUs generally occurred during the first 24 h of exposure, some species, such as *R. borbori* (Figure 3.8 B), *S. fusca* (Figure 3.8 C) and *Acinetobacter* sp. 409 (Figure 3.8 H), showed a further reduction over the subsequent 24 h period. In addition, most species were observed to decrease by approximately 60% (*S. fusca*) with maximum reductions of up to 90% (*P. stewartii*, Figure 3.8 I) in the first 24 h. In contrast, *S. maltophilia* strain CCF0025 showed < 50% CFUs reduction after 24 h (Figure 3.8 K).

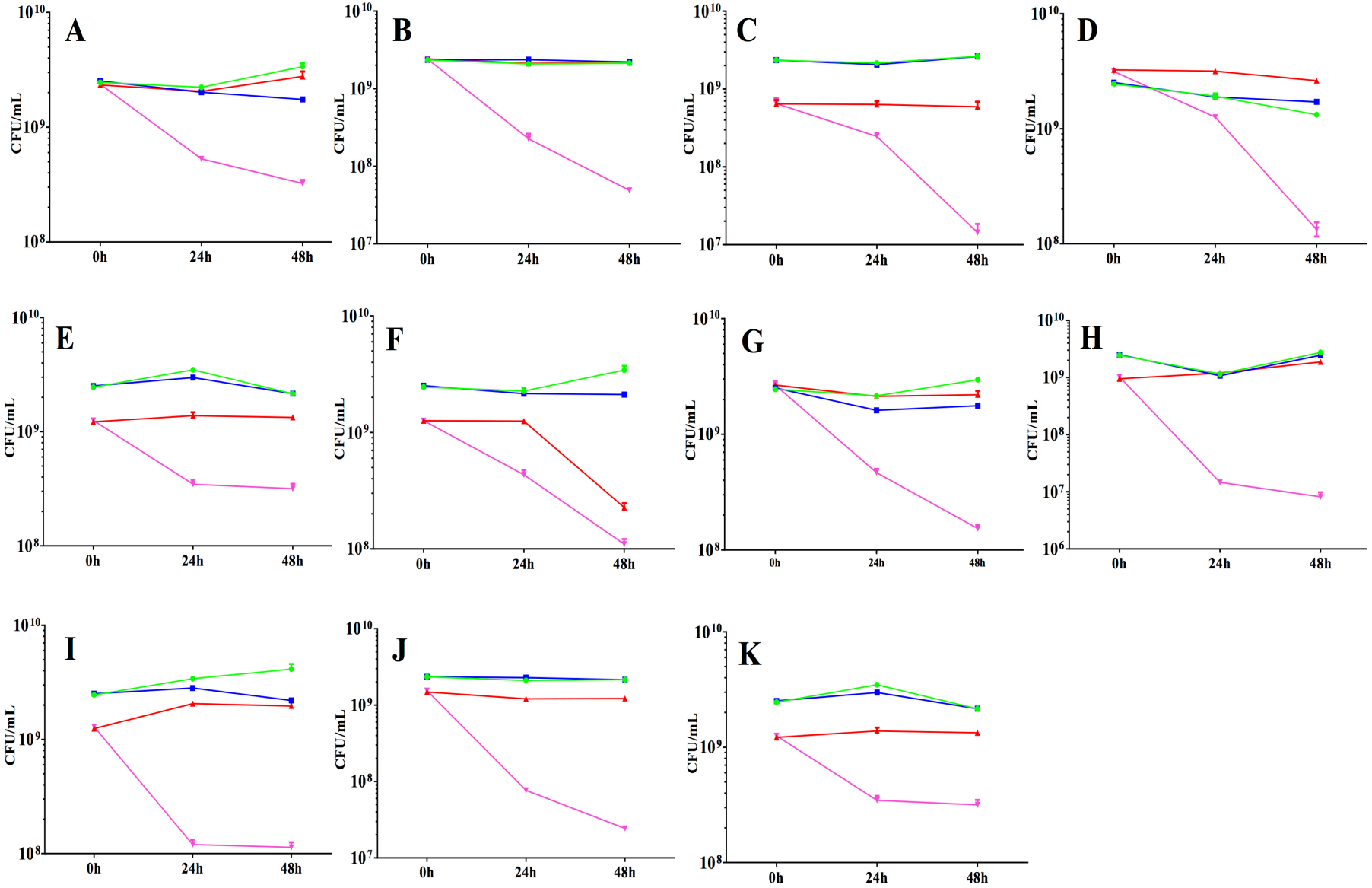
A
B
C
D
E
F
G
H
I
J
K
CFU/mL
0h
24h
48h

Figure 3.8 (Previous page) Impact of predation by *B. bacteriovorus* UP on dual species, planktonic communities. The communities were formed by *O. anthropi* and one additional species, (A) *Roseomonas* Unc. bacterium clone, (B) *Rhizobium borbori*, (C) *Shinella fusca*, (D) *Chryseobacterium* sp., (E) *Sphingobacterium multivorum* strain DW-1, (F) *Delftia tsuruhatensis*, (G) *Achromobacter* sp., (H) *Acinetobacter* sp.409, (I) *Pantoea stewartii*, (J) *Stenotrophomonas* sp. and (K) *Stenotrophomonas maltophilia* strain CCF0025, in the absence (control) or presence (treated) of *B. bacteriovorus* UP. Colony forming units (CFUs) of *O. anthropi* were shown in either green (control) or blue (treated), whilst the CFUs of other community member were shown either in red (control) or purple (treated). Data represent the means of three replicates from one representative experiment with error bars representing the standard error of the mean. The experiment was conducted three times with similar results.

The CFU results of the dual-species, planktonic communities showed that all of the selected bacteria that were vulnerable to *B. bacteriovorus* UP in mono-culture remained susceptible to *B. bacteriovorus* UP, even when co-incubated with a predation resistant bacterium. To further test for shared protection to predation, dual-species biofilms of the same combinations were also tested for sensitivity to predation. When grown as dual-species biofilms, it was hypothesized that the growth of *O. anthropi* would protect the other member of the dual-species biofilm community. However, as observed for the dual-species planktonic cultures, there was a significant ($P < 0.05$) reduction for the co-occurring species in the presence of *B. bacteriovorus* UP for all of the bacteria tested (Figure 3.9 A – K).

Eleven combinations of dual-species biofilms were developed to investigate the effects of predation by *B. bacteriovorus* UP. Irrespective of whether there was a synergistic effect on biofilm formation or not, the results showed biofilm reduction for all of the dual-species biofilms incubated with *B. bacteriovorus* UP, however, the extent of reduction was different. The biomass of some dual-species biofilms, such as those formed by *O. anthropi* with *Chryseobacterium* sp., *Acinetobacter* sp.409 and *P. stewartii*, respectively (Figure 3.9 D, H, and I) were reduced as much as 50%. In contrast, the biofilms formed by *O. anthropi* with *Roseomonas*, *R. borbori*, *S. fusca* and *Achromobacter* sp. (Figure 3.9 A, B, C and G) were reduced by only ~20%.

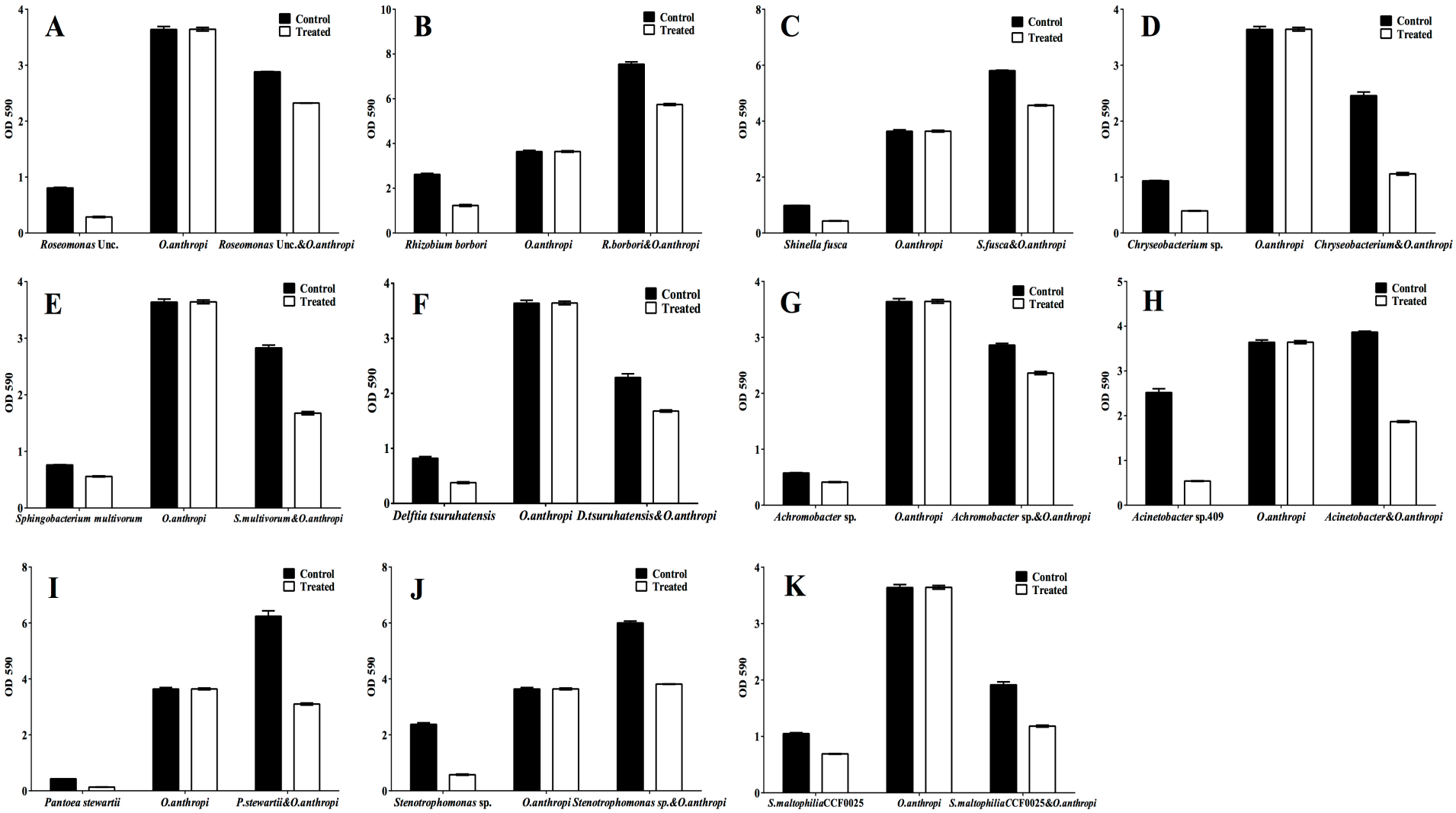
A
Control
Treated
OD 590
Roseomonas Unc.
O.anthropi
Roseomonas Unc.&O.anthropi
B
Rhizobium borbori
O.anthropi
R.borbori&O.anthropi
C
Shinella fusca
O.anthropi
S.fusca&O.anthropi
D
Chryseobacterium sp.
O.anthropi
Chryseobacterium&O.anthropi
E
Sphingobacterium multivorum
O.anthropi
S.multivorum&O.anthropi
F
Delftia tsuruhatensis
O.anthropi
D.tsuruhatensis&O.anthropi
G
Achromobacter sp.
O.anthropi
Achromobacter sp.&O.anthropi
H
Acinetobacter sp.409
O.anthropi
Acinetobacter&O.anthropi
I
Pantoea stewartii
O.anthropi
P.stewartii&O.anthropi
J
Stenotrophomonas sp.
O.anthropi
Stenotrophomonas sp.&O.anthropi
K
S.maltophiliaCCF0025
O.anthropi
S.maltophiliaCCF0025&O.anthropi

Figure 3.9 (Previous page) Impact of predation by *B. bacteriovorus* UP on dual-species biofilm communities. Biofilms were formed by *O. anthropi* and one additional species, (A) *Roseomonas* Unc. bacterium clone, (B) *Rhizobium borbori*, and (C) *Shinella fusca*, (D) *Chryseobacterium* spp., (E) *Sphingobacterium multivorum* strain DW-1, (F) *Delftia tsuruhatensis*, (G) *Achromobacter* spp., (H) *Acinetobacter* sp.409, (I) *Pantoea stewartii*, (J) *Stenotrophomonas* spp., (K) *Stenotrophomonas maltophilia* strain CCF0025, in the absence (control) or presence (treated) of *B. bacteriovorus* UP. Total biomass of biofilms was determined by crystal violet staining and measured at 590 nm. Data represent the means of three replicates from one representative experiment, with error bars representing the standard error of the mean. The experiment was conducted three times with similar results.

3.4 DISCUSSION

It is well accepted that in almost all environments bacteria tend to form highly organized and differentiated structures, called 'biofilms', and the characteristics and properties of biofilms have been extensively investigated [67, 107, 108]. Biofilms are typically noted for their significant resistance to a variety of stressors, especially to predation pressure from other microbial predators, such as viruses and protozoa [107]. In contrast to the general protection of biofilms to a range of stressors, studies have reported that biofilms are not always protective against predation by *Bdellovibrio* species [162, 178]. However, those studies mainly focused on biofilms formed by a single species and relatively few studies have focused on mixed species communities. Some studies have shown that biofilms with mixed species communities can better protect members from antimicrobial agents [132, 167, 190]. Therefore, it remains possible that mixed species biofilms may similarly provide protection against predation by *Bdellovibrio* species.

Laboratory model bacteria were tested for the predation effects of *B. bacteriovorus* UP (Chapter II), and the results showed that these model bacteria both in planktonic and biofilm growth were vulnerable to *B. bacteriovorus* UP. Therefore, effects of predation by of *B. bacteriovorus* UP were subsequently evaluated on biofilms formed by around 80 individual microorganisms, including bacterial and fungal species, from activated sludge samples. Most of the bacteria were *Proteobacteria*, whilst a few

belonged to the *Bacteroidetes*, *Actinobacteria* and *Firmicutes*. Bacteria from *Actinobacteria* and *Firmicutes* are Gram-positive, which are known to be resistant to *Bdellovibrio* spp. The results showed that, with the exception of *O. anthropi*, all of the *Proteobacteria*, and *Bacteroidetes* were vulnerable to predation by *B. bacteriovorus* UP. This may suggest that members of these classes could be one of the main food sources for *B. bacteriovorus* UP in wastewater treatment systems [73, 163, 164].

In general, the biofilm biomass reduction results showed that almost all of the Gram-negative bacteria were preyed upon by *B. bacteriovorus* UP, whilst none of the Gram-positive or fungal species were affected and this is consistent with previous reports that BALOs do not prey upon Gram-positive bacteria or fungi [95, 164] but rather feed exclusively on Gram-negative bacteria.

Interestingly, biofilms of one Gram-negative species, *O. anthropi*, were resistant to predation by *B. bacteriovorus* UP. Given that all of the species tested for this genus were resistant, it is likely that resistance is an attribute of this genus more generally. *O. anthropi* is ubiquitously distributed in soil as well as aquatic environments, and can also be found in antiseptic solutions and dialysis fluids in hospitals [191]. Whilst this species is generally found in clinical environments, it is not considered to be a human pathogen [192]. The mechanism of resistance was not defined in this study, however it may relate to the properties of the cell structure or the attributes of biofilm formation. Most known *Bdellovibrio* species require a 'periplasm' of prey cells to for the parasitic life-style, and the lack of 'periplasm' would prevent *Bdellovibrio* cells from entering into prey cells [95]. Whilst *O. anthropi* is a Gram-negative bacterium and as such is expected to have a typical Gram-negative cell wall structure, it nonetheless remains to be demonstrated that this is the case or that there are not other cell wall related differences that might account for the resistance of this species to predation. Additionally, the production of large amounts of extracellular polymeric substances that comprise the biofilm matrix may protect the prey cells embedded when within biofilms. For example, the biofilms formed by a mucoid isolate of *P. aeruginosa* were better protected from protozoan grazing than the isogenic, non-mucoid strain [125]. It has been shown that *O. anthropi* produces copious amounts of EPS [192] and this may potentially explain the complete resistance to predation observed in this study. Alternatively, it is possible that *O. anthropi* produces defensive compounds that inhibit the activity of the BALOs, as it was observed that

V. cholerae produced toxins to deter protozoan grazing [105], and this possibility should be investigated in the future.

Mixed species biofilms of *K. pneumoniae*, *P. aeruginosa* PAO1 and *P. protegens* Pf-5 have been shown to be protective of sensitive species when grown as mixed species biofilms [167]. Some studies of multi-species biofilms have investigated the synergistic interaction between biofilm members, so that the members can be more protected from a variety of stresses [132, 167, 190]. Therefore, it was interesting to investigate dual species biofilms here that *O. anthropi* was inoculated with other species sensitive to *B. bacteriovorus* UP and then subjected to this predator, to determine whether the inter-species protection will occur or not.

It has been demonstrated in one study that the presence of a Gram-positive bacterium slowed down the predation speed of *B. bacteriovorus* HD100 upon another Gram-negative species in planktonic culture [193]. Therefore, the predation effects of *B. bacteriovorus* UP were examined on dual-species communities both in planktonic and biofilm growth. In the planktonic dual-species communities, two members were mixed with equal numbers, and the viable cells numbers in controls showed that most of the selected species could grow together with *O. anthropi* without evidence of antagonistic effects, for example, reduced cell numbers (with one exception). Whilst *O. anthropi* maintained stable viable cell numbers under predation pressure in the dual species communities, the co-occurring species showed a significant reduction in viable cell numbers (Figure 3.8). However, it remains possible that even though *O. anthropi* does not cross protect the second member of these planktonic dual-species communities, it may decrease the rate at which that species is consumed relative to when it is present at a single species. Additionally, the dual-species biofilms were also reduced by *B. bacteriovorus* UP, some of which were reduced > 50% biomass. This indicated that the interspecies protection from predation did not occur between *O. anthropi* and the second member of the dual-species biofilms as shown in other studies [167, 190]. Therefore, the mechanism by which *O. anthropi* is protected from predation by *B. bacteriovorus* UP is not extendable to the other species within the dual-species biofilms. Thus, there was no evidence for inter-species protection from predation by *B. bacteriovorus* UP for either planktonic or mixed species biofilms.

In general, the effects of predation by *B. bacteriovorus* UP on different bacterial species isolated from the activated sludge were assessed both in planktonic cultures

and biofilms. Except for those fungal species, it was observed that most of the bacteria belong to *Proteobacteria*, which along with members of *Bacteroidetes* indicate that the Gram-negative bacteria are the predominant microbial communities in the activated sludge [194-196]. Biofilms of single species formed by species of *Proteobacteria* and *Bacteroidetes* showed collective reductions to *B. bacteriovorus* UP attack, additionally, biofilms of some strains were shown more reductions than other strains. The results may therefore suggest that *B. bacteriovorus* UP (or BALOs) would have predation preferences on specific spectrum of bacteria, and was further observed that the biofilms formed by two mixed species were also shown to be vulnerable to the predation by *B. bacteriovorus* UP. The mechanisms that prey species grown in biofilms were also vulnerable to the predation by *B. bacteriovorus* UP were not focused here, however, it is believed to relate to the predation nature of *Bdellovibrio* [95, 178, 197]. Due to their dependence upon prey cells to survive and proliferate, the *Bdellovibrio* cells actively search and attack the prey species upon encounter. Therefore, it is because that *B. bacteriovorus* UP cells can actively prey and lyse the prey cells grown in biofilms, so that the biofilms showed reduction in the presence of predators. Whilst the results were more likely due to the predation, other mechanisms could lead to inhibition or dispersal of biofilms. For example, secondary metabolites secreted by some microorganisms (especially bacteria) could affect the formation of biofilms and/or lead to the dispersal of biofilms [198-200]. However, there are no studies showing that *Bdellovibrio* species encode genetic elements typically associated with secondary metabolites production, e.g. non-ribosomal peptide synthases [95]. Further experiments are needed to address the underlying mechanisms of biofilms biomass reduction in the presence of *B. bacteriovorus* UP.

Additionally, the susceptibility of mixed species biofilms towards *B. bacteriovorus* UP' attack suggested that bacteria in more complex communities, such as floccular and granular activated sludge, would not be efficiently protected from the predation by *B. bacteriovorus* UP. Therefore, strong predation pressure may ultimately select for community members that are inherently resistant to BALOs, such as Gram-positive bacteria or fungi. Furthermore, the activated sludge is primarily comprised of microorganisms from the *Proteobacteria* and *Bacteroidetes* clades and given their overall sensitivity to predation, it is possible that the predatory activity of *B. bacteriovorus* UP could have a significant impact on the function of the activated

sludge by selectively removing these sensitive, but functionally important groups of microorganisms.

Chapter IV. THE EFFECTS OF *BDELLOVIBRIO* PREDATION ON FLOCCULAR AND GRANULAR ACTIVATED SLUDGE

4.1 INTRODUCTION

Wastewater treatment plants typically rely on the activated sludge process to treat wastewater, and this activated sludge is normally composed of floccular biomass containing suspended microorganisms that are responsible for remediation of the used water. The floccular biomass is typically held together by a self-produced EPS, which in this case represents a specific type of suspended biofilm [148]. The activated sludge based wastewater treatment systems have been used for over 100 years, with little modification to the biomass component of the process. A relatively recent development in wastewater treatment technology has been the conversion of floccular biomass into granules [145-147]. Granules are more structurally compact biomass with increased biomass density and granules have some distinct advantages over flocs that make them attractive for application in the wastewater remediation process. For example, granules are much more compact than floccular biomass and therefore the granules settle faster, which makes them easy to remove at the end of wastewater treatment process [149, 150, 155]. In addition, the closer spatial organization facilitates co-metabolism amongst the microorganisms and allows for the formation of gradients within the granules that enables aerobic and anaerobic organisms to co-exist within the same niche. A good example of this is the simultaneous nitrification and denitrification process facilitated by the formation of granules. The nitrate and nitrite generated through nitrification by microorganisms in the outer aerobic layer can be further used by microorganisms in the inner anaerobic core of granules, as a result of which the nitrate and nitrite accumulation in the wastewater is prevented [201].

Whilst there are clear advantages for the granular biomass and indeed some wastewater treatment plants have now begun to use granules for waste remediation [149, 150], the biological process of granule formation is not well understood [152]. One possible explanation for their formation is that granulation is a response of the

microbial communities to predation pressure from microbial predators, where the microbial communities embedded in the granules gain increased protection [15, 51, 105]. For example, it has been shown that the removal of protozoan predators from floccular biomass can result in disintegration of the flocs [202]. Therefore, it is also possible that *Bdellovibrio* spp. also represent strong predation pressures that drive the selection for formation of granules.

Although floccular and granular activated sludge differ from each other in size, morphology and microbial compositions [203], both floccular and granular activated sludge systems perform important functions, such as degradation of organic matter, nitrogen removal through nitrification and denitrification and phosphorus removal, to ultimately recover clean water from the wastewater [154, 155]. The proper function of the activated sludge is dependent on the presence and activity of specific groups of microorganisms carrying out these functions. It has generally been shown that *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were the predominant groups of functional bacteria in both floccular and granular activated sludge [129, 135, 141]. The effectiveness and efficiency of biological treatment on wastewater is therefore highly dependent on the metabolic activity of these groups of bacteria.

The effects of viral infection and protozoan grazing on both floccular and granular activated sludge have been broadly addressed [13, 25, 51, 55], which have been shown to have strong impacts on the functions of microbial communities. For example, the selective removal of protozoa from a laboratory-scale wastewater treatment system resulted in turbid effluent with a high content of suspended bacteria [55]. Bacteriophages have the similar effects in controlling the content of suspended bacteria in the effluent of wastewater, however bacteriophages may also affect the performance of wastewater treatment by killing the key functional groups of bacteria [24, 26]. Therefore, the presence of viral and protozoan predators can potentially result in disruption of the proper functioning of flocs and granules. Given the role of predation in controlling floccular biomass organization and function of the community, it is also possible that the *Bdellovibrio* spp. could also have a significant impact on sludge community composition and function.

The results from Chapter III showed that most of the Gram-negative species collected from activated sludge mainly belonged to *Proteobacteria* and *Bacteroidetes*, and these species were sensitive to predation by *B. bacteriovorus* UP. Additionally, whilst those Gram-negative species were generally sensitive to predation, some species were

more susceptible than others, which suggested that there was some predation preference for the *B. bacteriovorus* UP. Therefore, it is of particular interest to investigate whether the predation on flocs or granules will result in the selective removal of specific species. Such predation effects could ultimately disrupt the functions of both flocs and granules.

In this chapter, the effects of predation by *B. bacteriovorus* UP on microbial communities in floccular and granular activated sludge were tested. Additionally, the impact of predation on the overall microbial community diversity was determined through total RNA sequencing analysis on whole community. In brief, the results showed that the viability and total microbial biomass of both floccular and granular activated sludge was greatly affected due to the predation by *B. bacteriovorus* UP. However, the granular sludge was less sensitive to predation compared with floccular sludge. In addition, there were significant shifts in the microbial community composition of both sludge types due to predation by *B. bacteriovorus* UP.

4.2 MATERIALS AND METHODS

4.2.1 *Bdellovibrio* preparation and activated sludge maintenance

Bdellovibrio bacteriovorus UP (isolated and described in Chapter II) was incubated with *Pseudomonas protegens* Pf-5, which served as a food source for the predator. Activated sludge collected from the Ulu Pandan Wastewater Reclamation Plant (in Singapore) was used to seed laboratory reactors in which the community was maintained as floccular biomass or was converted into granular biomass. Floccular and granular activated sludges (Figures 4.1 A and B, respectively) were incubated and continuously maintained in either a laboratory-scale enhanced biological phosphorus removal (EBPR) reactor or a sequencing batch reactor (SBR), respectively.

The EBPR reactor with floccular sludge was fed with synthetic wastewater with organic matter, ammonia and phosphate. The organic source was a mixture of acetate and propionate of 200 mg COD/L ($COD_{Acetate}$: $COD_{Propionate}$, 3:1) and the ammonia and phosphate concentrations were 20 mg $N\text{-}NH_4^+$/L and 10 mg $P\text{-}PO_4^{3-}$/L. This reactor was operated with a working volume of 4 L and a cycle time of 5 h. Each cycle included stages of: 58 min anaerobic, 105 min aerobic and 25 min anoxic

periods in the first phase, and 38 min anaerobic, 40 min aerobic and 20 min anoxic periods in the second phase [204].

The SBR reactor incubating the granular sludge was also fed with the same synthetic wastewater as the floccular community and had a volume of 4 L. The operation of the bioreactor involved a 6 h cycle comprised of two different phases: Phase I - feeding (8 min), anaerobic (60 min), aerobic (80 min at day 0 and gradually increased to 95 min by the end of week 5) and anoxic (40 min at day 0 and gradually increased to 50 min by the end of week 5); Phase II - feeding (2 min), anaerobic (30 min), aerobic (40 min at day 0 and gradually increased to 70 min by the end of week 5) and anoxic (30 min). Each cycle was completed with a settling stage (60 min at day 0 and gradually decreased to 5 min by the end of week 5) and a 10 min decanting stage. The settling time was 5 min per cycle from week 6 onwards [203].

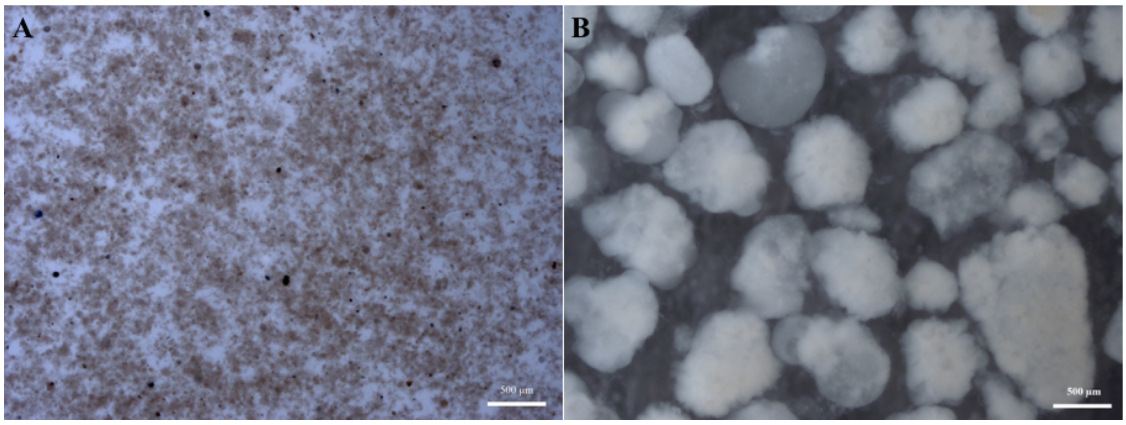

Figure 4.1 The activated sludge samples used in this study. Floccular sludge (A) and granular sludge (B) were observed with stereotype microscopy (Stereo V8, ZEISS). Images were taken at 10 × magnification, with scale bars representing 500 μm.

4.2.2 Effects of predation by *B. bacteriovorus* UP on floccular and granular sludge

Floccular activated sludge was incubated and maintained in an EPBR reactor and 25 mL of the evenly mixed liquor volatile suspended solids (MLVSS) was collected during the anoxic stage for experiments. The microbial biomass within the MLVSS was collected by centrifugation at 6,000 *g* for 8 min, and the pellet was re-suspended in liquid DNB ('Lab-Lemco' power 0.1 g/L, yeast extract 0.2 g/L, peptone 0.5 g/L and NaCl 0.5 g/L, amended by 3 mM $MgCl_2$ and 2 mM $CaCl_2$) for subsequent

experiments (final volume, 25 mL). The floccular sludge was incubated with *B. bacteriovorus* UP cell suspensions in a 1:1 ratio (v/v) as the 'Treated' group, whilst the negative control consisted of floccular sludge incubated in the absence of *B. bacteriovorus* UP.

The granular sludge was also collected in 25 mL aliquots from the anoxic stage. The experimental procedure to prepare granular sludge for experiments was the same as for the floccular sludge described above. In addition, the prepared *Bdellovibrio*-free medium and *B. bacteriovorus* UP cell suspensions were also mixed evenly with the liquid DNB medium ('Lab-Lemco' power 0.1 g/L, yeast extract 0.2 g/L, peptone 0.5 g/L and NaCl 0.5 g/L, amended by 3 mM $MgCl_2$ and 2 mM $CaCl_2$), which were used to determine the background of the negative control and *Bdellovibrio*-treated group, respectively. These samples were incubated in 24 well micro-titer plates at 30°C with shaking at 200 rpm.

At 0, 24 and 48 h, the activated sludge samples for the negative control and *Bdellovibrio*-treated groups as well as the background controls (no predator and *Bdellovibrio*-treated groups) were collected to determine the microbial activity (ATP) and total microbial biomass (protein content) with and without the predators. A small amount of the activated sludge samples of 'control' and 'treated' groups were stained with the Live-Dead staining reagent mixture, respectively, to determine the viability of microbial communities in the sludge samples.

Total ATP was quantified as a proxy for microbial activity and viability to determine the impact of predation on flocs and granules. Briefly, 100 µL of the floccular or granular sludge (negative control and *Bdellovibrio*-treated) were mixed evenly with 100 µL of the ATP measurement reagent (Promega, BacTiter-Glo™). Additional controls included samples with only the predator cells in the absence of floccular or granular sludge and an additional control consisted of the medium with no added cells (predator, prey or sludge). These background contributions (predator cells and medium with no cells) to the ATP concentration were subtracted from the live samples to determine the actual concentration of ATP in activated sludge samples.

The total microbial biomass was quantified by determining the amount of protein in the samples using the Bradford method (Bio-Rad™). Briefly, the floccular and granular sludge samples were lysed completely using a probe sonicator (SM Vibracell VCX750, Sonics & Materials, Inc.) for 5 min at 35% magnitude. The lysed cell debris were then removed by centrifugation at 8,000 *g* at 4°C for 10 min and the supernatant

was collected for determining the protein concentration. The supernatant, 10 µL, was mixed with 200 µL of the prepared dye reagent for protein determination, incubated at room temperature for 10 min and the absorbance was measured at 595 nm. The protein concentrations of samples were determined by comparison of the OD_{595} readings with a standard curve developed using bovine serum albumin. The background contributions of predator cells and medium with no cells to protein concentrations were also subtracted from the sample reading.

The viability of the microbial community in activated sludge was additionally assessed by using the Live/Dead staining reagents (LIVE/DEAD®, *Bac*Light™ Cell Viability Assays, Thermo Fisher Scientific Inc.). Briefly, 100 µL of floccular or granular sludge was evenly mixed with 100 µL of SYTO9/propidium iodide working solution (final concentration 6.68 µM and 40 µM, respectively), and the mixture was then kept in the dark at room temperature for at least 30 min for subsequent confocal microscopy. Stained samples were imaged at 488 nm excitation (SYTO9) as well as 561 nm excitation (propidium iodide) using confocal laser scanning microscopy. Multiple images (two-dimensional images for floccular sludge and three-dimensional images for granular sludge) were taken randomly for quantitative image analysis (IMARIS, Version 7.6.4. Bitplane, Oxford Instruments). The ratios of live and dead cells (L/D ratio) were determined from the image quantification.

4.2.3 Microbial composition analysis on floccular and granular sludge

To determine the impact of *B. bacteriovorus* UP predation on floccular and granular sludge communities, total RNA sequencing was performed. The floccular and granular sludge samples with and without predator were collected after 24 h incubation by centrifugation at 8,000 *g* at 4°C for 8 min, fixed in liquid nitrogen for 30 min and immediately stored at -80°C for subsequent RNA extraction and analysis. Total RNA was collected from flocs and granules using the RNA Clean and Concentrator™ -5 kit (ZYMO Research Corporation). A small aliquot of the purified RNA was used to determine the concentration and integrity of RNA, and the rest of total RNA was stored immediately at -80°C for RNA sequencing.

The concentration of RNA in the samples was determined using the Qubit® Assay Kits and Qubit® Fluorometer (Life Technologies, Thermo Fisher Scientific, Inc.). Briefly, 2 µL of purified RNA sample was mixed evenly with 198 µL of the Qubit®

reagent working solution and incubated at room temperature in the dark for 2 min. The fluorescence was measured using a Qubit® Fluorometer and the RNA concentrations were determined by comparison of the fluorescent readings with a standard curve. The integrity of RNA samples was determined using the RNA Analysis ScreenTape and 2200 TapeStation Instrument (Agilent Technologies, Inc.). Briefly, 1 μL of RNA samples was mixed evenly with 5 μL of sample buffer of RNA Analysis ScreenTape. The mixture was then denatured at 72°C for 3 min, followed by incubation on ice for 2 min. The samples were subsequently loaded into the 2200 TapeStation instrument for RNA integrity examination.

For sequencing, 200 ng RNA samples were used. In brief, the RNA samples were broken into fragments of ~150 bp by heating in a suitable buffer (containing Mg^{2+}) and were subsequently used to synthesize the corresponding complimentary DNA (cDNA) by reverse transcription. The second strand of DNA was then synthesized using the cDNA as templates and the synthesized double-strand DNA samples (dsDNA) were washed with AMPure XP PCR Purification systems (Beckman Coulter Inc., Singapore) to remove the dsDNA fragments less than 150 bp. Subsequently, adenine was added to both 3'-ends of the purified dsDNA fragments, which were then ligated with suitable adaptors, amplified by PCR and sequenced (Illumina HiSeq 2500, Illumina Inc.) using the extended 2×100 bp paired-end sequencing mode.

The sequence data were subsequently processed with RiboTagger [203] using a fast and unbiased tag-based approach established. This sequencing approach required no PCR amplification process, which was advantageous in minimizing the amplification bias [205]. In brief, a universal primer (5'-RGGATTAGATACCC) targeting the hypervariable region (V5) of the 16S rRNA genes was used to anchor every sequencing read to generate sequences of 33 nucleotides downstream of the primer. All of the targeted sequencing reads (33 nucleotides) were defined as the V5 tags of the 16S rRNA genes and sequencing reads shorter than 33 nucleotides were discarded. The universal primer used here matched 83% of the 16S rRNA sequences in the database of the Ribosomal Database Project (RDP) [205, 206]. Every different V5 tag was used as an individual sequence representing one operational taxonomic unit (OTU), and only those V5 tags that were detected in at least two different sequencing reads were included for analysis.

The abundance of microbial populations in the floccular and granular sludge samples was reflected in the numbers of the sequencing reads detected. The difference in

sequencing reads for OTUs for both floccular and granular microbial communities between negative controls and *Bdellovibrio*-treatment was then plotted using multi-dimensional scaling (MDS) algorithm analysis based on the Euclidean distance similarity or dissimilarity [203]. The changes of floccular and granular microbial communities due to predation by *B. bacteriovorus* UP were furthermore compared using both the Menhinick's index (showing 'richness' of microbial communities) and Shannon-Wiener index (showing 'diversity' of microbial communities), respectively. The Menhinick's index was calculated using the formula D = *s*/sqrt(N), where '*s*' was the total number of V5 tags detected in the samples, whilst 'N' was the total counts of all sequencing reads. The Shannon-Wiener index was calculated using the formula $H = \sum_{i=1}^{s}(Pi \times |\ln Pi|)$, where '*Pi*' was the proportion of each individual OTU making up of the total counts of all sequencing reads, '*s*' was the total numbers of detected V5 tags.

The microbial populations present in the sequence data at > 0.5% of the total microbial community were subsequently examined as the dominant species (communities) in the floccular and granular activated sludge. OTUs present at < 0.5% of the total community were omitted from further analysis. Within these dominant species, the sequencing reads in the negative controls and *Bdellovibrio*-treatment groups were additionally compared to determine the predation effects by *B. bacteriovorus* UP on microbial communities in both floccular and granular activated sludge. All of the statistical analyses above were performed with Prism (GraphPad) and PRIMER 6 (PRIMER-E Ltd., Plymouth Marine Laboratory).

4.3 RESULTS

4.3.1 Effects of *Bdellovibrio bacteriovorus* UP on microbial activity and biomass of floccular and granular activated sludge

Flocs utilized in this study were defined as small (< 100 μm), loosely aggregated microbial communities, whilst granules were highly compact aggregates with larger sizes (> 100 μm) and both were comprised of microbial communities characterized as having high species diversity. Biofilm formation, e.g., flocs and granules, is thought to protect bacteria from predation by physically excluding the predators through the production of extracellular EPS matrix.

The effects of predation by *B. bacteriovorus* UP on floccular and granular activated sludge were determined here by monitoring cell numbers as well as by quantifying ATP content and biofilm biomass, which was measured as total protein concentration of the microbial communities. The ATP content of the *Bdellovibrio*-free controls for both the floccular and granular sludge increased across the 48 h of the experiment, indicating that the sludge biomass remained viable and increased in cell numbers (Figures 4.2 A and C). In the presence of predators, the microbial activity (viability, as determined by ATP content) of the floccular sludge decreased over 80% in the first 24 h of predation compared to the controls with no predators (Figure 4.2 A). At 48 h, the total ATP content was approximately 70% of the controls. The microbial activity of the granular sludge (Figure 4.2 C) was reduced more than 60% in the first 24 h of predation and was further reduced another 15% (75% overall reduction) at 48 h.

Since ATP content was used here as an indication of increased cell numbers or changes in the cell energetics, the total biomass of the community was also determined by quantifying the total protein content of the floccular and granular communities. In the first 24 h, there was a ~50% reduction in the protein concentration for both floccular and granular sludge samples incubated with *B. bacteriovorus* UP (Figure 4.2 B and D). The protein concentrations of the floccular sludge were further reduced to approximately 30% of the control at 48 h (Figure 4.2 B). In contrast, there was no further reduction in biomass after the first 24 h for the granular communities (Figure 4.2 D). During the 48 h of the experiment, there were no significant changes to the total protein concentrations of floccular and granular sludge in the negative controls (Figure 4.2 B and D), suggesting that the total biomass

of the microbial communities within the flocs and granules was not affected under these experimental conditions.

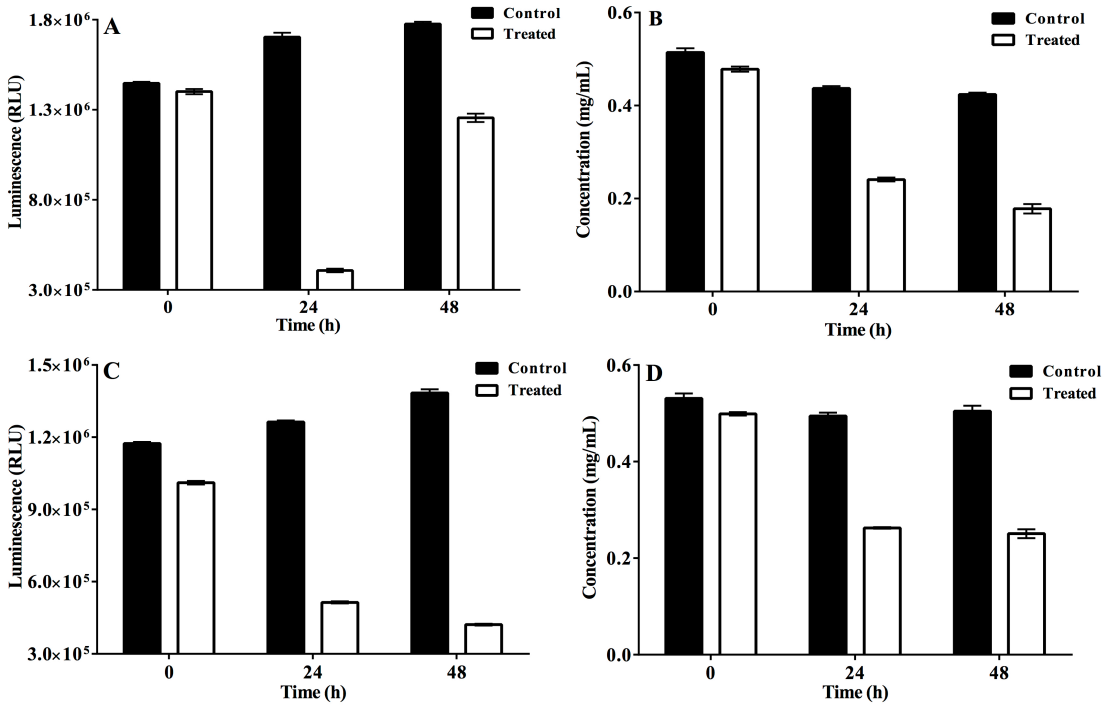

Figure 4.2 The effects of predation by *B. bacteriovorus* UP on floccular and granular sludge communities. (A) Microbial activity (ATP) and (B) protein concentration of floccular sludge respectively. (C) Microbial activity (ATP) and (D) protein concentrations for the granular sludge respectively. Each set of data represents the means of three replicates from one representative experiment, with error bars representing the standard error of the mean (error bars are present for all data points, however in some cases are difficult to observe due to the relatively small standard errors). Experiments were repeated three times with similar results.

4.3.2 Impacts of *Bdellovibrio bacteriovorus* UP on the viability of microbial communities in floccular and granular activated sludge

The results above clearly demonstrated that both microbial activity (viability) and total biomass (protein content) of floccular and granular activated sludge were significantly ($P < 0.05$) affected by predation of *B. bacteriovorus* UP. It was also possible that the predators lysed the prey cells within the sludge biofilms that remained associated with the suspended particles and such lysed biomass would still be detected in the protein measurement. Therefore, the viability of the sludge biomass

was determined using the *Bac*Light® Live/Dead reagents and quantitative image analysis on floccular and granular sludge.

In the negative controls (Figure 4.3 A – C), most of the community in the floccular sludge appeared to be viable (green cells) for the 48 h duration of the experiment, with a slightly increased portion of dead cells over time (red color). In contrast, the viability of the floccular sludge communities incubated with *B. bacteriovorus* UP decreased dramatically (Figure 4.3 D – F) at both 24 h and 48 h.

At 0 h, the floccular sludge for both the negative controls and *Bdellovibrio*-treatment groups showed a high level of viability (Figure 4.3 A and D). After 24 h of incubation, most of the cells in the negative controls appeared to remain intact and viable (Figure 4.3 B). In contrast, the samples incubated in the presence of the predators were comprised almost entirely of dead cells (Figure 4.3 E). Similar results were observed at 48 h, where the control biomass was composed largely of viable cells (Figure 4.3 C) and the samples incubated with *B. bacteriovorus* UP contained a high frequency of dead cells (Figure 4.3 F). Although the microbial communities showed high proportions of dead cells after predation, the morphological structure of the floccular sludge appeared unaffected by the comparison between negative controls and *Bdellovibrio*-treatment samples (Figure 4.3 B and E, C and F).

Multiple images (n = 30) of the flocs were analyzed to calculate the bio-volumes of both viable (live) and non-viable (dead) cells. In the negative controls, the bio-volume of the viable cells gradually increased from 2.7×10^5 μm^3 to 3.2×10^5 μm^3 over the 48 h, whilst the bio-volume of the non-viable cells, less than 1.0×10^5 μm^3, did not change significantly over the 48 h (Figure 4.5 A). The live/dead (L/D) ratios of the floccular sludge in the negative controls fluctuated from ~4 to ~6, suggesting that most of the biomass in the negative controls was viable (Figure 4.5 B). In contrast, in the *Bdellovibrio*-treatment groups the bio-volumes of viable and dead cells changed dramatically (Figure 4.5 A). The viable cell bio-volume decreased from 2.6×10^5 μm^3 at 0 h to 0.2×10^5 μm^3 at 24 h, and increased slightly to 0.3×10^5 μm^3 at 48 h. The abundance of dead cells increased from 1.0×10^5 μm^3 at 0 h to 2.0×10^5 μm^3 after 24 h, and to 3.0×10^5 μm^3 at 48 h (Figure 4.5 A). The dramatic changes of bio-volumes were further demonstrated in the L/D ratios, which decreased from ~3.0 at 0 h to ~0.12 at 24 h (Figure 4.5 B).

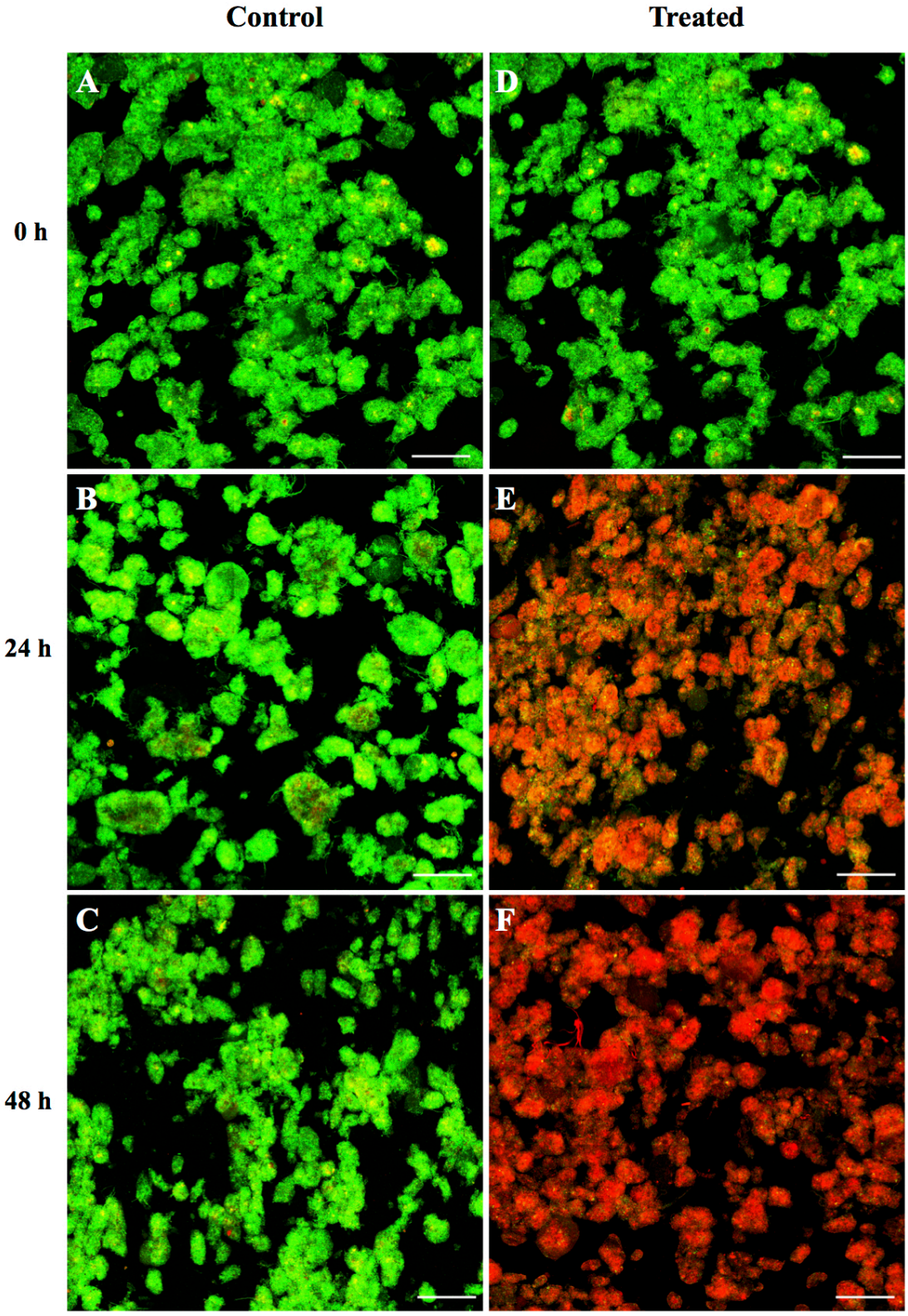

Figure 4.3 The effects of predation by *B. bacteriovorus* UP on floccular sludge. Floccular sludge was incubated over 48 h in the absence (A – C) and presence (D – F) of *B. bacteriovorus* UP. Viability of the microbial communities was observed by Live/Dead staining and confocal microscopy exanimation. Images were taken at 200× magnification with scale bars representing 500 µm.

Similar results were also observed for the granular sludge, where the negative control samples were composed predominantly of viable cells over the entire 48 h period (Figure 4.4 A – C). In contrast, in the *Bdellovibrio*-treatment groups, the viability of granular communities decreased dramatically at both 24 h and 48 h (Figure 4.4 E and F).

In the negative controls, the bio-volume of viable cells gradually decreased from 2.25×10^7 μm^3 to 1.5×10^7 μm^3 over the 48 h, whilst the bio-volume of dead cells, lower than 1.0×10^6 μm^3, did not change significantly ($P > 0.05$) over the 48 h (Figure 4.5 C). The live/dead (L/D) ratios of microbial communities in the negative controls decreased from ~20 at 0 h to ~13 at 24 h and then to 10 at 48 h (Figure 4.5 D). Similar with the floccular sludge incubated with *B. bacteriovorus* UP, there was a statistically significant ($P < 0.01$) change in the bio-volume of live and dead cells in the treated groups. The bio-volume of live cells decreased significantly from 2.25×10^7 μm^3 at 0 h to 5.0×10^6 μm^3 at 24 h and 4.0×10^6 μm^3 at 48 h. The bio-volume of dead cells increased accordingly from 1.0×10^6 μm^3 to 2.0×10^7 μm^3 and 1.7×10^7 μm^3 at 24 h and 48 h (Figure 4.5 C).

Although a significant portion of the granular community appeared to be dead, the L/D ratio of ~0.4 (Figure 4.5 D) at 24 h was higher than for the floccular sludge (0.12) at 24 h (Figure 4.5 B), which might suggest that the granular communities were better protected than the floccular communities. Additionally, it was observed that the Live/Dead staining reagents fully penetrated the floccular sludge in contrast to the granules, which were not completely stained. This observation might also suggest that the density of highly compact granules was such that it was difficult for *B. bacteriovorus* UP to penetrate deep beyond the outer layer of the granules.

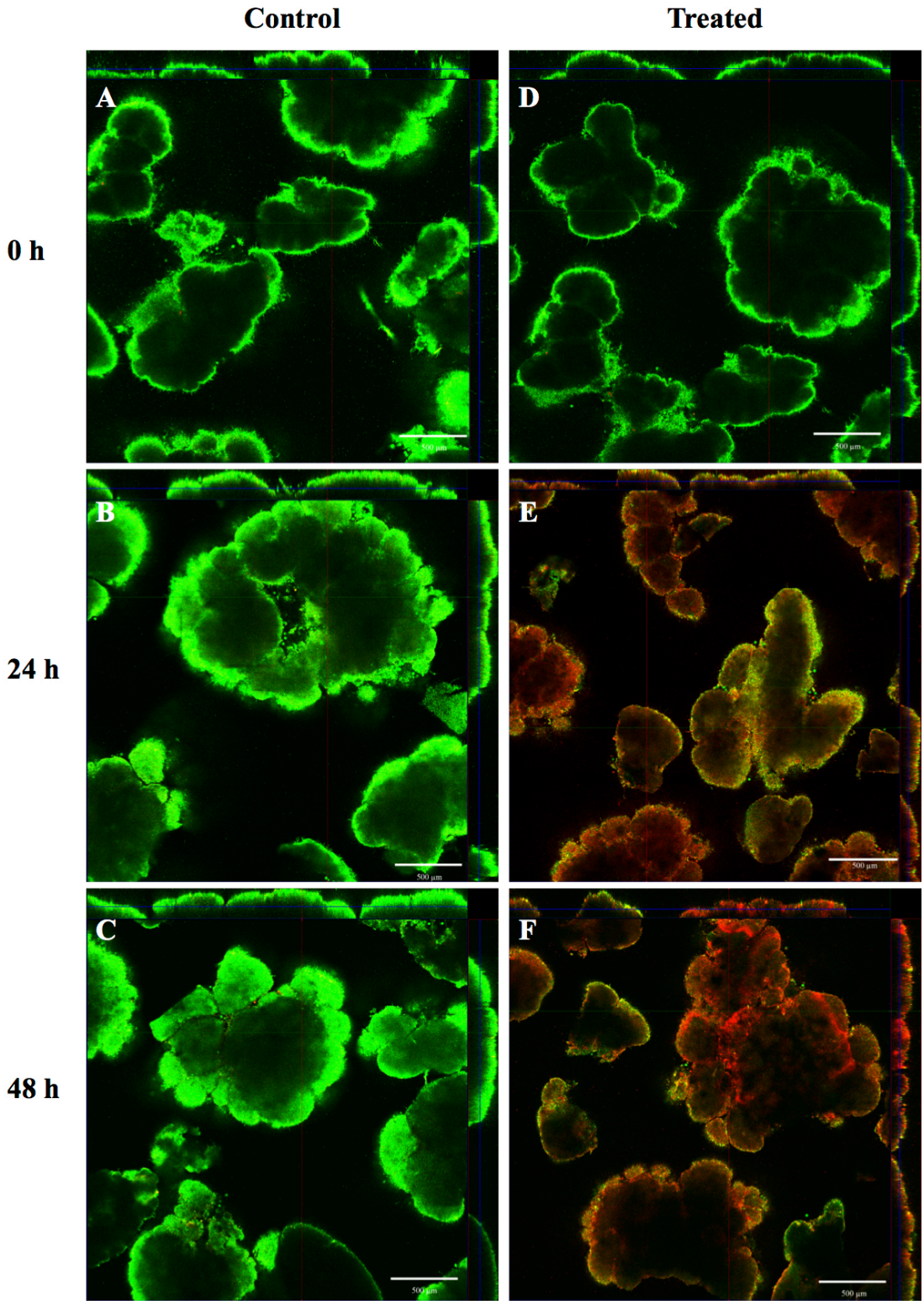

Figure 4.4 The effects of predation by *B. bacteriovorus* UP on granular sludge. Granular sludge was incubated over 48 h in the absence (A – C) and presence (D – F) of *B. bacteriovorus* UP. Viability of microbial communities in granular sludge was observed by Live/Dead staining and confocal microscopy exanimation. Images were taken at magnification 200× with scale bars representing 500 µm.

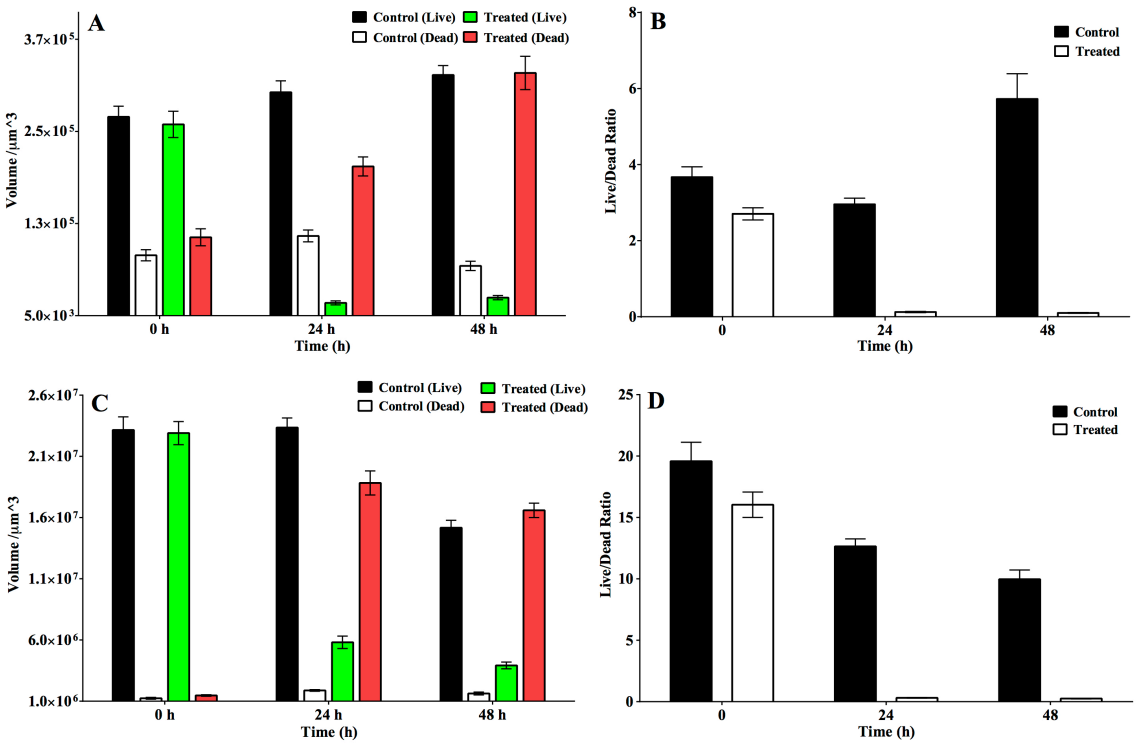

Figure 4.5 The effects of predation by *B. bacteriovorus* UP on viability of floccular and granular microbial communities. The floccular and granular sludges were incubated in the absence (control) and presence (treated) of *B. bacteriovorus* UP, and the viability of microbial biomass was assessed through Live/Dead staining. The total bio-volume of viable (live) and non-viable (dead) communities in (A) floccular and (C) granular sludge were calculated, and the ratios of live to dead communities in (B) floccular and (D) granular sludges were accordingly calculated. Data represent the means of multiple replicates (n = 30 for floccular sludge and n = 20 for granular sludge) from one representative experiment, with error bars representing the standard error of the mean.

4.3.3 The effects of *B. bacteriovorus* UP predation on microbial community composition in floccular and granular sludge

Predation was shown above to have a significant impact on ATP content, protein biomass, as well as biomass viability (Live/Dead staining) and bio-volumes for the floccular and granular sludges. Although a great proportion of the activated sludge was reduced in viability and total biomass, it remains unclear what impact predation by *B. bacteriovorus* UP has on the community composition. Therefore, changes in the

microbial communities in response to predation were determined by whole community RNA sequencing.

Based on MDS plots of the community composition and abundance, there were significant differences ($P < 0.05$) between the negative controls and *Bdellovibrio* treatment groups (Figure 4.6). In general, the floccular communities in the *Bdellovibrio* treatment groups showed more than 70% dissimilarity to the negative controls (Figure 4.6 A), whilst the granular communities in the treatment groups showed more than 60% dissimilarity to the corresponding negative controls (Figure 4.6 B). Although there were minor dissimilarities amongst the three biological replicates (Figure 4.6, 'PCO2') of both floccular (15.3% dissimilarity) and granular activated sludge (29.2% dissimilarity), these dissimilarities were quite low. These data therefore showed that *B. bacteriovorus* UP can have a significant impact on both floccular and granular sludge communities.

The Menhinick's index and the Shannon-Wiener index were subsequently used to analyze the richness and diversity of the sludge communities, respectively. By comparing the negative controls for the floccular and granular sludges, it was observed that the floccular sludge communities were more diverse than the granular sludge communities (Table 4.1 'Control'). Additionally, after incubation with *B. bacteriovorus* UP, the richness and diversity of communities in both floccular and granular sludge decreased (Table 4.1). The diversity reductions for the floccular and granular community were 11.5% and 23.2%, respectively. Therefore, the diversity of the granular sludge community was more strongly impacted by predation than the floccular sludge.

Table 4.1 The richness and diversity of microbial communities in activated sludge

	Floccular sludge		Granular sludge	
	Control	Treated	Control	Treated
Menhinick's Index (Richness)	7.77±0.216	7.03±0.211	6.27±0.230	5.65±0.310
Shannon-Wiener Index (Diversity)	4.16±0.339	3.68±0.064	3.59±0.109	2.76±0.147

* All of the data represented the mean values of three biological replicates with standard deviations.

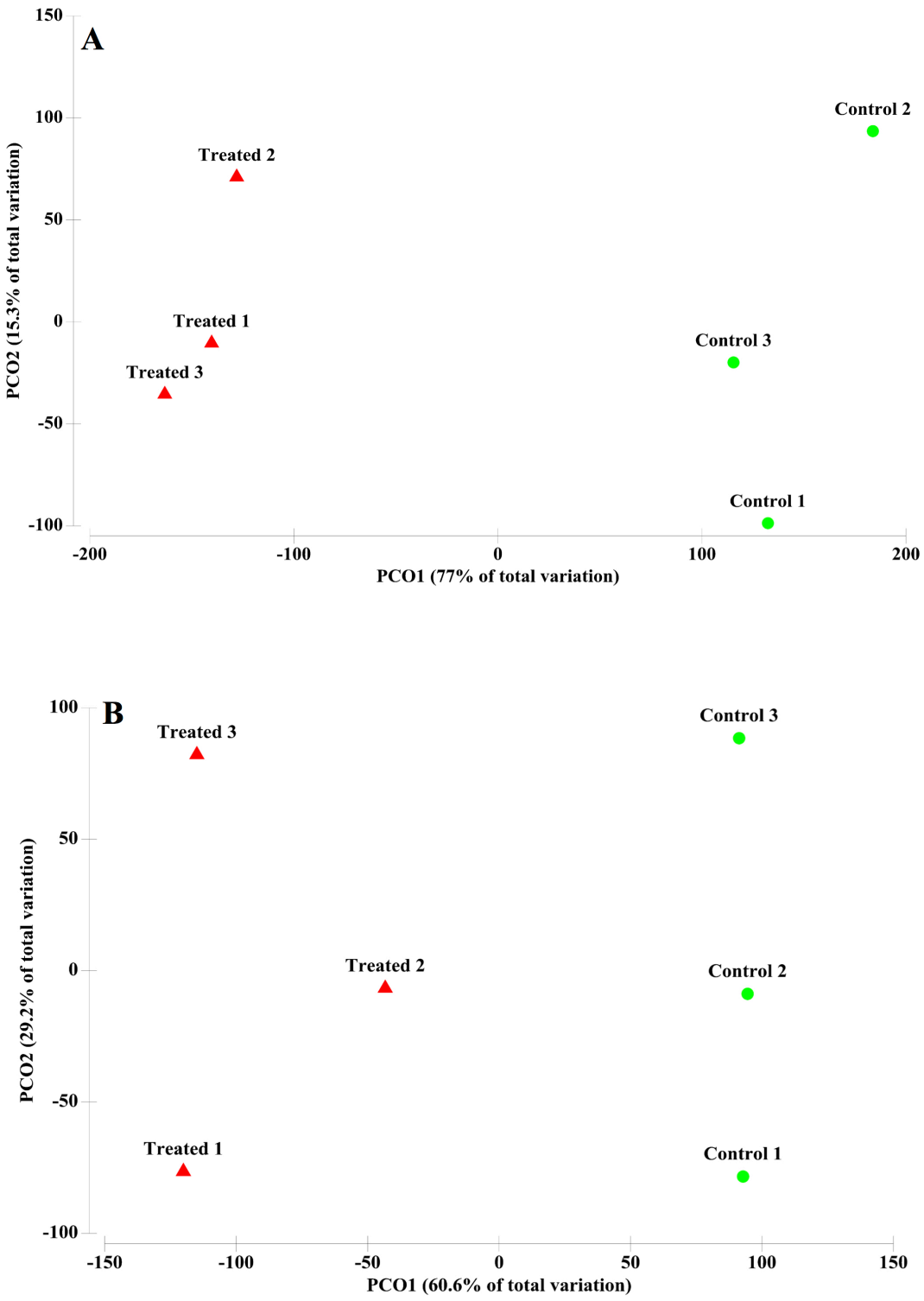

Figure 4.6 Multi-dimensional scaling plots for (A) floccular and (B) granular activated sludge. The data were square root transformed for the principal coordinates analysis (PCO), which was used to demonstrate the difference between negative controls ('Control') and *Bdellovibrio* treatment groups ('Treated'). Data represented three independent biological replicates.

The results of the MDS analysis as well as quantification of microbial community richness and diversity showed that *B. bacteriovorus* UP can greatly affect the microbial community as a whole for both floccular and granular activated sludge. To determine which microorganisms were associated with the observed changes in community diversity as a consequence of predation, the dominant OTUs for the floccular and granular sludge communities were interrogated. A total of 400 OTUs were present in the sequence data at > 0.5% of the total community and were therefore used here to investigated the impact of predation on prey species.

The results showed that members of the *Acidobacteria* (22 out of 400), *Bacteroidetes* (44 out of 400), *Chloroflexi* (15 out of 400), *Planctomycetes* (14 out of 400) and *Proteobacteria* (106 out of 400) were the predominant groups of identifiable microorganisms in the floccular activated sludge (Figure 4.7 A). Similarly, *Bacteroidetes* (33 out of 400), *Chloroflexi* (12 out of 400), *Planctomycetes* (10 out of 400) and *Proteobacteria* (103 out of 400) were the predominant microorganisms in granular activated sludge (Figure 4.7 B). It should be noted that between 45 – 50% of both community members that comprised the top 400 OTUs could not be taxonomically assigned with any confidence. Interestingly, the relative abundance of the community members with unknown members in the databases were higher in the granular sludge (48.75%, Figure 4.7 B) compared to the floccular sludge (38.25%, Figure 4.7 A). Amongst those community members that could be identified, *Bacteroidetes* and *Proteobacteria* were the most dominant groups in both floccular and granular sludge. By comparing the dominant microbial communities in the negative control floccular and granular sludges, it was observed that these two groups were the most significantly affected by predation. Interestingly, it was observed that a number of *Eukaryota* members both in floccular and granular sludges were also reduced in relative abundance due to the predation. Gram-positive bacteria (mainly from *Actinobacteria*, 6 out of 400 for floccular sludge and 3 out of 400 for granular sludge) were also included, and their relative abundances were not affected (neither increased nor decreased).

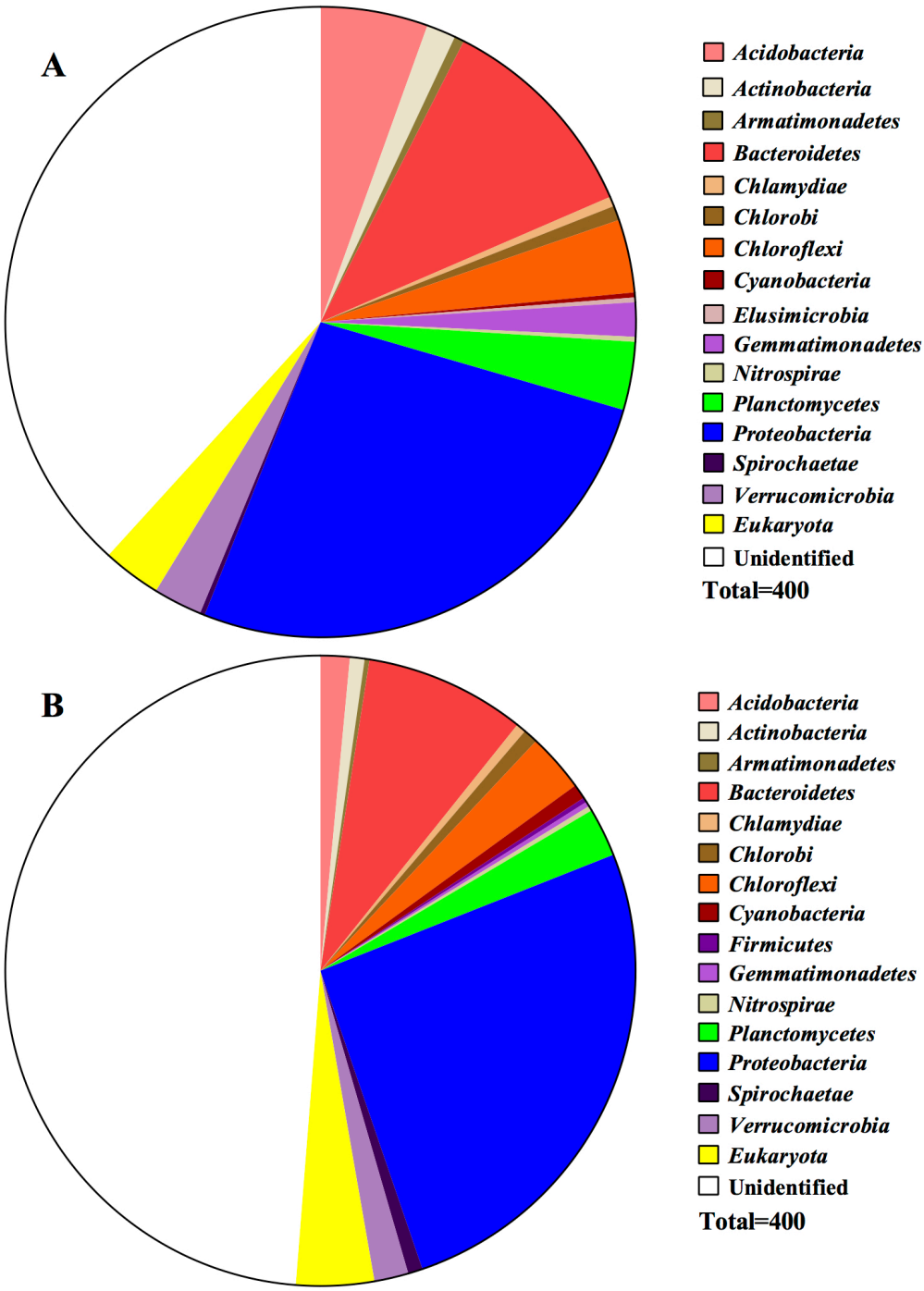

Figure 4.7 Dominant microbial communities in (A) floccular sludge and (B) granular sludge. In total, 400 operational taxonomic units (OTUs) present at > 0.5% of the total community were analyzed for their taxonomic assignment and relative abundance.

The effects of predation by *B. bacteriovorus* UP on the floccular and granular sludge communities were then assessed by comparing the relative abundance of each OTU in

the controls and treatment groups and have been presented as their ratios in the treatment groups relative to control communities. The base 10 logarithms (Log_{10}) of the ratios were subsequently taken to show the effects of predation by *B. bacteriovorus* UP on the dominant microbial communities in floccular and granular activated sludges (Figures 4.8 and 4.9, respectively).

If the Log_{10} of the ratio was lower than 0, this indicated that *B. bacteriovorus* UP showed a positive predation effect on those community members, as their relative abundance decreased due to predation. Conversely, if the Log_{10} of the ratio was higher than 0, this indicated that *B. bacteriovorus* UP showed a negative predation effect. The most positively and negatively affected microorganisms in floccular and granular dominant communities were additionally plotted to show the predation effects of *B. bacteriovorus* UP on the microbial communities.

Amongst the dominant 400 OTUs from the floccular sludge, more than 320 OTUs were positively affected, whilst ~30 OTUs were negatively affected by the predation by *B. bacteriovorus* UP. Seventy OTUs that were the most positively affected and 18 OTUs that were the most negatively affected were plotted to show the predation effects and preferences of *B. bacteriovorus* UP on the floccular sludge communities (Figure 4.8). Amongst the most positively affected OTUs, the majority of the clearly taxonomically assigned OTUs were from *Bacteroidetes* (11 OTUs) and *Proteobacteria* (28 OTUs). The *Bacteroidetes* were mainly from two phylogenetic orders: *Flavobacteriales* (4 OTUs, including *Cloacibacterium*, *Chryseobacterium* and *Flavobacterium*) and *Sphingobacteriales* (6 OTUs, including *Chitinophagaceae*) (Figure 4.8). The *Flavobacteriales* seemed to be more vulnerable to predation than the *Sphingobacteriales* members. For example, the Log_{10} values for *Cloacibacterium* and *Chryseobacterium* were -1.39 and -0.93, respectively, whilst the Log_{10} values for *Sphingobacteriales* members ranged from -0.30 to -0.24.

The 28 *Proteobacteria* were mainly from the Alpha- (12 OTUs), Beta- (10 OTUs) and Gamma-*Proteobacteria* (5 OTUs) clades. The 12 Alpha-*Proteobacteria* were distributed amongst the orders of *Caulobacterales* (2 OTUs, including *Brevundimonas*), *Rhizobiales* (4 OTUs, including *Mesorhizobium*, *Devosia*, *Bradyrhizobium* and *Hyphomicrobium*), *Rhodobacterales* (2 OTUs), *Rhodospirillales* (2 OTUs, including *Reyranella*) and *Sphingomonadales* (2 OTUs). The Log_{10} values for these microorganisms ranged from -0.82 (one *Sphingomonadales*) to -0.23 (one *Rhodobacterales*). Most of the Beta-*Proteobacteria* OTUs (7 out of 10) were

attributed to the order of *Burkholderiales*, including *Comamonadaceae* (4 OTUs), *Oxalobacteraceae* (2 OTUs) and *Burkholderiaceae* (1 OTU). The Log_{10} values for the members of Beta-*Proteobacteria* also showed considerable variation, ranging from -1.07 (one *Oxalobacteraceae*) to -0.25 (one *Comamonadaceae*). Five of the Gamma-*Proteobacteria* belonged to two orders: *Pseudomonadales* (3 OTUs) and *Xanthomonadales* (2 OTUs). Of these 5 OTUs, the Log_{10} values ranged from -0.81 (one *Pseudomonas*) to -0.60 (one *Xanthomonadaceae*).

Interestingly, 3 OTUs belonging to *Eukaryota* were also reduced in relative abundance. These three fungal species were members of the *Microascaceae* (1 OTU) and *Tremellales* (2 OTUs, including *Trichosporon*). There were also many OTUs in the floccular sludge communities that could not be taxonomically assigned but that were also vulnerable to *B. bacteriovorus* UP, such as OTU42, OTU133, OTU487 and OTU403 (Log_{10} values for these OTUs were -1.60, -1.53, -1.18 and -1.11, respectively). This would suggest these unknown microorganisms were some of the most strongly impacted by predation.

Additionally, the relative abundance of some microorganisms within the floccular sludge communities increased after the sludge was incubated with *B. bacteriovorus* UP, as shown in the increases of Log_{10} values. These included bacteria from the *Proteobacteria*, such as one *Brevundimonas* species (Alpha-*Proteobacteria*), one *Candidatus* species (Beta-*Proteobacteria*) and one of *Stenotrophomonas* species (Gamma-*Proteobacteria*). Furthermore, there were no changes observed in the relative abundance of Gram-positive bacteria (mainly from *Actinobacteria*) in these dominant floccular communities.

In general, the results showed that members of the *Bacteroidetes* and *Proteobacteria* were dominant in the floccular sludge, and were highly sensitive to predation by *B. bacteriovorus* UP. Additionally, species of other bacterial phyla, such as *Chloroflexi*, and even some species of *Eukaryota* (fungi), were also sensitive to the predation. The reductions of relative abundance of different bacteria may indicate the predation preference of *B. bacteriovorus* UP on the floccular sludge microbial communities. Conversely, some species of both bacteria and fungi were observed to increase in relative abundance in the floccular communities incubated with the predator suggesting that they were resistant to predation.

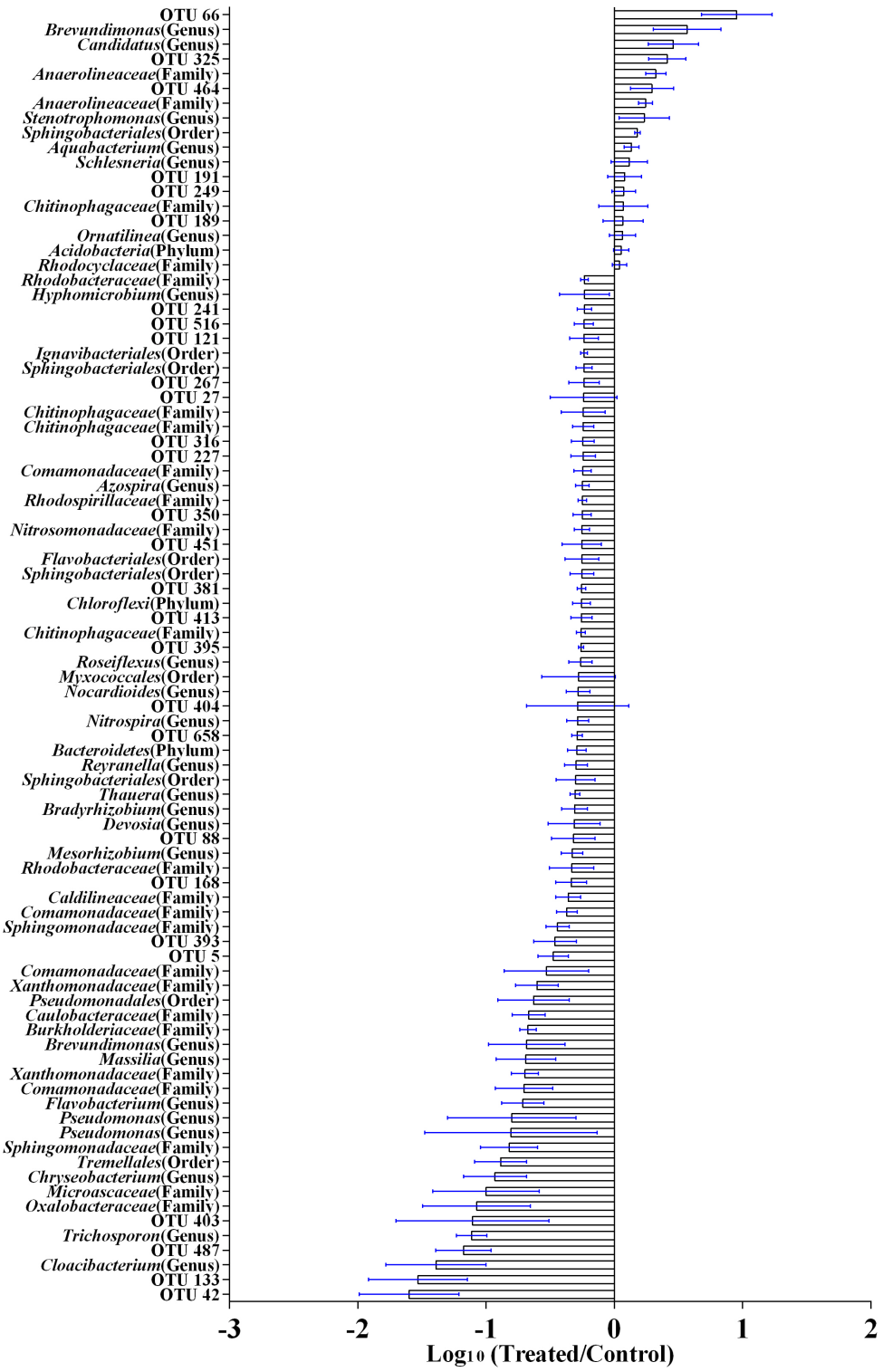

Figure 4.8 The effects of predation by *B. bacteriovorus* UP on the dominant microbial communities in floccular sludge. The Log_{10} of the ratios of sequencing reads of *Bdellovibrio*-treatment group to negative control were taken. All the OTUs were assigned to the closest taxonomical level with > 99% confidence, whilst those not be assigned taxonomically were shown as 'OTU'. Data represent the means of three biological replicates, with error bars representing the standard error of the mean.

Similarly, amongst the dominant 400 OTUs from the granular sludge, more than 320 OTUs were significantly preyed upon, whilst more than 30 OTUs (less than 40) were either resistant to predation or increased in numbers upon predation by *B. bacteriovorus* UP (data not shown). Sixty-nine OTUs that were the most reduced and 16 OTUs that increased the most were plotted to show the predation effects and preferences of *B. bacteriovorus* UP on the granular sludge communities (Figure 4.9).

The most positively affected OTUs (reduced upon predation) included members of the *Bacteroidetes* (5 OTUs) and *Proteobacteria* (33 OTUs). The *Bacteroidetes* were mainly distributed into two phylogenetic orders: *Flavobacteriales* (1 OTU, *Chryseobacterium*) and *Sphingobacteriales* (4 OTUs, mainly *Chitinophagaceae*) (Figure 4.8). The *Flavobacteriales* and *Sphingobacteriales* groups were also vulnerable to predation by *B. bacteriovorus* UP with reductions in relative abundance between -0.32 to -0.19.

The 33 *Proteobacteria* were also mainly distributed into three groups: Alpha-, Beta- and Gamma-*Proteobacteria*. Twenty-four OTUs were attributed to Alpha-*Proteobacteria*, which were largely distributed across the orders of *Rhizobiales* (12 OTUs, including *Shinella*, *Nitratireductor*, *Mesorhizobium*, *Bosea*, *Devosia* and *Hyphomicrobiaceae*), *Rhodobacterales* (4 OTUs), *Rhodospirillales* (5 OTUs, including *Ferrovibrio* and *Dongia*) and *Sphingomonadales* (1 OTU). The Log_{10} values for these microorganisms ranged from -1.01 (*Shinella*) to -0.15 (one *Rhodospirillales*).

The 5 Beta-*Proteobacteria* were attributed into the order of *Burkholderiales*, including the members of *Comamonadaceae* (2 OTUs, including *Delftia* and *Rhizobacter*), *Oxalobacteraceae* (1 OTU), *Burkholderiaceae* (1 OTU, *Chitinimonas*) and *Neisseriaceae* (1 OTU). The Log_{10} values for the members of Beta-*Proteobacteria* ranged from -0.43 (*Delftia*) to -0.17 (*Rhizobacter*). The 4 Gamma-*Proteobacteria* all belonged to *Xanthomonadales*, including *Thermomonas*, *Dokdonella* and *Aquimonas*, and the Log_{10} values ranged from -0.53 (one *Xanthomonadales*) to -0.20 (one *Aquimonas*).

There were also 4 OTUs in the granular communities that were assigned to *Eukaryota* (fungi) and were reduced in relative abundance including *Chromulinales* (1 OTU), *Pinnularia* (1 OTU), *Neobodo* (1 OTU) and *Filamoeba* (1 OTU). As for the granular sludge, a significant number of the OTUs also could not be taxonomically assigned that were vulnerable to *B. bacteriovorus* UP, such as OTU643, OTU616, OTU528

and OTU519 (Log_{10} values for these OTUs were -0.29, -0.29, -0.26 and -0.25, respectively).

Additionally, the relative abundance of some microorganisms within the granular sludge communities also increased after the sludge was incubated with *B. bacteriovorus* UP. Most of these OTUs could not be taxonomically assigned, such as OTU373, OTU66, OTU556 and OTU3 (Log_{10} values for these OTUs were 0.65, 0.62, 0.53 and 0.41, respectively). There were no changes observed on the relative abundance of the Gram-positive bacteria (mainly *Actinobacteria*) in these dominant granular communities (data not shown).

By comparing the compositions of the dominant communities in floccular and granular sludge, it was observed that there was significant variation in the community composition. For example, of *Bacteroidetes* in the dominant communities, 4 species of *Flavobacteriales* and 6 species of *Sphingobacteriales* were detected in the floccular communities, whilst only one species of *Flavobacteriales* and 4 species of *Sphingobacteriales* were detected in the granular communities. The changes in the *Proteobacteria* community compositions were more dramatic. Twelve Alpha-, 10 Beta- and 5 Gamma-*Proteobacteria* members were detected in floccular communities, whilst 24 Alpha-, 5 Beta- and 4 Gamma-*Proteobacteria* members were detected in granular communities. The Alpha-*Proteobacteria*, members were relatively evenly distributed across this clade in the floccular sludge, whilst the Alpha-*Proteobacteria* found in the granular sludge were mainly assigned to *Rhizobiales* (12 out of 24), *Rhodobacterales* (4 out of 24) and *Rhodospirillales* (5 out of 24).

In general, the results showed that bacterial communities of *Bacteroidetes* and *Proteobacteria* were also dominant in the granular sludge, especially the communities of *Proteobacteria*, which were the most dominant in the granular sludge. Members of *Bacteroidetes* and *Proteobacteria* also showed the highest sensitivity to predation by *B. bacteriovorus* UP. However, as the reductions in relative abundance for most bacterial populations in granular sludge did not vary greatly, it was possible that *B. bacteriovorus* UP did not show a strong feeding preference for the members of the granular sludge community (Figure 4.9). Additionally, the relatively low overall reductions in relative abundance (compared with results of floccular communities, Figure 4.8) may suggest that the granular sludge was generally better protected than the floccular sludge.

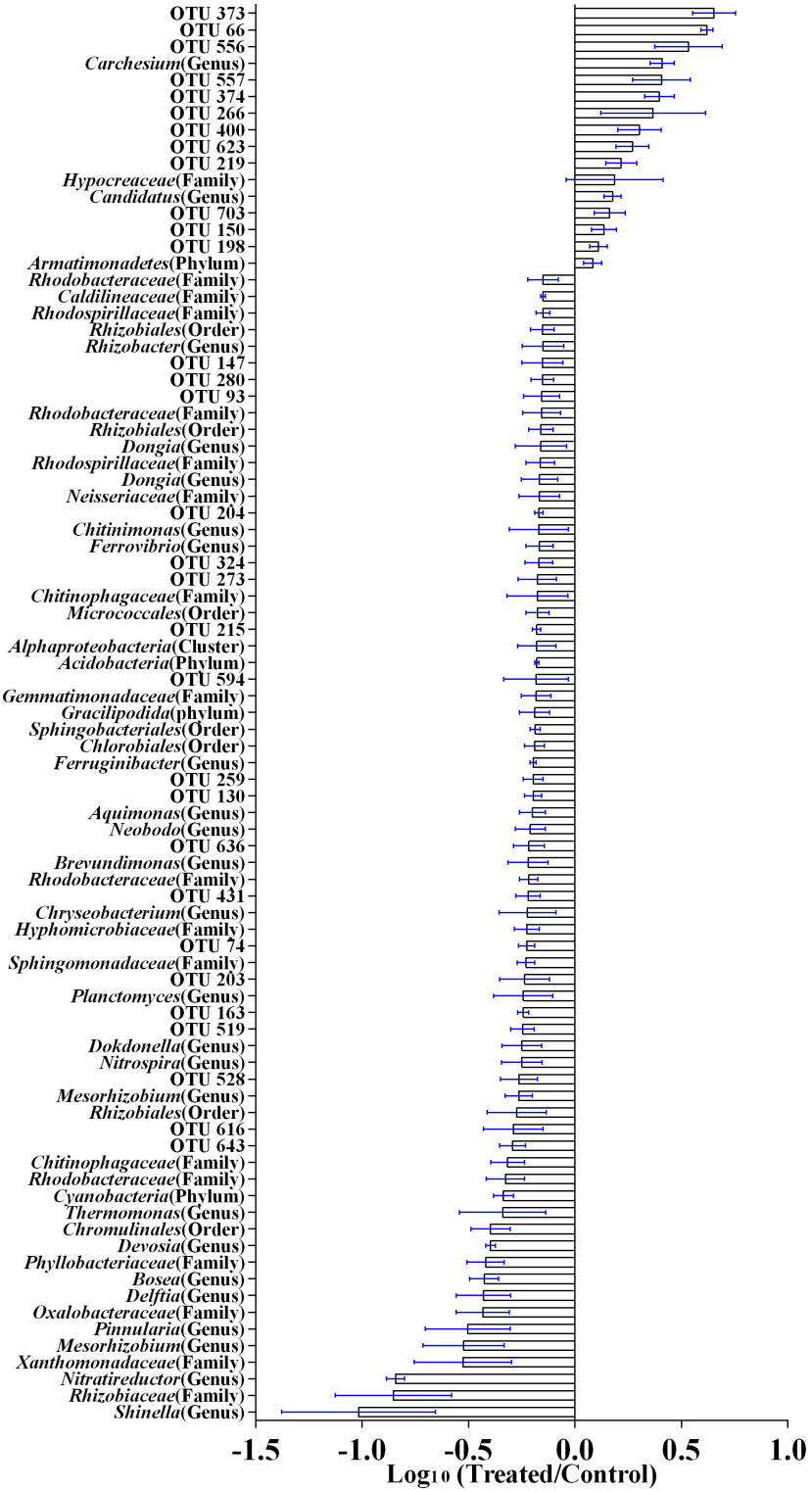

Figure 4.9 The effects of predation by *B. bacteriovorus* UP on the dominant microbial communities in granular sludge. The Log_{10} of the ratios of sequencing reads of *Bdellovibrio*-treatment group to negative control were taken. All the OTUs were assigned to the closest taxonomical level with > 99% confidence, whilst those not be assigned taxonomically were shown as 'OTU'. Data represented the means of three biological replicates, with error bars representing the standard error of the mean.

4.4 DISCCUSION

The effects of *Bdellovibrio bacteriovorus* UP predation on individual microbial isolates from activated sludge were investigated (Chapter III) and almost all of the Gram-negative isolates were shown to be sensitive to predation. Multi-species communities composed of two bacterial isolates in planktonic and biofilm growth were also sensitive to *B. bacteriovorus* UP. Therefore, biofilms composed of more complex microbial communities, floccular and granular activated sludge that represented the isolation source for *B. bacteriovorus* UP, were subsequently investigated here to determine the impact of predation on community compositions and viability.

The physiological properties of floccular and granular sludge communities, such as microbial activity and total biomass, were first measured to demonstrate the effects of predation by *B. bacteriovorus* UP. The microbial activity was determined by ATP content [207], whilst the total biomass was reflected by protein concentration of the activated sludge communities [208]. The results generally showed that both floccular and granular sludge communities were significantly affected by predation of *B. bacteriovorus* UP. The reductions in microbial activity were generally correlated to the reductions of total biomass of sludge communities, and these findings were further strengthened by the viable cell assay with Live/Dead staining, which collectively suggested that the reductions in microbial activity and total biomass were primarily due to massive death of microbial communities caused by *B. bacteriovorus* UP predation.

The granules, which by definition are more compact biomass aggregates, have been suggested to be better protected from other microbial predators, such as bacteriophages and protozoa relative to floccular sludge [13, 25, 51, 55]. However, both floccular and granular sludges were sensitive to *B. bacteriovorus* UP predation and showed similar reductions in biomass and viability. Interestingly, the image based analysis suggested that predation impacted the floccular biomass more than the granular biomass. This either could be due to the failure of the Live/Dead staining reagents to penetrate the outer layer of the granular sludge or the limitation of emission of the staining reagents, which might indicate that the predator could not access the bacteria in the inner core (usually deeper than 80 – 130 μm) of the granular sludge [209].

The effects of predation by *B. bacteriovorus* UP on the community composition of the floccular and granular sludges were assessed through total RNA sequencing of the communities [210]. The MDS analysis showed significant differences between the communities in negative controls and *Bdellovibrio*-treatment groups of both floccular and granular sludge. The Menhinick's index and Shannon-Wiener index were additionally used to quantify the variances of activated sludge communities [188]. Both indices showed that the richness and diversity of the activated sludge communities decreased due to the predation by *B. bacteriovorus* UP. A large portion of the sludge communities was preyed upon by *B. bacteriovorus* UP (Figure 4.3 and 4.4), which will lead to the decreases of richness and diversity of microbial communities. Additionally, it was observed that the floccular sludge community was more diverse relative to granular sludge. This decreased community diversity was also observed in a related study investigating the shifts of microbial communities during granulation from floccular sludge [203]. Additionally, the reductions in granular community diversity were greater relative to the reductions of floccular communities. This reduction difference might be related to the community composition in different types of sludge in that communities with more diverse compositions would better withstand the pressure of predation and maintain the stability of community [211-213]. Interestingly, almost half of both the floccular and granular communities were comprised of organisms that could not be identified and this may suggest that the development of both types of suspended biofilms is likely to be strongly influenced by novel bacteria [214, 215].

Some studies have shown that viruses and protozoa can significantly affect the marine microbial communities. As a result of viral predation, both microbial biomass production and composition of communities were remarkably influenced, which affected the biomass and energy transfer in the ecosystems [45, 216]. The effects of protozoan grazing on microbial communities and their performances were also broadly investigated [15, 105]. In a study, protozoan predation was shown to cause a clear population shift of nitrite-oxidizing bacteria, however, the nitrification performance of the microbial community was not significantly affected and this may be due to redundancy of nitrite oxidation capacity in other community members [217].

In this study, it was shown that the sequenced OTUs were primarily assigned to the classes of Alpha-*Proteobacteria*, Gamma-*Proteobacteria* and *Bacteroidetes*, which

were also consistent with bacterial groups discovered in other activated sludge communities [129, 218, 219]. Within these classes, a significant number of important bacterial groups, such as ammonia-oxidizing bacteria, nitrite-oxidizing bacteria, phosphate accumulating bacteria, as well as bacteria that hydrolyze diverse organic matter in wastewater, were found to carry out essential functions [219-221]. Some members of these functional bacterial groups in the activated sludge communities experimented in this study, such as *Nitrospira* (containing nitrite-oxidizing bacteria) and *Acinetobacter* (containing phosphate accumulating bacteria) [222], were observed here to be reduced in relative abundance due to predation by *B. bacteriovorus* UP. Therefore, the biological removal of nitrogen and phosphate by activated sludge communities may potentially be reduced correspondingly. However, it may be that, as shown above for the effect of protozoa predation on nitrite oxidation, other members of the community may be able to perform these functions in place of the sensitive strains found here [13, 25, 51, 55]. Further experiments are hence required to verify the correlation between the reduction of specific bacterial groups and the performance of sludge communities, which can further demonstrate the effects of predation by *B. bacteriovorus* UP on the functions of activated sludge.

Additionally, compared with protozoan grazing and viral lysis on microorganisms, *B. bacteriovorus* UP showed stronger predation effects on microbial communities given that one single predator isolate can cause the dramatic reductions on a broad spectrum of microorganisms [45, 216, 217]. Although most of the communities were reduced in relative abundance due to the predation, there was also a small portion of microorganisms increased relative abundance in both floccular and granular sludge. This would be expected since the results are presented as the relative abundances of sequences observed. To address the overall impact in more detail, quantitative data that indicate the exact numbers of specific organisms would be required. It is also not clear if those organisms that were observed to increase in relative abundance were explicitly resistant to predation, as was observed for the *O. anthropi*, or if they were simply less accessible by being present in the interior of the flocs or granules [194, 209]. Nonetheless, it was shown here that under the pressure of predation by *B. bacteriovorus* UP, the microbial communities of both floccular and granular sludges were re-structured.

In this chapter, the effects of predation by *B. bacteriovorus* UP on complex microbial communities in both floccular and granular activated sludge were investigated.

Through determining the changes in biomass and activity of the floccular and granular sludge communities, as well as analyzing the shifts of compositions of sludge communities, it can be shown that *B. bacteriovorus* UP exerted a significant impact on the microbial communities. The shifts in microbial communities may also suggest that there is no specific predation preference of *B. bacteriovorus* UP but that some species were more readily affected than others [164]. More importantly, the significant changes of microbial communities in activated sludge are likely to affect the performance of wastewater treatment plant via activated sludge [223]. To address the real effects of predation by *Bdellovibrio*, a series of important laboratory experiments is needed in the future to monitor the alterations of some essential parameters, such as carbon removal, nitrogen cycling and phosphate removal, during the wastewater treatment processes [129, 195, 196].

Chapter V. GENERAL DISCUSSION

Activated sludge in the wastewater treatment process is comprised of complex microbial communities that carry out a variety of functions necessary to remove the nutrients from the wastewater before it can be discharged into the environment for recycling. The removal of nutrients, such as nitrogen, phosphate and organic matter is largely dependent on the performance of specific groups of bacteria, such as ammonia-oxidizing bacteria, nitrite-oxidizing bacteria, phosphate accumulating bacteria and hydrolyzers [129, 196]. The composition and hence the function of these microbial communities can be significantly altered as a consequence of different stressors representing a risk for wastewater treatment plants [219, 224]. The causes of the disturbance are well understood in some cases, such as the input of industrial contaminations, whilst in some cases, the proximal causes are not apparent. One suspected factor in the disturbance of these complex communities is the predatory effects of viruses and protozoa. Whilst these two classes of predators have been well studied in terms of the impacts on microbial communities in wastewater systems, their analogous bacterial predator, the *Bdellovibrio*-and-like organisms (BALOs) have been less well characterized.

BALOs are a group of predatory bacteria, first described in the 1960s, which are ubiquitously distributed across a wide variety of environments [95]. They are obligate predators of a broad spectrum of Gram-negative bacteria. Because of their dependence upon prey species to survive and proliferate, they tend to be closely associated with high-density microbial communities, such as biofilms in both natural and man-made environments [69, 70]. Whilst BALOs have been studied in the laboratory to define their prey range and mechanisms of feeding, few studies have focused on their impacts on the communities from which they have been isolated. In contrast, the viruses and protozoa have been broadly investigated on their ecological roles. For example, viruses and protozoa both have been proposed as important causes of microbial mortality [225, 226], and such predation has been linked to changes in nutrient recycling, such as carbon and nitrogen [226, 227]. Because of the capacity of BALOs to prey upon a broad range of bacteria, it is therefore likely that this group of predators can also have a significant impact on complex microbial communities and are likely to play a similarly important role as a selective pressure affecting the selection for dominant organisms across the various habitats where they are endemic.

In contrast to bacteriophages, which can persist as quiescent particles in the absence of a host, the BALOs are highly dependent on being able to infect and parasitize prey bacteria, without which, the BALOs rapidly become energy exhausted and may lose viability. Therefore, BALOs are most likely to persist in habitats where microbial cell densities are high, e.g. biofilms [66]. In this regard, the activated sludge of wastewater treatment systems represents a potentially ideal niche for these predators. Therefore, the present study has investigated the presence and impact of BALOs on the high cell density and species rich, activated sludge community of a wastewater treatment system.

The relatively low abundance of *Bdellovibrio* populations makes it difficult to quantify their exact numbers or density in the wastewater treatment systems. Therefore, to fulfill the objectives of this study, experiments were first designed to isolate *Bdellovibrio* species from a wastewater treatment plant system (Ulu Pandan Wastewater Reclamation Plant, Singapore). One of the *Bdellovibrio* strains was collected and clearly identified, which was referred to here as '*Bdellovibrio bacteriovorus* UP' (Chapter II). To increase experimental reproducibility for subsequent work (Chapter II, III and IV), the numbers of *Bdellovibrio* that were used for each experiment should be consistent. Therefore, the growth pattern of *B. bacteriovorus* UP was characterized to determine when the predators were present the highest number for collection and to ensure the same number of predators were used at the start of each experiment (Chapter II).

The effects of predation by *B. bacteriovorus* UP on microorganisms both in single and mixed species communities were assessed to determine the prey range and predation preference. The results showed that *B. bacteriovorus* UP was effective at growing on a range of Gram-negative bacteria (Chapter II and III). Additionally, the mixed species communities did not demonstrate enhanced predation resistance (Chapter III). The impacts of *B. bacteriovorus* UP on more complex microbial communities, such as communities in floccular and granular sludge, were further investigated (Chapter IV). The results showed that the composition of the microbial communities significantly changed by the predation by *B. bacteriovorus* UP.

5.1 *Bdellovibrio*-and-like organisms (BALOs) isolated from activated sludge

Generally, BALOs are in low abundances relative to the whole microbial community in both the natural and man-made environments, due to their unique predator-parasite life-cycle [95] For example, in this study, the meta-genomic sequencing results in Chapter IV indicated that in the activated sludge samples the abundance of *Bdellovibrio* relative to the total microbial communities was approximately 1:10,000 (unpublished data). The relatively low abundance makes it difficult to investigate their ecological roles in situ, and hence, isolation of these predators from their habitats is required [171, 173]. Isolation experiments resulted in the selection of two different types of predatory bacteria from the floccular activated sludge. One was identified as a member of the genus *Bdellovibrio*, whilst the other one was taxonomically assigned to the Alpha-*Proteobacteria* clade, with no close matches in the database. Both predatory isolates could prey on multiple species of bacteria, which is consistent with the general feeding behavior of this class of organisms.

Within the Alpha-*Proteobacteria* clade, two groups of predatory bacteria have been identified and studied for which *Ensifer adhaerens* and *Micavibrio aeruginosavours* are the representative members [104, 185]. The 16S rRNA sequence of the Alpha-proteobacterial strain found in this study showed < 85% identity to both of these two species of bacteria and hence is likely to represent a novel predatory bacterium. The closest match for the *Bdellovibrio* strain was '*B. bacteriovorus* strain Tiberius' and the isolated strain was named as '*Bdellovibrio bacteriovorus* UP' [183]. The *B. bacteriovorus* strain Tiberius has previously been shown to display prey-independent growth on nutrient agar in addition to its predatory feeding behavior. However, there was no evidence that *B. bacteriovorus* UP was capable of prey-independent growth in this study (data not shown). For some BALOs, prey-independent growth appears to result from a rare mutation within the population (1 out of 10^6 – 10^8) and this phenotype is not stable, suggesting it is either strongly selected against or represents a bi-stable state of the predator, which may be exclusive to laboratory experiments [95, 228]. Given that *B. bacteriovorus* UP did not display this behavior, it may be a distinct strain or species from *B. bacteriovorus* Tiberius. The differences between the two strains may be resolved by whole genome sequencing of *B. bacteriovorus* UP for comparison with other members of *Bdellovibrio* spp.

5.2 *Bdellovibrio bacteriovorus* UP preys upon a broad spectrum of species

Whilst *B. bacteriovorus* UP did not display prey-independent growth, it was effective at growing on a range of Gram-negative bacteria, which is consistent with the behavior of this class of predators [73, 229]. The predation ability of *B. bacteriovorus* UP was first tested on a group of well-studied model laboratory strains, which were all from the Gamma-*Proteobacteria* clade (Chapter II). All of these bacteria both in planktonic and biofilm growth were shown to be vulnerable to *B. bacteriovorus* UP attack, and these results are consistent with other studies [162]. Whilst all of the model laboratory prey species were sensitive to predation, it was observed that cultures of *P. aeruginosa* PAO1 were less sensitive relative to other bacteria. *P. aeruginosa* has been shown to be resistant to protozoa grazing through the production of excessive EPS as well as the secretion of defensive compounds [105]. It is therefore possible that *P. aeruginosa* uses similar defensive strategy to reduce predation by *B. bacteriovorus* UP.

The effects of predation by *B. bacteriovorus* UP were further investigated using microorganisms isolated from activated sludge, the original source of *B. bacteriovorus* UP. These experiments also showed that *B. bacteriovorus* UP was capable of feeding on a broad spectrum of prey bacteria and this was true irrespective of whether the bacteria were presented as planktonic cells or were grown as biofilms. This was particularly surprising since it has been shown that for a range of stresses, including phage or protozoan predation, the biofilm can be protective of the prey [105, 230]. Most of the microbial species were taxonomically assigned to *Proteobacteria* and *Bacteroidetes*, and the dominance of these organisms in this study was consistent with previous reports on the microbial community composition in activated sludge [194, 196]. Almost all of the members of these two phyla, when grown as single-species biofilms were vulnerable to the predation by *B. bacteriovorus* UP. One exception was *Ochrobactrum anthropi*, a Gram-negative bacterium from Alpha-*Proteobacteria* showing resistance to predation. The mechanism of resistance was not studied here, however it would be of particular interest to determine this mechanism since few if any other Gram-negative species have been shown to be completely resistant to predation by BALOs [191].

When grown on mono-species populations of prey, the growth pattern of *B. bacteriovorus* UP tended to reflect the well-defined Lotka-Volterra equation that is usually used to describe the predator-prey interaction for higher organisms [10]. In the

experiments presented here for mono-species prey cultures, there appeared to be a minimum number of 10^4 CFU/mL, below which *B. bacteriovorus* UP numbers also began to decline. This was also observed in other study that prey cell concentrations of at least 10^5 – 10^6 CFU/mL are required for BALOs survival [71]. Whilst this was true when mono-species cultures of prey were used, it would be particularly interesting to determine whether similar patterns would be observed when exposed to high diversity communities. For example, would one prey species preferentially become depleted before the other species was attacked or would both species be equally reduced until the overall cell number was below that required to support *B. bacteriovorus* UP growth.

5.3 Mixed species communities do not show enhanced predation resistance

In addition to presenting a choice of prey species, some of which may be preferred by the predator [229], it has been suggested that growth in a mixed species community may be protective where resistance mechanisms can be shared between different community members. For example, mixed-species biofilms comprised of *Candida albicans* and *Staphylococcus epidermidis* showed that extracellular polymers produced by *S. epidermidis* could inhibit penetration of the antifungal drug fluconazole whilst *C. albicans* could protect the EPS-negative *S. epidermidis* from vancomycin [132]. Hence, mixed communities with two species in both planktonic and biofilm growth were tested and no cross protection between species was observed. In these experiments, the dual species cultures included the predation resistant species *O. anthropi* and an additional sensitive strain. The sensitive member of the two-membered community was still preyed upon by *B. bacteriovorus* UP, as it was clearly demonstrated in the reduction of viable cell numbers in the two-membered planktonic cultures. Although the two-membered biofilms were also reduced in biomass content due to exposure to *B. bacteriovorus* UP, the proportion of each member within the biofilms was not clearly delineated. Thus, whilst it is clear from the planktonic data that only the sensitive strain was removed, it can only be inferred that this was also the case in the dual species biofilms. Quantitative measurement of each species, e.g. using quantitative PCR, in these multi-species biofilms may further elucidate if there is some degree of cross protection or if indeed, there is selective feeding on the sensitive strain only.

5.4 *Bdellovibrio bacteriovorus* UP has a significant impact on floccular and granular sludge communities

It has been proposed that the formation of granules is a response of the microbial communities to predation pressure from microbial predators, where the microbial communities embedded in the granules gain increased protection [203]. For example, in one study, the removal of protozoa from the floccular biomass can lead to disintegration of the flocs [231]. Therefore, *Bdellovibrio* spp. may also represent predation pressures that drive the formation of granules. In agreement with the data presented for the mono- and dual-species communities, the microbial communities in floccular and granular sludge were both significantly affected by predation. Due to predation, the vast majority of the microbial communities appeared to be dead (Chapter IV, Live/Dead staining and ATP content) and this was accompanied by significant reductions in the total biomass (based on changes in protein content). Collectively, the data showed that neither biofilm community was strongly protected from predation by BALOs.

Whilst there was no general protection from predation, detailed examination of the microbial communities suggested that, as was observed for the mono-species cultures, there were subtle differences in the effects of predation on different community members. For both communities, 38.25% and 48.75% of the most abundant OTUs could not be identified in the databases and thus potentially represent novel species or strains. Interestingly, for the floccular sludge community, members of this unidentified fraction were some of the most sensitive to predation. This is consistent with other reports of sludge communities. These results also suggest that the current expectation that BALOs do not have a feeding preference may in part be due to limitations in the prey species used. Therefore, experiments using isolated representatives of these unknown OTUs may be informative in showing whether there are indeed preferred prey species in this complex community. Amongst the vulnerable populations, some populations were more significantly reduced in relative abundance than others, which indicated that *B. bacteriovorus* UP might have a predation preference when there is a choice of prey in highly diverse microbial communities. This preferential predation suggests that predation pattern of *B. bacteriovorus* UP might be applicable to the 'kill-the-winner' hypothesis that has been proposed and studied on the dynamic interaction between virus and bacterial communities [232]. Whilst it is supported by data collected from diverse aquatic environments [225], this

hypothesis is generally applicable to simplified ecosystems under laboratory conditions. It may even be possible to further test this hypothesis using floccular or granular sludge communities in laboratory based experiments, where the predator has a greater prey range to further refine this ecological theory.

It has been shown here and in other studies that *B. bacteriovorus* UP was capable of feeding on a broad spectrum of prey species [95, 162]. Therefore, the predation pattern for *B. bacteriovorus* UP when presented with complex microbial communities might be better represented as a 'kill-them-all' strategy. Given the capacity of BALOs to feed on these community members and the lack of protection afforded by biofilm growth, it is somewhat surprising that they appear to be rare members of the community as determined by sequence analysis (Prof. Yehuda Cohen, personal communication). Given the isolation of at least one BALOs that had no known relative identified in the databases, it may be that there is a high diversity of predators present in these species rich and high density sludge communities that are yet to be identified.

Additionally, functional bacterial populations in both floccular and granular sludges were shown to be vulnerable to predation. For example, microorganisms associated with nitrogen and phosphate cycling were observed to be significantly reduced upon predation. Similarly, previous studies have shown clear shifts of functional populations due to protozoan grazing. Whilst there were changes in the relative abundances of functional classes of bacteria, the performance of the whole community was not significantly affected due to the replacement by other species carrying out the same functions [217]. In the work presented here, representatives of most of the functional populations were significantly reduced due to predation, and therefore, it remains possible that the performance of the community is affected as a consequence of predation.

5.5 Concluding remarks and future work

This study has shown that two predatory bacteria were isolated from the activated sludge collected at the Ulu Pandan Wastewater Reclamation Plant (Singapore). One of these isolated predators was taxonomically identified as *Bdellovibrio bacteriovorus* strain UP. The growth pattern of this *Bdellovibrio* isolate on specific prey species was investigated and was shown to be able to feed on all of the Gram-negative prey

species tested (Chapter II). With one exception of one species, all of the Gram-negative bacteria isolated from activated sludge, grown in biofilms were also shown to be vulnerable to *B. bacteriovorus* UP. Prey species grown in mixed communities were also significantly preyed upon by *B. bacteriovorus* UP, irrespective of whether they were grown as planktonic cells or as biofilms. Collectively, the data demonstrate that *B. bacteriovorus* UP was able to prey on a broad spectrum of bacteria not only grown as single species cultures but also as mixed species communities (Chapter III). Complex microbial communities in both floccular and granular sludges were also significantly impacted by the predatory activity of *B. bacteriovorus* UP resulting in significant killing of these microbial communities (Chapter IV). Therefore, the fuller isolation and identification of the complete range of BALOs present in sludge systems will be important in understanding their ability to influence the stability of microbial communities in terms of both composition and function as it relates to the performance of wastewater treatment systems.

Some areas of further study are suggested here.

1. Full genome sequencing and analysis of BALOs will allow for a more complete identification and comparison with known isolates.
2. Fluorescence in situ hybridization with specific probes was used to demonstrate the presence of *Bdellovibrio* and related organisms in the activated sludge from the Ulu Pandan Wastewater Reclamation Plant. Therefore, this technique could be further used to quantify BALOs in the wastewater treatment plant, especially if coupled with fluorescence assisted cell sorting (FACS).
3. Mixed species biofilms that were shown to be vulnerable to *Bdellovibrio* were only comprised of two species, which were quite simplified systems. Biofilms with multiple species (more than two) can be further explored with their resistance/sensitivity to *Bdellovibrio* attack.
4. The functional bacterial populations in floccular and granular sludges were significantly reduced due to predation. Therefore, future work can be carried out to determine if these changes also impact the performance of activated sludge, such as the carbon removal, nitrogen cycling and phosphate removal.
5. Whilst the results presented here showed that floccular and granular sludges were both sensitive to predation by BALOs, the floccular sludge was more significantly impacted. Therefore, it remains possible that BALOs represent a

significant selective pressure that encourages granule formation. This could potentially be tested by specifically infecting floccular sludge systems with BALOs in the laboratory to determine if such predation pressure leads to the conversion of the biomass into a granular community.

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